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Plasmid-Mediated Dissemination of the Metallo-β-Lactamase Gene *bla*$_{IMP}$ among Clinically Isolated Strains of *Serratia marcescens*

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The distribution of strains producing metallo-β-lactamase among 105 strains of *Serratia marcescens* was investigated. All of these strains were isolated in seven general hospitals located in Aichi Prefecture, Japan, from April to May 1993. Southern hybridization analysis suggested that four *S. marcescens* strains, AK9373, AK9374, AK9385, and AK9391, had a metallo-β-lactamase genes similar to the *bla*$_{IMP}$ gene found by our laboratory (E. Osano, Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato, Antimicrob. Agents Chemother. 38:71–78, 1994), and these four strains showed resistance to carbapenems as well as to the other broad-spectrum β-lactams. In particular, strains AK9373, AK9374, and AK9391 showed an extraordinarily high-level resistance to imipenem (MICs, ≧64 μg/ml), whereas strain AK9385 demonstrated moderate imipenem resistance (MIC, 8 μg/ml). The imipenem resistance of AK9373 was transferred to *Escherichia coli* CSH2 by conjugation with a frequency of 10^{-5}. The DNA probe of the *bla*$_{IMP}$ gene hybridized to a large plasmid (approximately 120 kb) transferred into the *E. coli* transconjugant as well as to the large plasmids harbored by AK9373. On the other hand, although we failed in the conjugational transfer of imipenem resistance from strains AK9374, AK9385, and AK9391 to *E. coli* CSH2, imipenem resistance was transferred from these strains to *E. coli* HB101 by transformation. A plasmid (approximately 25 kb) was observed in each transformant which acquired imipenem resistance. The amino acid sequence at the N terminus of the enzyme purified from strain AK9373 was identical to that of the metallo-β-lactamase IMP-1. In contrast, strains ES9348, AK9386, and AK93101, which were moderately resistant to imipenem (MICs, ≧4 to ≦8 μg/ml), had no detectable *bla*$_{IMP}$ gene. As a conclusion, 19% of clinically isolated *S. marcescens* strains in Aichi Prefecture, Japan, in 1993 were resistant to imipenem (MICs, ≥2 μg/ml), and strains which showed high-level imipenem resistance because of acquisition of a plasmid-mediated *bla*$_{IMP}$-like metallo-β-lactamase gene had already proliferated as nosocomial infections, at least in a general hospital.

Carbapenems, such as imipenem and panipenem (22), are relatively stable agents against extended-spectrum β-lactamases produced by gram-negative rods (11, 14, 31, 32). Most of these β-lactamases have a serine residue at the active center of the enzymes, and they belong to class A β-lactamases (1), which include SHV- and TEM-related β-lactamases (3, 4, 12, 19). Moreover, some AmpC-type β-lactamases which gave resistance to various broad-spectrum β-lactams were also ineffectual against carbapenems (8, 9, 15, 23, 26). Therefore, imipenem has been used freely in Japan for the chemotherapy of infectious diseases caused by gram-negative bacteria. Under such circumstances, however, several clinical isolates showing resistance to carbapenems and broad-spectrum β-lactams have emerged (6, 24, 25, 27, 36, 38). These resistant strains of the family *Enterobacteriaceae* or *Pseudomonas* spp. sometimes cause opportunistic or nosocomial infections in compromised hosts, especially in general hospitals that use huge amounts of broad-spectrum β-lactams. Recently, we isolated an imipenem-resistant *Serratia marcescens* isolate, strain TN9106, and confirmed that this strain produced a metallo-β-lactamase, IMP-1 (25). This finding warned us that imipenem-resistant strains which produce metallo-β-lactamases may be prevalent in the near future if the present style of chemotherapy is continued without precautions or careful consideration. Strains producing metallo-β-lactamase usually show high-level resistance to almost every broad-spectrum β-lactam (7, 36). Therefore, it became urgent for us to investigate the distribution of bacteria producing metallo-β-lactamase among general hospitals in Japan.

