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Molecular Characterization of an Enterobacterial Metallo β-Lactamase Found in a Clinical Isolate of *Serratia marcescens* That Shows Imipenem Resistance

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A clinical isolate of *Serratia marcescens* (TN9106) produced a metallo β-lactamase (IMP-1) which conferred resistance to imipenem and broad-spectrum β-lactams. The bla_{IMP} gene providing imipenem resistance was cloned and expressed in *Escherichia coli* HB101. The IMP-1 was purified from *E. coli* HB101 that harbors pSMBNU24 carrying bla_{IMP}, and its apparent molecular mass was calculated to be about 30 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Kinetic studies of IMP-1 against various β-lactams revealed that this enzyme hydrolyzes not only various broad-spectrum β-lactams but also carbapenems. However, aztreonam was relatively stable against IMP-1. Although clavulanate or cloxacillin failed to inhibit IMP-1, Hg^{2+}, Fe^{2+}, or Cu^{2+} blocked the enzyme's activity. Moreover, the presence of EDTA in the reaction buffer resulted in a decrease in the enzyme's activity. Carbapenem resistance was not transferred from *S. marcescens* TN9106 to *E. coli* CSH2 by conjugation. A hybridization study confirmed that bla_{IMP} was encoded on the chromosome of *S. marcescens* TN9106. By nucleotide sequencing analysis, bla_{IMP} was found to encode a protein of 246 amino acid residues and was shown to have considerable homology to the metallo β-lactamase genes of *Bacillus cereus*, *Bacteroides fragilis*, and *Aeromonas hydrophila*. The G+C content of bla_{IMP} was 39.4%. Four consensus amino acid residues, His-95, His-97, Cys-176, and His-215, which form putative zinc ligands, were conserved in the deduced amino acid sequence of IMP-1. By determination of the amino acid sequence at the N terminus of purified mature IMP-1, 18 amino acid residues were found to be processed from the N terminus of the premature enzyme as a signal peptide. These results clearly show that IMP-1 is an enterobacterial metallo β-lactamase, of which the primary structure has been completely determined, that confers resistance to carbapenems and other broad-spectrum β-lactams.

Many extended-spectrum β-lactamases conferring high levels of resistance to broad-spectrum β-lactam antibiotics have been found worldwide with the increasing use of newly developed broad-spectrum β-lactam antibiotics (10, 11). They are mostly R plasmid-mediated TEM- or SHV-related class A enzymes (4, 12, 18, 22, 29, 32). However, plasmid-mediated AmpC-type β-lactamases that belong to class C have recently been reported (9, 21). These plasmid-mediated extended-spectrum β-lactamases have a serine residue at the active center of the enzyme, and they effectively hydrolyze broad-spectrum β-lactams, except carbapenems. Although it was reported by genetic analyses that bacteria belonging to the family *Enterobacteriaceae*, except *Klebsiella* spp. (1, 2) and *Pseudomonas* spp., have chromosomally encoded inducible AmpC β-lactamases (8, 14, 16, 20, 26, 34), these strains are usually susceptible to imipenem because of its stability against AmpC enzymes. Therefore, imipenem is now widely used in Japan for the treatment of infectious diseases caused by gram-negative bacteria. On the other hand, *Bacteroides fragilis* and *Aeromonas hydrophila* sometimes show resistance to imipenem and broad-spectrum β-lactams (7, 17, 37). The imipenem resistances of these bacteria are mainly due to the production of metallo β-lactamases that belong to class B. The class B enzymes require zinc ions for enzyme activity and demonstrate a primary structure quite different from those of the class A and class C enzymes belonging to the group of serine β-lactamases. Recently, several strains of *Serratia marcescens* were reported to show resistance to carbapenems as well (35). In this study, we clearly show that a clinically isolated strain of *S. marcescens* (TN9106) showing resistance to imipenem has a chromosomally encoded metallo β-lactamase gene.

(This study was presented in part at the General Meeting of the American Society for Microbiology, Atlanta, Ga., 16 to 20 May 1993 [20a].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used for the analysis of IMP-1 are listed in Table 1. *S. marcescens* TN9106, showing a high level of resistance to various broad-spectrum β-lactam antibiotics, was isolated from a patient with a urinary tract infection at Aichi Hospital, Okazaki, Japan, in 1991. Bacteria were grown in Luria-Bertani medium or 2× YT medium (25) supplemented with the appropriate antibiotics.