(This study was presented in part at the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy, Orlando, Fla., 4 to 7 October 1994 [2].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains tested were collected from seven general hospitals located in Aichi Prefecture from April to May 1993 (Table 1). Aichi Prefecture is located in the midland of Japan, and its approximate population is 6,850,000. Both Anjyo and Okazaki are located adjacent to Nagoya in Aichi Prefecture. A single strain was isolated from each patient to avoid the duplicate isolation of the same strain. *Escherichia coli* JM109 that harbors pSMBNU24 (25) or pHIP29 (Fig. 1) carrying the DNA probe of *bla*$_{IMP}$ was cultured in Luria-Bertani (LB) broth supplemented with 30 μg of chloramphenicol per ml. Mueller-Hinton II agar and Mueller-Hinton II broth (BBL Microbiology Systems, Cockeysville, Md.) were used for susceptibility testing.

Antibiotics. The following antibiotics were provided by the indicated sources:

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TABLE 1. Distribution of level of resistance to imipenem among 105 strains isolated in seven general hospitals

Hospital	No. of isolated strains for which the imipenem MIC (μg/ml) is:		
	<2	≧2–≦32	≧64
A	9	0	0
B	5	0	0
C	10	7	0
D	7	3	0
E	17	3	0
F	19	1	0
G[a]	18	3	3

[a] Hospital where the strains that harbored a plasmid carrying a metallo-β-lactamase gene were isolated.

ampicillin, Meijiseika Kaishya, Ltd., Tokyo, Japan; piperacillin and cefoperazone, Toyama Chemical Co., Ltd., Toyama, Japan; aztreonam, Eizai Co., Ltd., Tokyo, Japan; ceftizoxime, Fujisawa Pharmaceutical Co., Osaka, Japan; cefotetan, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan; cefmenoxime, Takeda Chemical Industries, Ltd., Osaka, Japan; cefotaxime, Farbwerke Hoechst AG, Frankfurt, Germany; ceftazidime, Japan Glaxo Co., Tokyo, Japan; cephaloridine and moxalactam, Shionogi and Co., Ltd., Osaka, Japan; chloramphenicol, Sigma Chemical Co., St. Louis, Mo.; clavulanate, SmithKline Beecham Pharmaceuticals, Surrey, United Kingdom; imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; meropenem, Sumitomo Pharmaceuticals Ltd., Osaka, Japan; panipenem and cefmetazole, Sankyo Co., Ltd., Tokyo, Japan; rifampin, Nippon CIBA-GEIGY Co., Ltd., Hyogo, Japan; norfloxacin, Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan.

Susceptibility testing. MICs were determined by the agar dilution method with Mueller-Hinton II agar (BBL) plates supplemented with graded concentrations of antibiotics. A 5-μl aliquot of culture (approximately 10^6 CFU/ml) grown to the logarithmic phase in Mueller-Hinton broth (BBL) was inoculated onto the agar plates. Plates were incubated for 18 h at 37°C, and the MIC of each β-lactam antibiotic was judged according to the approved standard of the National Committee for Clinical Laboratory Standards (21).

Purification of metallo-β-lactamase, inhibition study, and determination of amino acid sequence. For inhibition studies, crude metallo-β-lactamase was prepared from each imipenem-resistant strain as described elsewhere (25). The activity of metallo-β-lactamase was assayed by hydrolysis against imipenem. The inhibitory effects of Hg^{2+}, Cu^{2+}, and EDTA were tested by the method of Watanabe et al. (37) by using imipenem as the reporter substrate. The metallo-β-lactamase of strain AK9373 was highly purified by the method of Osano et al. (25) and was used for sequencing analysis at the N terminus of the protein with a peptide sequencer (model 437A; Applied Biosystems Inc., Foster City, Calif.).

DNA manipulation and Southern hybridization analysis. Plasmid DNA was prepared by the rapid alkaline extraction method (30) and was purified by low-melting-point agarose (agarose-L; Nippon Gene Co., Ltd., Toyama, Japan) gel electrophoresis and then electroelution. Restriction endonucleases were also supplied by Nippon Gene Co., Ltd. A recombinant plasmid, plasmid pHIP29, carrying the DNA probe of the metallo-β-lactamase gene bla_{IMP} was constructed as shown in Fig. 1. As a hybridization probe, the 0.5-kb *Hin*dIII-*Hin*dIII fragment containing the internal region of bla_{IMP} was excised from the low-melting-point agarose gel after *Hin*dIII digestion of pHIP29, and this was followed by agarose gel electrophoresis. The gel block containing the DNA probes was labeled with [α-^{32}P]dCTP directly by using the Klenow fragment and the labeling buffer of the DNA labeling kit (Nippon Gene Co., Ltd.). DNA was blotted onto nylon mem-

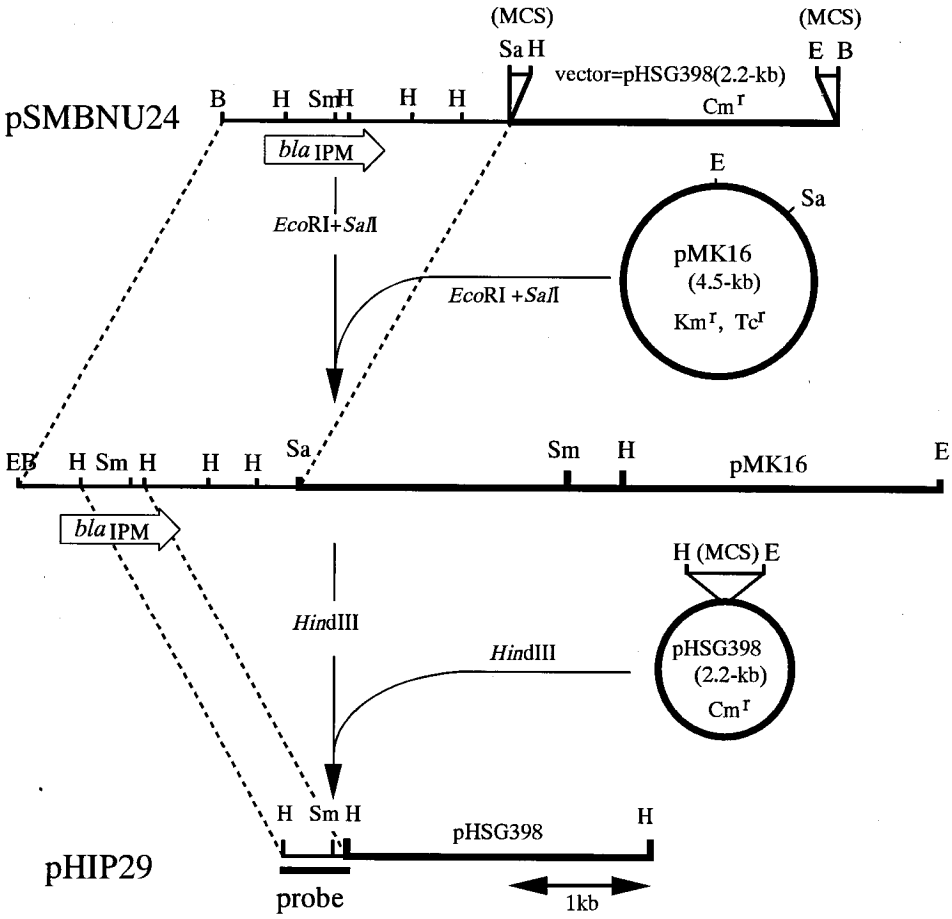

FIG. 1. Construction of pHIP29 carrying DNA probe for metallo-β-lactamase gene. The *Eco*RI-*Sal*I fragment of pSMBNU24 (25) carrying the metallo-β-lactamase gene bla_{IMP} was transferred to pMK16, and then the 0.5-kb *Hin*dIII-*Hin*dIII fragment carrying the inner region of bla_{IMP} gene was subcloned with pHSG398 (35), a derivative of the pUC vector (39). The resulting plasmid was designated pHIP29. The nucleotide sequences of both terminal regions of the insert DNA of pHIP29 were confirmed by DNA sequencing. Abbreviations: B, *Bam*HI; E, *Eco*RI; Sa, *Sal*I; Sm, *Sma*I; H, *Hin*dIII; MCS, multicloning site; bla_{IMP}, metallo-β-lactamase gene.

branes (Hybond-N; Amersham, Buckinghamshire, United Kingdom) after agarose gel electrophoresis by the method of Southern (33). DNA hybridization was achieved under the high-stringency conditions described elsewhere (4), by which a sequence similarity greater than 60% is detected. The preparation of large plasmids was performed by the method of Kado and Liu (13).

Transfer of imipenem resistance. Conjugational transfer of imipenem resistance from *S. marcescens* AK9373 was performed by using *E. coli* CSH2 as the recipient. After conjugation, the bacterial suspension was spread onto the LB agar plates containing 50 μg of ampicillin per ml and 100 μg of rifampin per ml. The colonies of transconjugants that appeared were replica plated onto the agar plate supplemented with 2 μg of imipenem per ml to confirm the transfer of imipenem resistance.