Antibiotics. Antibiotics were obtained from the following sources: ampicillin, Meiji-seika Kaishya, Ltd., Tokyo, Japan; aztreonam, Eizai Co. Ltd., Tokyo, Japan; cefazolin and ceftizoxime, Fujisawa Pharmaceutical Co., Osaka, Japan;

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TABLE 1. List of bacterial strains, phage, and plasmids used

Bacterial strain, phage, plasmid, or clone	Characteristics[a]	Source or reference
Strains		
S. marcescens		
TN9106	Clinical isolate; an imipenem-resistant strain isolated in Aichi Hospital, Okazaki, Japan (1991)	This study
IMPS08	Clinical isolate; an imipenem-sensitive strain isolated in Nagoya University Hospital, Nagoya, Japan (1986)	This study
E. coli		
HB101	F$^-$ *hsdS20*(r$^-$, m$^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* (λ^-)	25
JM109	*recA1* Δ(*lac pro*) *endA1 gyr-96 thi-1 hsdR* mutant *supE44 relA1* F'(*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *ZΔM15*)	25
Plasmids and phage		
pMK16	Cloning vector; Km^r Tc^r	A. Ohta
pHSG 398	Cloning vector; Cm^r	31
M13 (mp18 and mp19)	Vector for DNA sequencing	36
Clones		
pSMBNU2	Recombinant plasmid encoding β-lactamase gene of *S. marcescens* TN9106	This study
pSMBNU24	Recombinant plasmid encoding β-lactamase gene of *S. marcescens* TN9106	This study

[a] Abbreviations: Km^r, kanamycin resistance; Tc, tetracycline resistance; Cm^r, chloramphenicol resistance.

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 and cloxacillin, 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, Sankyo Co., Ltd., Tokyo, Japan; rifampin, Nippon CIBA-GEIGY Co., Ltd., Hyogo, Japan.

Susceptibility testing. MICs were determined by the agar dilution method with Mueller-Hinton II agar (BBL Microbiology Systems, Cockeysville, Md.) containing graded concentrations of antibiotics. A 5-μl aliquot of culture (approximately 10^6 CFU/ml) grown to log phase in Mueller-Hinton broth (BBL) was inoculated on the agar plates. Plates were incubated for 18 h at 37°C for determination of the MIC of each β-lactam antibiotic. The resistance breakpoint of *S. marcescens* against imipenem was based on the MIC data of the National Committee for Clinical Laboratory Standards (19).

Purification of IMP-1. *Escherichia coli* HB101(pSMB NU24) was cultured overnight in 2 liters of Luria-Bertani broth with shaking at 37°C. Proteins in the cultured supernatant were precipitated in a 90% saturated concentration of ammonium sulfate [$(NH_4)_2SO_4$]. The precipitate was resolved in 50 mM phosphate buffer (pH 7.0). The solution containing IMP-1 was applied to a Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.) column for gel filtration, and then fractions with β-lactamase activity were condensed by ultrafiltration. The crude enzyme solution was used for assay of the inhibitory effects of metal ions, EDTA, cloxacillin, and clavulanate on enzyme activity. Enzyme activity was assayed in phosphate buffer (pH 7.0) containing 100 μM $ZnCl_2$. For further purification of IMP-1, the enzyme solution was also chromatographed through a DEAE-Sephacel column equilibrated with 50 mM phosphate buffer (pH 7.0). Most of the intermixed proteins were trapped by the DEAE-Sephacel column, although IMP-1 passed through the column, and then the enzyme solution was condensed by ultrafiltration with a Centricut U-10 (Kurabo Co., Osaka, Japan). The purity of the preparation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Kinetic study of IMP-1. The purified IMP-1 preparation was assayed against various β-lactam substrates at 30°C in 50 mM phosphate buffer (pH 7.0) containing 100 μM $ZnCl_2$ by a spectrophotometer (UV2200; Shimadzu, Kyoto, Japan). The absorption maxima of the substrates used were as follows: ampicillin, 235 nm; aztreonam, 315 nm; ceftazidime, 272 nm; ceftizoxime, 250 nm; cephaloridine, 295 nm; moxalactam, 274 nm; imipenem, 297 nm; meropenem, 299 nm; panipenem, 299 nm. The molar extinction coefficients were calculated by the method of Seeberg et al. (27). K_m and maximum rate of metabolism (V_{max}) values were obtained by a double-reciprocal (Lineweaver-Burk) plot of the initial steady-state velocities at different substrate concentrations. Relative V_{max} and V_{max}/K_m values were calculated for comparison of enzyme activities, as recommended by Bush and Sykes (6).