The transfer of imipenem resistance by transformation was also performed. Plasmid DNA was prepared from each strain which demonstrated positive hybridization, and the DNA was introduced into *E. coli* HB101 by an electroporation method. Transformants were isolated on the LB agar plate supplemented with 16 μg of ceftazidime per ml. The MICs of imipenem for each transconjugant or transformant were also tested as described above.

Profiles of outer membrane proteins. Bacterial outer membrane proteins were prepared from strains showing imipenem resistance (MICs, >2 μg/ml) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method described by Inokuchi et al. (10).

RESULTS

Susceptibility testing. The numbers of *S. marcescens* strains isolated from each general hospital in Aichi Prefecture are given Table 1. Strains which showed imipenem resistance were isolated from every hospital except hospitals A and B. In particular, the highly imipenem-resistant strains were all isolated from hospital G. The percentage of imipenem-resistant strains appeared to be diverse among the seven hospitals, and the highest percentage was found in hospital C. In this hospital, seven of 17 clinical isolates showed imipenem resistance. The cumulative resistance of 105 clinical isolates against 13 β-lactams are also given in Fig. 2. The *S. marcescens* strains tested were classified into three groups according to their susceptibility to imipenem. Eighty-five strains (81%) for which the imipenem MIC was less than 2 μg/ml were judged to be susceptible to imipenem according to the approved standard of the National Committee for Clinical Laboratory Standards (21). Then, 20 strains (19%) were judged to be resistant to imipenem. Among them, 3 strains (AK9373, AK9374, and AK9391) for which the imipenem MIC was greater than 32 μg/ml (Table 2) were classified as the highly resistant group. Thus, the other 17 strains (16% of tested strains) were classified as the group moderately resistant to imipenem, and they still showed high-level resistance to various cephems and penicillins. The MICs of each β-lactam antibiotic, together with those of chloramphenicol and norfloxacin, for nine multi-β-lactam-resistant strains for which the imipenem MIC was greater than 2 μg/ml are given in Table 2. The sources of the imipenem-resistant strains listed in Table 2 were all urine. Patients from whom these highly resistant strains were isolated were all medicated with combinations of various antibiotics including imipenem for several weeks.

Plasmids and transfer of imipenem resistance. Each imipenem-resistant strain harbored several plasmids. The plasmid profiles of strains for which the imipenem MIC was greater than 2 μg/ml appeared to be divergent (Fig. 3A), even though some of them were isolated in the same hospital. The imipenem resistance of strain AK9373 was transferred to *E. coli* CSH2 by conjugation, but the MIC of imipenem for this transconjugant was only 4 μg/ml. Transfer of a large plasmid (about 120 kb) into *E. coli* CSH2 (Fig. 4) was involved in the transfer of imipenem resistance and the concomitant transfer of trimethoprim resistance. On the other hand, we failed to transfer the imipenem resistance of strains AK9374, AK9385, and AK9391 to *E. coli* CSH2 by conjugation. Then, transformation by electroporation with the plasmids prepared from these strains was performed. Each transformant was found to be resistant to 16 μg of ceftazidime per ml. These transformants also showed imipenem resistance (MICs, 4 μg/ml), and each of these transformants harbored a plasmid of similar size (approximately 25 kb) (data not shown).

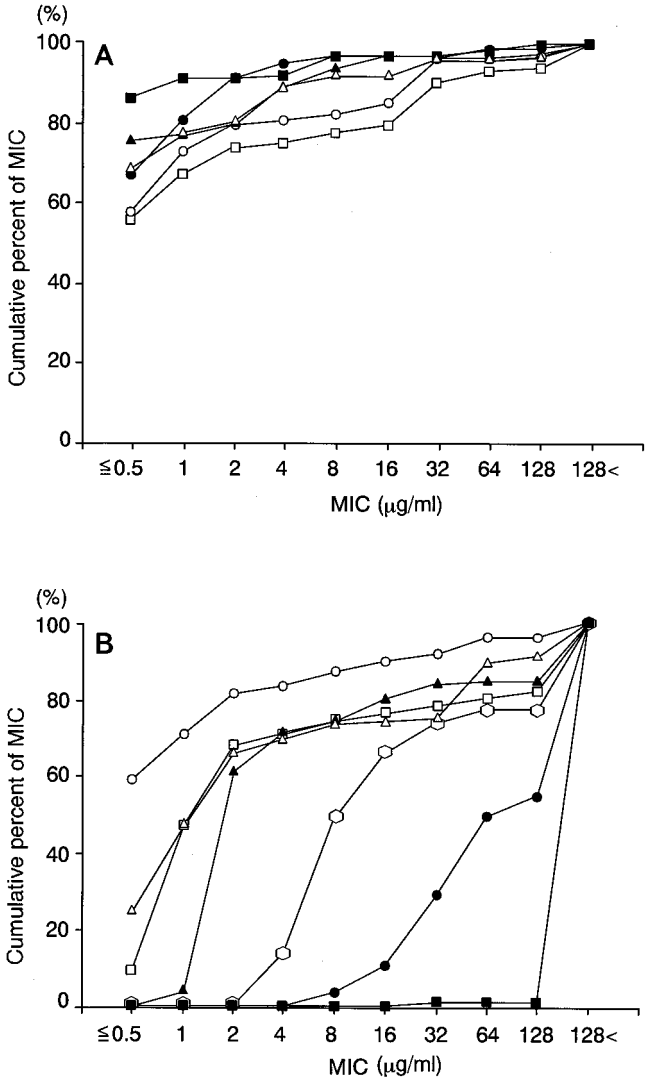

FIG. 2. Distribution of resistance levels against various β-lactams. One hundred five clinical isolates of *S. marcescens* were subjected to susceptibility testing against 13 β-lactams. The cumulative percentages of strains inhibited are shown. (A) ●, imipenem; ▲, panipenem; ■, meropenem; ○, cefotaxime; △, ceftazidime; □, moxalactam. (B) ●, ampicillin; ▲, piperacillin; ■, cephaloridine; ⬡, cefmetazole; ○, cefmenoxime; △, cefotetan; □, cefoperazone.

Southern hybridization analyses. The metallo-β-lactamase-specific DNA probe that was derived from the bla_{IMP} gene of *S. marcescens* TN9106 hybridized to plasmids prepared from four imipenem-resistant strains, strains AK9373, AK9374, AK9385, and AK9391 (Fig. 3B). However, no detectable hybridization to the chromosomal positions of the strains tested was observed. Hybridizations to plasmids similar in size were detected among strains AK9374, AK9385, and AK9391. Two distinct hybridization signals to different positions were observed in strain AK9373, although these two signals might come from different forms of the same plasmid (Fig. 4, lane 3). A single hybridization signal to a large plasmid that was trans-

TABLE 2. Susceptibilities of strains showing imipenem resistance

S. marcescens strain	MIC (μg/ml) of various antibiotics[b]															
	IPM	PPM	MPM	ABPC	PIPC	CER	CMZ	CMX	CTT	CPZ	CTX	CAZ	MOX	AZT	CP	NOR
MH9337	4	16	8	>128	>128	>128	>128	64	>128	>128	32	32	128	2	32	16
MH9338	4	8	8	>128	>128	>128	>128	64	64	>128	32	32	32	2	128	1
EK9348	4	16	8	>128	>128	>128	>128	>128	>128	>128	>128	>128	64	32	32	8
AK9373[c]	>128	>128	128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	8	8	2
AK9374[c]	64	128	64	>128	32	>128	>128	>128	>128	>128	>128	>128	>128	4	128	32
AK9385[c]	8	16	8	>128	32	>128	>128	64	>128	>128	32	32	>128	2	4	16
AK9386	8	4	4	>128	>128	>128	>128	32	>128	>128	32	8	>128	4	128	128
AK9391[c]	64	>128	64	>128	>128	>128	>128	64	>128	>128	128	>128	>128	2	32	16
MH93101	4	8	8	>128	>128	>128	>128	64	>128	>128	32	32	>128	2	32	32

[a] Strains for which the MIC of imipenem was greater than 2 μg/ml are listed.
[b] IPM, imipenem; PPM, panipenem; MPM, meropenem, ABPC, ampicillin; PIPC, piperacillin; CER, cephaloridine; CMZ, cefmetazole; CTT, cefotetan; CPZ, cefoperazone; CTX, cefotaxime; CAZ, ceftazidime; MOX, moxalactam; AZT, aztreonam; CP, chloramphenicol; NOR, norfloxacin.
[c] Strains to which plasmid the metallo-β-lactamase gene-specific DNA probe hybridized.

ferred to *E. coli* CSH2 was detected as shown in Fig. 4, lane 4. Moreover, the plasmids introduced in *E. coli* HB101 from AK9374, AK9385, and AK9391 by transformation were also hybridized with the DNA probe (data not shown).