Molecular mass determination and isoelectric focusing. Molecular mass was determined by SDS-PAGE with the purified enzyme preparation. Isoelectric focusing of the enzyme was carried out with a Multiphor II (Pharmacia-LKB) with an Ampholine gel (Pharmacia-LKB) with a pH range of 3.5 to 9.5. The pH gradient was determined with an isoelectric focusing calibration kit (pH 3 to 10) according to the recommendations of the manufacturer (Pharmacia).

Inhibition tests. The inhibitory effects of Hg^{2+}, Cu^{2+}, EDTA, clavulanate, and cloxacillin on IMP-1 were tested by the method of Watanabe et al. (33). Enzyme was preincubated with 10 μM inhibitor in 50 mM phosphate buffer (pH 7.0) containing 100 μM $ZnCl_2$ at 30°C for 5 min, and the remaining activity was assayed spectrophotometrically with 100 μM cephaloridine as the substrate.

Amino acid sequencing at the N terminus of IMP-1. Twenty-four amino acid residues in the N terminus of mature IMP-1 were sequenced by a peptide sequencer (model 473A; Applied Biosystems Inc., Foster City, Calif.) with the purified enzyme preparation.

DNA techniques. Plasmid DNA was prepared by the rapid

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TABLE 2. Antibiotic susceptibility of *S. marcescens*, transformants, and *E. coli*

Antibiotic	MIC (μg/ml) for:					
	S. marcescens			*E. coli*		
	TN9106	IMPS08	IMPS08(pSMBNU2)	HB101(pSMBNU2)	HB101(pSMBNU24)	HB101
Ampicillin	>128	128	>128	64	>128	4
Piperacillin	32	2	4	4	8	<0.5
Cephaloridine	>128	>128	>128	64	64	4
Cefazolin	>128	>128	>128	64	64	2
Ceftazidime	>128	0.5	64	64	>128	0.5
Cefoperazon	>128	2	64	64	>128	0.5
Cefotaxime	128	0.5	32	32	128	<0.5
Cefmenoxime	>128	<0.5	16	16	64	<0.5
Ceftizoxime	64	<0.5	32	16	128	<0.5
Moxalactam	>128	0.5	>128	>128	>128	<0.5
Aztreonam	8	<0.5	<0.5	<0.5	<0.5	<0.5
Imipenem	32	0.5	4	2	4	0.5
Panipenem	64	<0.5	16	2	4	0.5
Meropenem	32	<0.5	4	2	2	<0.5

alkaline lysis method (25) and was purified by low-melting-point agarose (agarose-L; Nippon Gene Co. Ltd., Toyama, Japan) gel electrophoresis followed by electroelution. Restriction endonucleases, exonuclease III, mung bean nuclease, Klenow's fragment, and T4 DNA ligase were also supplied by Nippon Gene Co. Ltd. As a hybridization probe, the 0.5-kb *Hin*dIII-*Hin*dIII fragment of pSMBNU24 containing the coding frame of bla_{IMP} was subcloned into pHSG299 (31) and excised from the low-melting-point agarose gel after *Hin*dIII digestion and agarose gel electrophoresis. The DNA fragments were labeled with [α-^{32}P]dCTP with a multiprime labeling kit (Nippon Gene Co. Ltd.). DNA was blotted onto nylon membranes (Hybond-N; Amersham, Buckinghamshire, United Kingdom) by the method of Southern (28). DNA hybridization was achieved under high-stringency conditions as described elsewhere (2).

Analyses of data. Analyses of homologies among the deduced amino acid sequences of metallo β-lactamases were carried out with Swiss-Prot data base release 22. Analyses of data were assisted by GENETYX system version 6.0 software (SDC, Tokyo, Japan).

Nucleotide sequence accession number. Information regarding the sequences of *Bacillus cereus* 5/B/6 and the *B. fragilis* and *A. hydrophila* strains used in this study may be obtained from the Swiss-Prot data base under accession no. P04190, P25910, and P26918, respectively.

RESULTS

Susceptibility testing. The MICs of β-lactam antibiotics for *S. marcescens* TN9106 (parental strain), IMPS08, and IMPS08(pSMBNU24) and *E. coli* HB101, HB101 (pSMBNU2), and HB101(pSMBNU24) are listed in Table 1. IMPS08(pSMBNU24) and HB101(pSMBNU24) harboring bla_{IMP} showed higher levels of resistance to various broad-spectrum β-lactam antibiotics than IMPS08 and HB101, respectively. Although IMPS08(pSMBNU24) and HB101 (pSMBNU24) harbor multicopy bla_{IMP} genes, the resistance of these transformants to imipenem was lower than that of parental strain TN9106 (Table 2). The MICs of aztreonam for TN9106 and transformants were relatively lower than those of the other broad-spectrum β-lactams.