The hybridization patterns of the DNA probe to *Eco*RI- or *Hin*dIII-digested DNA fragments were similar among AK9393, AK9374, AK9385, and AK9391 (Fig. 5B and C). However, DNA hybridization to *Bam*HI-digested fragments of strain AK9373 was different from those to fragments of the other three strains. In strain AK9373, a strong hybridization was detected at about 9 kb, although among the other three strains hybridizations were detected at positions longer than 20 kb (Fig. 5A).

Inhibition of enzyme activity. A crude enzyme solution was prepared from strains AK9373, AK9374, AK9385, and AK9391 to which the DNA probes of bla_{IMP} hybridized. The presence of Hg^{2+} and Cu^{2+} in the reaction buffer blocked the enzyme activity against imipenem in each preparation, and furthermore, the addition of EDTA also resulted in a significant reduction in enzyme activity. However, the enzyme activity against imipenem was not inhibited detectably by clavulanate or tazobactam, which generally block the serine β-lactamase activity efficiently (6) (data not shown).

Amino acid sequence of the metallo-β-lactamase prepared from strain AK9373. The sequence of 15 amino acid residues at the N terminus of the purified enzyme prepared from *S. marcescens* AK9373 was determined. The amino acid sequence determined was equal to that of the N terminus of the IMP-1 metallo-β-lactamase of *S. marcescens* TN9106.

Profiles of outer membrane proteins. The electrophoretic patterns of outer membrane proteins prepared from strains for which the imipenem MICs were greater than 2 μg/ml were classified into several groups (data not shown). A clear band corresponding to the 40- or 41-kDa outer membrane protein of *S. marcescens* (17, 34) was observed among the strains tested. Although the patterns of minor outer membrane proteins appeared to be different among the strains tested, considerable similarity was found among strains AK9373, AK9374, AK9385, and AK9391. However, it was difficult to find a relationship between the levels of resistance to norfloxacin or chloramphenicol and the profiles of the outer membrane proteins.

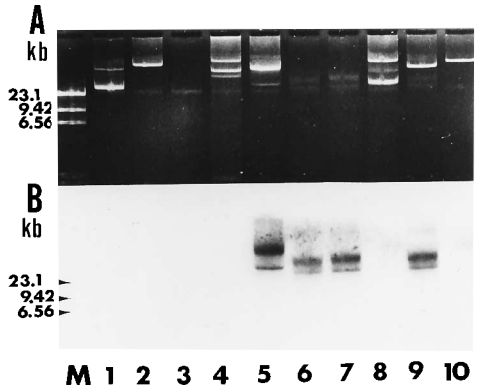

FIG. 3. Plasmid profiles and Southern hybridization analysis. Plasmids were prepared from the imipenem-resistant strains of *S. marcescens* listed in Table 2 by the method of Kado and Liu (13). (A) Plasmid profiles of each strain. (B) Result of DNA hybridization analysis with bla_{IMP}-specific DNA probe. The 0.5-kb *Hin*dIII fragment of pHIP29 (Fig. 1) was used as a DNA probe. Hybridizations to the large plasmids harbored by strain AK9373, AK9374, AK9385, and AK9391 were observed. Lanes: M, *Hin*dIII-digested DNA marker; 1, *S. marcescens* CS9310; 2, MH9337; 3, MH9338; 4, EK9348; 5, AK9373; 6, AK9374; 7, AK9385; 8, AK9386; 9, AK9391; 10, MH93101. An imipenem-susceptible strain, CS9310, for which the imipenem MIC was less than 0.25 μg/ml, is shown in lane 1 as a control.