Cloning of the bla_{IMP} gene. *Eco*RI-digested fragments of chromosomal DNA prepared from *S. marcescens* TN9106 were ligated into the *Eco*RI site of pMK16. These recombinants were introduced into *E. coli* HB101, and transformants showing resistance to ampicillin (50 μg/ml) and kanamycin (30 μg/ml) were isolated. A 13.5-kb recombinant containing about 9.5 kb of insert was selected out and termed pSMBNU2 because it provided *E. coli* HB101 with resistance to imipenem (Table 2). pSMBNU2 was then digested partially with *Sau*3AI and subcloned into the *Bam*HI site of pHSG398 (31). Colonies showing resistance to both ampicil-

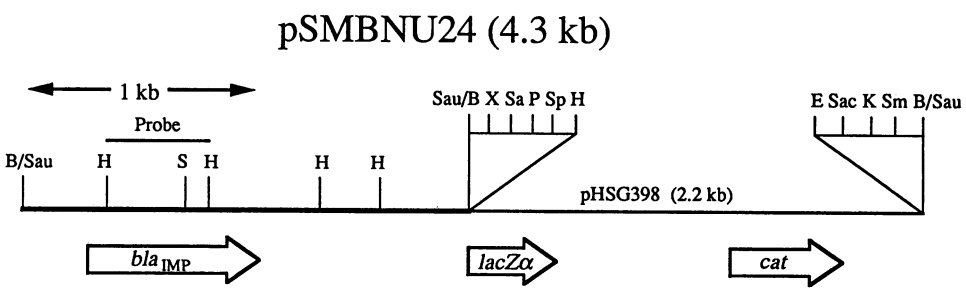


FIG. 1. Restriction map of pSMBNU24. The position and transcriptional direction of bla_{IMP} are shown by an open arrow, as are those of the chloramphenicol acetyltransferase gene (*cat*) and 3′-truncated *lacZ* gene (*lacZα*) on the vector plasmid pHSG398 (31), which is shown by a thick line. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Sa, *Sal*I; Sac, *Sac*I; Sau, *Sau*3AI; Sm, *Sma*I; Sp, *Sph*I; P, *Pst*I; X, *Xba*I.

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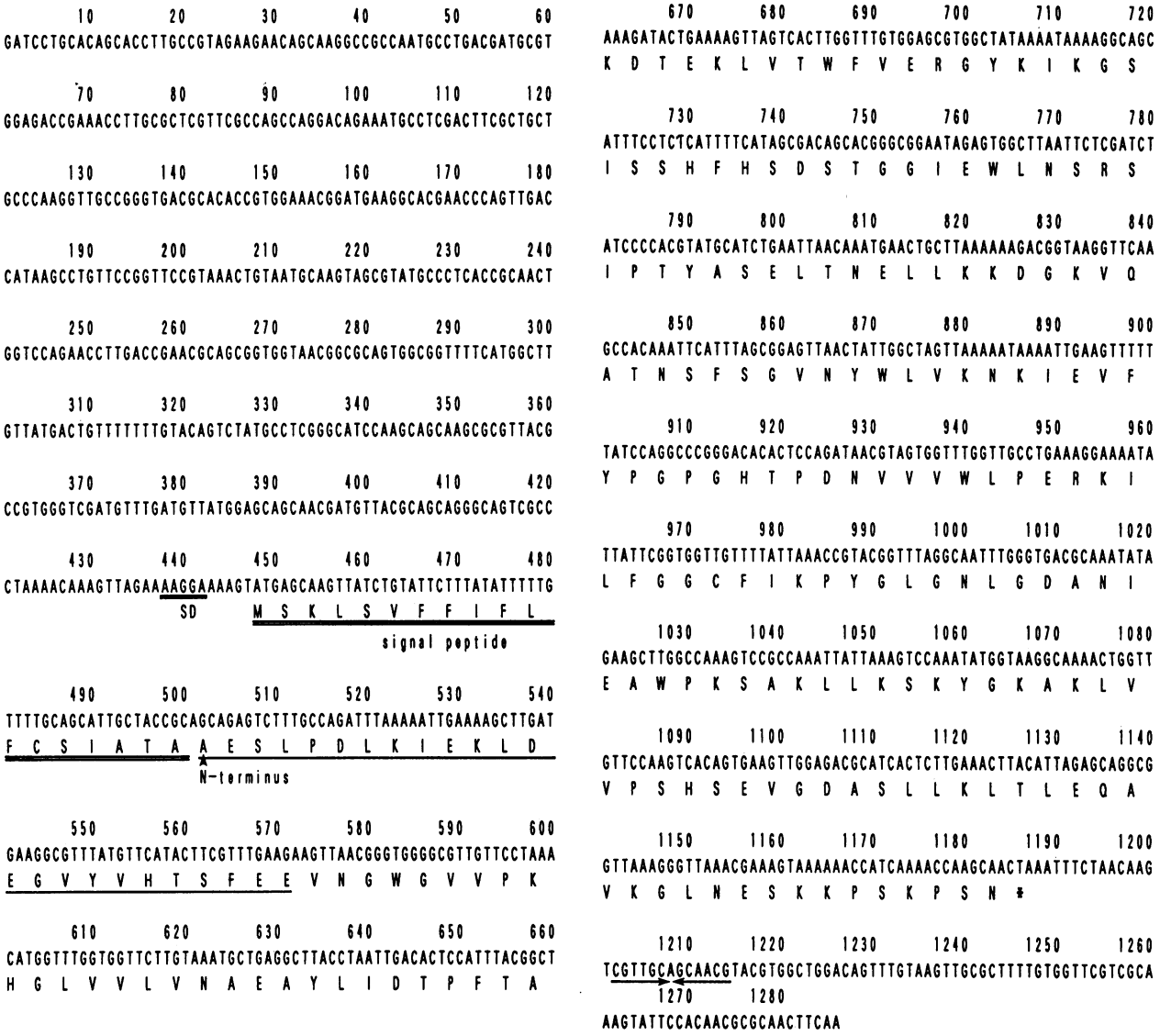


FIG. 2. Nucleotide sequence of bla_{IMP} and its flanking regions. The deduced amino acid sequence is shown in the usual single-letter amino acid code below the nucleotide sequence. The putative Shine-Dalgarno sequence (SD) for bla_{IMP} is underlined with a thick line, a 24-amino-acid sequence determined at the N terminus of a purified IMP-1 preparation is underlined, and the signal sequence of IMP-1 is double underlined. The N terminus of mature IMP-1 is marked with an arrowhead. Horizontal arrows indicate regions of dyad symmetry.

lin and chloramphenicol were isolated, and the recombinant plasmid pSMBNU24 carrying a 2.1-kb insert was selected for further DNA analysis (Fig. 1). The 2.1-kb insert of pSMBNU24 was transferred into the *Bam*HI site of the M13 phages mp18 and mp19 (36), and deleted subclones were constructed with a deletion kit according to the manufacturer's protocol. DNA sequencing was performed with a DNA sequencer (model 373A; Applied Biosystems). A 738-bp open reading frame encoding a possible protein of 246 amino acid residues was identified in the 2.1-kb insert of pSMBNU24 (Fig. 2). Comparisons of amino acid sequences among the metallo β-lactamase genes are also shown in Fig. 3. The G+C content of bla_{IMP} was calculated to be 39.4%, and this value is relatively lower than the species average of *S. marcescens*.

Purification of IMP-1 and enzymological analyses. IMP-1 was purified from the culture supernatant of *E. coli* HB101(pSMBNU24) as described in Materials and Methods. The purified enzyme appeared as a single protein band (30 kDa) by SDS-PAGE and was stained with Coomassie brilliant blue (Fig. 4). The final purity of the enzyme was considered to be >95%. The pI of IMP-1 was estimated to be >9.5, since no focus was observed between pHs ranging from 3.5 to 9.5. The kinetic parameters (K_m and relative V_{max}) and hydrolytic efficiencies (V_{max}/K_m) of IMP-1 against various β-lactams are given in Table 3. All of the K_m values for the substrates tested were quite low compared with those of the other metallo β-lactamases reported. IMP-1 hydrolyzed carbapenems detectably; however, the hydrolytic efficiencies for carbapenems are lower than those for cephems. Interestingly, the hydrolytic efficiency for aztreonam

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IMP-1   1 M----SK---------LSVFFIFLFCS-I-ATAAES---LPDLK----------IE
B. c.   1 MKNTLLK-----LGVCVSLLGITPFVSTISSVQAER---TVEHKVIKNETGTISIS
B. f    1 M----KT---------VFILISMLFPV--AVMAQKSVKISDDIS----------IT
A. h    1 M----MKGWMKCG---L-AGAVVLMAS--FWGGSVR---AAGMS----------LT