DISCUSSION

A number of metallo-β-lactamases that confer resistance to carbapenems were initially reported in nonenterobacteria such as *Bacteroides fragilis* (5, 7, 28), *Aeromonas hydrophila* (18), *Xanthomonas maltophilia* (36), and *Pseudomonas aeruginosa*

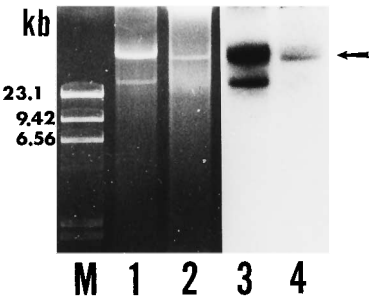

FIG. 4. Conjugational transfer of the large plasmid encoding the metallo-β-lactamase gene. Large plasmids prepared from strain AK9373 and the transconjugants by the method of Kado and Liu (13) were applied to the agarose gel electrophoresis. Lanes: M, *Hin*dIII-digested DNA marker; 1, *S. marcescens* AK9373; 2, transconjugant of *E. coli* CSH2; 3, strain AK9373 (two hybridization signals are observed); 4, transconjugant of *E. coli* CSH2. A hybridization signal corresponding to the large plasmid transferred to *E. coli* CSH2 is indicated by an arrow.

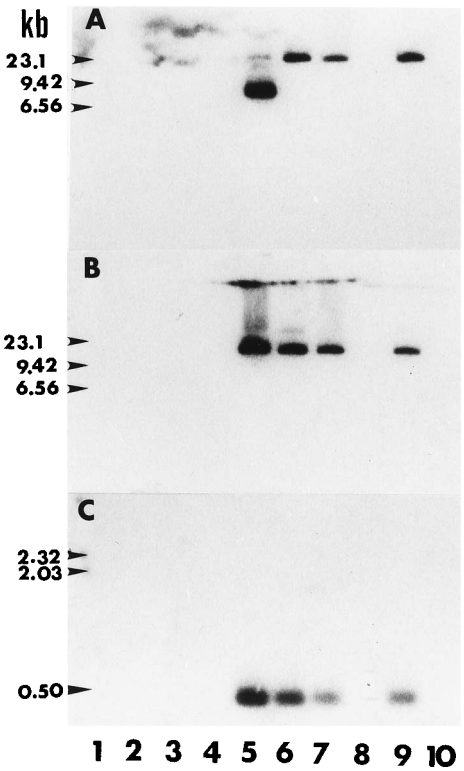

FIG. 5. Hybridization to restriction enzyme-digested DNA fragments. DNA prepared from each imipenem-resistant strain was digested with *Bam*HI, *Eco*RI, or *Hin*dIII. The 0.5-kb *Hin*dIII fragment was used as a DNA probe for the bla_{IMP} gene. (A to C) Hybridization patterns to *Bam*HI-, *Eco*RI-, or *Hin*dIII-digested DNAs, respectively. Lanes: 1, *S. marcescens* CS9310 (an imipenem-susceptible strain); 2, MH9337; 3, MH9338; 4, EK9348; 5, AK9373; 6, AK9374; 7, AK9385; 8, AK9386; 9, AK9391; 10, MH93101.

(37). However, recently, we found an enterobacterial metallo-β-lactamase, IMP-1, in a clinical isolate of *S. marcescens* that shows a high level of imipenem resistance (25). Notably, this strain demonstrated high-level resistance to various broad-spectrum β-lactams. The isolation frequency of imipenem-resistant strains of *S. marcescens* is speculated to have been increasing. Therefore, in the present study we collected 105 clinical isolates of *S. marcescens* and investigated the relationship between the level of imipenem resistance and the production of metallo-β-lactamases similar to IMP-1. The results confirmed that 4 of 105 strains of *S. marcescens* tested have the metallo-β-lactamase gene similar to the bla_{IMP} gene which was found in *S. marcescens* TN9106 (26). The N-terminal amino acid sequence of the metallo-β-lactamase prepared from *S. marcescens* AK9373 showed complete identity to that of IMP-1. Moreover, strain AK9373 was isolated from a general hospital in the city of Anjyo, next to the city of Okazaki, where strain TN9106 was isolated in 1991. These findings may suggest a local prevalence of imipenem-resistant *S. marcescens* strains that carry the bla_{IMP} gene.