IMP-1  29 KLDEGVYVHTSFEEVNGWGVVPKHGLVVLVNAEAYLIDTPFTAKDTEKLVTWFVER
B. c   49 QLNKNVWVHTELGYFSG-EAVPSNGLVLNTSKGLVLVDSSWDDKLTKELIE-MVEK
B. f   32 QLSDKVYTYVSLAEIEGWGMVPSNGMIVINNHQAALLDTPINDAQTEMLVNWVTDS
A. h   34 QVSGPVYVVEDNYYVQENSMVYFGAKGVTVVGATW---TPDTARELHKLIKR-VSR

IMP-1  85 GY--KIKGSISSHFHSDSTGGIEWLNSRSIPTYASELTNELLKKD----------
B. c  103 KFKKRVTDVIITHAHADRIGGMKTLKERGIKAHSTALTAELAKKN----------
B. f   88 LH-AKVTTFIPNHWHGDCIGGLGYLQRKGVQSYANQMTIDLAKEK----------
A. h   86 K---PVLEVINTNYHTDRAGGNAYWKSIGAKVVSTRQTRDLMKSDWAEIVAFTRKG

IMP-1 128 ------GKVQATNSFS-GV-NYWLVKNKIEVFYPGPGHTPDNVVVWLPERKILFGG
B. c  148 ------GYEEPLGDLQ-SVTNLKFGNMKVETFYPGKGHTEDNIVVWLPQYQILAGG
B. f  132 ------GLPVPEHGFTDSL-TVSLDGMPLQCYYLGGGHATDNIVVWLPTENILFGG
A. h  139 LPEYPDLPLVLPNVVH-DG-DFTLQEGKVRAFYAGPAHTPDGIFVYFPDEQVLYGN

IMP-1 176 CFIK---PYGLGNLGDANIEAWPKSAKLLKSKYGKAKLVVPSHSEVGDASLLKLTL
B. c  197 CLVKSASSKDLGNVADAYVNEWSTSIENVLKRYGNINLVVPGHGEVGDRGLLLHTL
B. f  181 CMLKDNQATSIGNISDADVTAWPKTLDKVKAKFPSARYVVPGHGDYGGTELIEHTK
A. h  193 CILK---E-KLGNLSFADVKAYPQTLERLKAMKLPIKTVIGGHDSPLHGPELIDHY

                                                    Source
IMP-1 229 EQAVKGLNESKKPSKPSN  246 ------  Serratia  marcescens
B. c  253 DLLK               256 ------  Bacillus cereus
B. f  237 QIVNQYIESTSKP       249 ------  Bacteroides fragilis
A. h  245 EALIKAAPQS          254 ------  Aeromonas hydrophila
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FIG. 3. Alignment of amino acid sequences of metallo β-lactamases. The amino acid sequence of IMP-1 is compared with those of other metallo β-lactamases found in *B. cereus* (B. c [13]), *B. fragilis* (B. f [24]), and *A. hydrophila* (A. f [17]). Amino acid residues of each β-lactamase identical to those of IMP-1 are marked by asterisks, and conservative amino acid substitutions are marked by dots. Gaps are indicated by hyphens. Amino acid residues conserved among four enzymes are marked with capital letters above the alignments. Histidine and cysteine residues which probably work as zinc ligands (30) at the active center of IMP-1 are boxed.

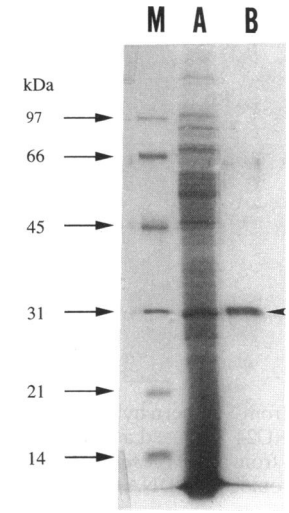


FIG. 4. Results from SDS-PAGE of purified IMP-1. Lanes: A, crude enzyme; B, purified IMP-1; M, molecular weight marker. The position of IMP-1 is indicated by an arrowhead.

was extraordinarily low compared with those for the other substrates tested. This is because of the low relative V_{max} of IMP-1 for aztreonam, although the K_m for aztreonam was similar to those of the other substrates.