Strains AK9373, AK9374, AK9385, and AK9391 were isolated in the same general hospital. The plasmid profiles of these four strains showed that the plasmids appeared to be different in size. However, these strains showed similar outer membrane protein profiles. The results of Southern hybridization analyses strongly suggested that the plasmids harbored by strains AK9374, AK9385, and AK9391 were derived from the same source. From these findings, it is speculated that introduction of a plasmid carrying the bla_{IMP} gene through mobilization into moderately imipenem-resistant strains, such as strain AK9386, might result in the emergence of highly imipenem-resistant strains such as AK9374 and AK9391. As a result of the proliferation of strains which had acquired the metallo-β-lactamase gene, an outbreak of nosocomial infections might occur in the general hospital from which these four strains were isolated.

The hybridization pattern of the *Bam*HI-digested fragment of AK9373 was different from those of the other three strains (Fig. 5A), although the hybridization patterns of *Eco*RI- or *Hin*dIII-digested fragments looked similar among the four strains (Fig. 5B and C). This observation suggests that the structures of the flanking regions of the metallo-β-lactamase gene of AK9373 is different from those of the other three strains. Furthermore, the plasmids harbored by strains AK9374, AK9385, and AK9391 were not transferred to *E. coli* CSH2 by conjugation. These findings reveal that the transferable large plasmid found in strain AK9373 has an origin different from those found in strains AK9374, AK9385, and AK9391. Therefore, it is possible to speculate that the bla_{IMP} gene carried by a plasmid might be transposed onto the other one. Transposon- or integron-like elements may play an important role in the transposition or translocation of the metallo-β-lactamase gene into various plasmids.

We found some differences in the level of imipenem resistance among strains which have the metallo-β-lactamase gene. Strains AK9374 and AK9391 demonstrated high-level imipenem resistance (MICs, 64 μg/ml), whereas strain AK9385 showed a moderate resistance level (MIC, 8 μg/ml), although these strains harbor similar plasmids to which the DNA probe hybridized. This finding might be explained in part by the permeation efficiency of imipenem through each bacterial outer membrane (16). It is well known that resistance to chloramphenicol and norfloxacin partly depend on the permeability of the bacterial outer membrane. However, we failed to find considerable differences in the outer membrane protein profiles among strains tested as mentioned above. To examine the membrane permeation of antibiotics, we tested the susceptibilities of strains AK9374, AK9385, and AK9391 to these agents. Indeed, the resistance level of strain AK9374 against chloramphenicol was very high (MIC, 128 μg/ml) compared with those of the other two strains. This is because of the production of chloramphenicol acetyltransferase in strain AK9374. However, no significant difference in the level of resistance to chloramphenicol was observed between AK9385 and AK9391. Moreover, nor was a considerable difference in the level of resistance to norfloxacin found among these three strains (Table 2). These findings suggest that minor outer membrane proteins might be involved in the permeation of imipenem through the bacterial outer membrane. Another possible explanation for the differences in resistance to imipenem among these three strains would be the varied expression levels of the metallo-β-lactamase gene in strain AK9385 compared with those of the genes in the other two strains (29). In other words, the promoter activity of the metallo-β-lactamase gene may be suppressed by unidentified regulatory systems in AK9385. Promoter analyses of metallo-β-lactamase genes should be continued to resolve this question.

Strains EK9348, AK9386, and MN93101 showed moderate levels of imipenem resistance, even though the metallo-β-lactamase-specific DNA probe did not hybridize to DNAs prepared from these strains. Indeed, the presence of different types of metallo-β-lactamase genes may not be excluded, but we failed to detect the production of any metallo-β-lactamase activity among these strains. On the other hand, these strains

also showed high-level resistance to chloramphenicol and norfloxacin (Table 2). Therefore, it may be possible to speculate that either decreased permeation of antibiotics through the bacterial outer membrane or the production of imipenem-hydrolyzing β-lactamases such as Sme-1 (20) or NOR-1 (24) may play an important role in the imipenem resistance among these strains.

A large amount of imipenem has been using clinically in Japan because it is a very stable agent against the extended-spectrum β-lactamases produced by gram-negative bacteria. Only imipenem was available when the imipenem-resistant strains tested were isolated in 1993. Quite recently, panipenem, another carbapenem, has also been released for clinical use in Japan. Other newly developed carbapenems such as meropenem and biapenem will soon be used clinically in Japan. However, as we demonstrated in the present work, bacteria have already been furnished with the tool to cope with these carbapenems. Therefore, we must give careful consideration to the clinical use of carbapenems to prevent the proliferation of carbapenem-resistant strains producing metallo-β-lactamase.

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