Inhibition analyses of IMP-1 activity. The enzyme activity of IMP-1 was not detected in the Zn^{2+}-free phosphate buffer at all; therefore, the effect of each inhibitor was tested in phosphate buffer containing 100 μM $ZnCl_2$. The presence of 100 μM Hg^{2+}, Fe^{2+}, and Cu^{2+} ions in the reaction buffer inhibited IMP-1 activity by 100, 100, and 86%, respectively. Furthermore, the addition of 100 μM EDTA also resulted in a reduction in enzyme activity (85% inhibition). Although enzyme activity was decreased by the addition of EDTA, it was recovered by the addition of 100 μM $ZnCl_2$. However, 100 μM Ca^{2+} and Mg^{2+} failed to recover enzyme activity under the same experimental conditions. On the other hand, 10 μM clavulanate and cloxacillin, β-lactamase inhibitors, failed to decrease enzyme activity. Ca^{2+} or Mg^{2+} did not block IMP-1 activity at all. Moreover, enzyme activity was not decreased detectably after 30 min of heating at 65°C at 1 atm (101.29 kPa).

Southern hybridization analysis. The 0.5-kb *Hin*dIII fragment containing bla_{IMP}, which is shown in Fig. 1, was used as a hybridization probe, and it hybridized with the chromosomal position of *S. marcescens* TN9106 on the DNA blot as shown in Fig. 5.

Amino acid sequence of IMP-1. The sequence of 24 amino acid residues at the N terminus (from Ala-19 to Glu-42) of purified mature IMP-1 was determined directly as shown in Fig. 2. The total amino acid sequence deduced from the nucleotide sequence of the bla_{IMP} gene is also shown in Fig. 2. The amino acid sequence determined at the N terminus of mature IMP-1 corresponded to the amino acid sequence deduced from the 19th to 42nd residues, so a typical processing of the signal peptide occurs between Ala-18 and Ala-19 in the premature IMP-1 (Fig. 2). Comparisons among metallo β-lactamases are also shown in Fig. 3. Although the deletion of 16 amino acid residues was observed in IMP-1 compared with the sequence in *A. hydrophila*, IMP-1 showed considerable homology to the metallo β-lactamases of *B. cereus*, *B. fragilis*, and *A. hydrophila* (Table 4). Among them, the highest level of homology was observed with the β-lactamase of *B. cereus* (38.9%). Four amino acid resides, His-95, His-97, Cys-176, and His-215, which probably form zinc ligands at the active center of the enzyme (30), were conserved in the deduced amino acid sequence of IMP-1

TABLE 3. Hydrolysis of various β-lactam antibiotics by β-lactamase produced from pSMBNU24

Substrate	K_m (μM)	Relative V_{max}	Relative V_{max}/K_m
Ampicillin	2.15	100	100
Cephaloridine	7.74	29.5	8.2
Ceftazidime	1.24	16.1	27.8
Cefoperazone	2.13	54.7	55.2
Ceftizoxime	2.07	17.4	18.2
Moxalactam	7.55	47.4	13.5
Imipenem	7.33	6.9	1.9
Panipenem	2.07	2.7	2.8
Meropenem	0.74	1.0	3.0
Aztreonam	3.97	0.078	0.43

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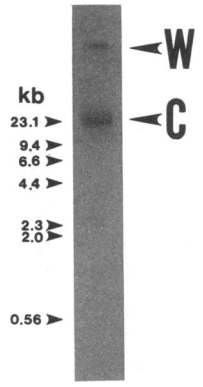


FIG. 5. Results from Southern hybridization. The 0.5-kb *Hin*dIII fragment of pSMBNU24 was used as a DNA probe. Chromosomal DNA was prepared from *S. marcescens* TN9106. C and W indicate the positions of chromosomal DNA and well, respectively, on the agarose gel.

(Fig. 3). Several amino acid residues, such as lysine (K), aspartic acid (D), and threonine (T), are also conserved generally among four metallo β-lactamases.

DISCUSSION

Carbapenems such as imipenem are quite stable agents in terms of withstanding the effects of chromosomally encoded or plasmid-mediated AmpC-type β-lactamases and extended-spectrum TEM-related β-lactamases. Therefore, imipenem is now widely used in Japan for the chemotherapy of infectious diseases caused by gram-negative bacteria. On the other hand, metallo β-lactamases found in some clinical isolates of *B. fragilis*, *A. hydrophila*, and *Xanthomonas malthophilia* are also becoming of great interest because they confer resistance to both carbapenems and newly developed broad-spectrum β-lactams. Among the species of the family *Enterobacteriaceae*, some strains of *S. marcescens* were also reported to produce a specific β-lactamase that provides imipenem resistance (35), but no molecular structure was shown. In this study, we have cloned and determined the nucleotide sequence of a metallo β-lactamase gene encoded by the chromosome of a clinically isolated imipenem-resistant strain of *S. marcescens*. The chromosomal genes could be transferred or exchanged among species belonging to the same bacterial family. Therefore, it is genetically possible for the metallo β-lactamase gene of *S. marcescens* to be disseminated to the other species of the family *Enterobacteriaceae* through conjugation or transduction. Furthermore, plasmid-mediated metallo β-lactamases have been reported in *B. fragilis* (3) and *Pseudomonas aeruginosa* (33). Thus, the increase in the frequency of isolation of gram-negative bacteria producing metallo β-lactamases which confer high levels of resistance to broad-spectrum β-lactams, including carbapenems, will be a threat in the near future.

TABLE 4. Matrix of amino acid sequence homologies among class B β-lactamases[a]

β-Lactamase source	% of amino acid sequence homology with β-lactamase from:		
	B. cereus	*B. fragilis*	*A. hydrophila*
IMP-1	38.9	35.9	22.1
B. cereus		30.0	23.5
B. fragilis			23.0

[a] β-Lactamases were derived from *S. marcescens* TN9106 (IMP-1 [this study]), *B. cereus* 5/B/6 (13), *B. fragilis* (24), and *A. hydrophila* (17).

The nucleotide sequences of the metallo β-lactamase genes in such species as *B. cereus* (13), *B. fragilis* (24), and *A. hydrophila* (17), which do not belong to the family *Enterobacteriaceae*, have been reported. Comparisons among these β-lactamases and IMP-1 of *S. marcescens* have revealed that IMP-1 may have an evolutionary history independent of those of the other metallo β-lactamases reported. However, the relatively lower G+C content of bla_{IMP} than those of the species average of *S. marcescens* still strongly suggests an extragenus origination of bla_{IMP} from an unknown organism with little G+C content. These results may suggest the clonal proliferation of a strain of *S. marcescens* which had acquired an imipenem resistance gene through lateral transfer of bla_{IMP} from an unknown source.

Massidda et al. proposed subclasses in class B β-lactamases (17). According to amino acid sequence analyses, a gap of 16 amino acid residues was found in the central region between the IMP-1 and the metallo β-lactamase of *A. hydrophila*. However, no such gap was observed among IMP-1 and metallo enzymes produced by *B. fragilis* and *B. cereus*. Furthermore, three histidine residues and a cysteine residue, which work as zinc ligands at the active center of the metallo enzymes of *B. cereus*, were also conserved in IMP-1. Therefore, it is concluded that IMP-1 has a much closer evolutionary relationship with the metallo β-lactamases produced by *B. fragilis* and *B. cereus* than it has with the β-lactamase produced by *A. hydrophila*.

The level of resistance of *S. marcescens* IMPS08 (pSMBNU2) to imipenem was not as high as that of parental strain TN9106, although multiple copies of bla_{IMP} existed in the transformant. The most likely explanation for this observation is the differences in the permeability of imipenem through the bacterial outer membrane. There have been several reports regarding the involvement of bacterial outer membrane permeability in imipenem resistance (15, 23). Changes in the relative ratio of outer membrane proteins, together with the production of metallo β-lactamase, may play an important role in the high level of imipenem resistance of *S. marcescens* TN9106. Analyses of the pattern and permeability of the outer membrane should be continued.

Enzymological analysis of IMP-1 demonstrated that this enzyme has K_m values for carbapenems lower than those of *S. marcescens* S6 metallo β-lactamase (35). Moreover, Sme-1 was weakly inhibited by clavulanate or sulbactam (5), while IMP-1 activity was not detectably inhibited by 10 μM clavulanate in the presence of 100 μM Zn^{2+}. CcrA was also hardly inhibited by the agents mentioned above (5). In this study, IMP-1 was highly purified from *E. coli* HB101 harboring a bla_{IMP}^{+} plasmid; however, the purity of Sme-1 was not shown. The most notable finding in terms of the substrate specificity of IMP-1 is the kinetic parameter against aztreonam. According to the results, the V_{max} and V_{max}/K_m values of IMP-1 for aztreonam are lower than those for the other agents tested, although the K_m is similar to those of the other agents. These results suggest that, although aztreonam has a binding affinity to the active center of IMP-1 similar to that of the other agents, it is poorly hydrolyzed by IMP-1. The MICs of aztreonam for the strains producing IMP-1 are also lower than those of the other broad-spectrum β-lactams.

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These findings may provide a clue for the improvement of new β-lactams. In other words, some derivatives of monobactams are potential candidates for treatment of infectious diseases caused by bacteria producing metallo β-lactamases.

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