ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2001, p. 2368–2371 Vol. 45, No. 8
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.8.2368–2371.2001

Identification of a Plasmid Encoding SHV-12, TEM-1, and a Variant of IMP-2 Metallo-β-Lactamase, IMP-8, from a Clinical Isolate of *Klebsiella pneumoniae*

JING-JOU YAN,[1] WEN-CHIEN KO,[2] AND JIUNN-JONG WU[3]*

Departments of Pathology[1] and Medicine,[2] National Cheng Kung University Medical Center, and Department of Medical Technology,[3] National Cheng Kung University Medical College, Tainan, Taiwan

Received 7 December 2000/Returned for modification 20 March 2001/Accepted 11 May 2001

A multidrug-resistant plasmid encoding TEM-1, SHV-12, and a variant of IMP-2 metallo-β-lactamase, designated IMP-8, was identified from a clinical isolate of *Klebsiella pneumoniae*. There are four nucleotide differences between bla_{IMP-2} and bla_{IMP-8}, resulting in two amino acid differences. bla_{IMP-8} was also found to be carried by an integron-borne gene cassette similar to the bla_{IMP-2} cassette.

The emergence of carbapenem-hydrolyzing metallo-β-lactamases in gram-negative bacteria has raised serious concern, since the enzymes usually possess a broad hydrolysis profile that includes carbapenems and extended-spectrum β-lactams (6, 8, 12, 13, 15). The genetic determinants of the metallo-β-lactamases are usually carried on mobile gene cassettes inserted in plasmid- or chromosome-borne integrons (1, 8, 13, 15). Since 1991, the IMP-1 metallo-β-lactamase has spread among isolates of various members of the family *Enterobacteriaceae, Pseudomonas aeruginosa*, and other nonfastidious, gram-negative nonfermenters in Japan (5, 6, 17, 18). Several novel metallo-β-lactamases have been described more recently. VIM-1 and VIM-2 were found to be produced by *P. aeruginosa* isolates from Italy (8) and France (13), respectively. IMP-2 was detected in an Italian isolate of *Acinetobacter baumannii* and was chromosomally encoded (15). IMP-3 was detected in a *Shigella flexneri* isolate in Japan and was plasmid mediated (7). Among these metallo-β-lactamases, only IMP-1 has been described in *Klebsiella pneumoniae* in Japan (5) and Singapore (T. H. Koh, G. S. Babini, N. Woodford, L.-H. Sng, L. M. C. Hall, and D. M. Livermore, Letter, Lancet **353:** 2162, 1999). In this report we describe a plasmid encoding TEM-1, the SHV-12 extended-spectrum β-lactamase, and a variant of the IMP-2 enzyme (designated IMP-8) from a clinical isolate of *K. pneumoniae*.

Bacterial strains and vectors. *K. pneumoniae* KPO787 was recovered from a central venous catheter tip that was removed from a patient with diabetes mellitus and acute pancreatitis at the intensive care unit of the National Cheng Kung University Medical Center in 1998. The isolate could be a colonizer, and the patient died of multiple organ system failure unrelated to the infection after 40 days of hospitalization. The recipient strain was *Escherichia coli* C600 (2), which is resistant to streptomycin. *E. coli* HB101 was the host for the cloning experiments (16). An *E. coli* C600 strain carrying a bla_{SHV-12}-containing plasmid (pEKPB657) that was transferred from a clinical isolate of *K. pneumoniae* was used for comparison in susceptibility tests (20). The cloning vectors used were pUC19 and pHP13 (4, 21). pHP13 is a bifunctional multicopy vector with erythromycin and chloramphenicol-resistance markers (4).

Susceptibility tests. MICs of antimicrobial agents were determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (11). The antibiotics used in the study were obtained from the following sources: amoxicillin, SmithKline Beecham Pharmaceuticals, Surrey, United Kingdom; aztreonam, Bristol-Myers Squibb, New Brunswick, N.J.; cefotaxime and cefuroxime, Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.; ceftazidime, Glaxo Group Research, Ltd., Greenford, United Kingdom; ceftriaxone, Hoffmann-La Roche, Inc., Nutley, N.J.; cefoxitin and imipenem, Merck Sharp & Dohme, West Point, Pa.; meropenem, Sumitomo Pharmaceuticals, Ltd., Osaka, Japan; cephalothin and cefaclor, Eli Lilly & Co., Indianapolis, Ind.; piperacillin, Lederle Laboratories, Pearl River, N.Y.; and streptomycin and chloramphenicol, Sigma Chemical Co., St. Louis, Mo.

Transfer of resistance determinants. Conjugation experiments were performed by broth mating as described previously (14, 20). Transconjugants were selected on tryptic soy agar plates supplemented with streptomycin (500 μg/ml) and ceftazidime (10 μg/ml).

Plasmid DNA preparation. Plasmid preparation was performed by the rapid alkaline lysis method (19). Plasmid DNA obtained from the transconjugant was restricted with *Eco*RI or *Pst*I (Roche Molecular Biochemicals, Mannheim, Germany), and its size was estimated by adding up the restriction fragment lengths.

Detection of the bla_{SHV} and bla_{TEM} genes. PCR was used to amplify the entire sequences of the bla_{SHV} and bla_{TEM} genes in plasmid preparations as described previously (20). The amplicons were purified with PCR clean up kits (Roche Molecular Biochemicals) and were sequenced on an ABI PRISM 377 sequencer analyzer (Applied Biosystems, Foster City, Calif.).

Cloning and sequencing of the bla_{IMP-8} gene. The resistance plasmid (pEKPO787) obtained from the transconjugant was digested with *Pst*I. A 5.6-kb fragment was cloned into vector

* Corresponding author. Mailing address: Department of Medical Technology, National Cheng Kung University Medical College, 1 University Rd., Tainan, Taiwan 70101. Phone: 886-6-2353535, ext. 5775. Fax: 886-6-2363956. E-mail: jjwu@mail.ncku.edu.tw.

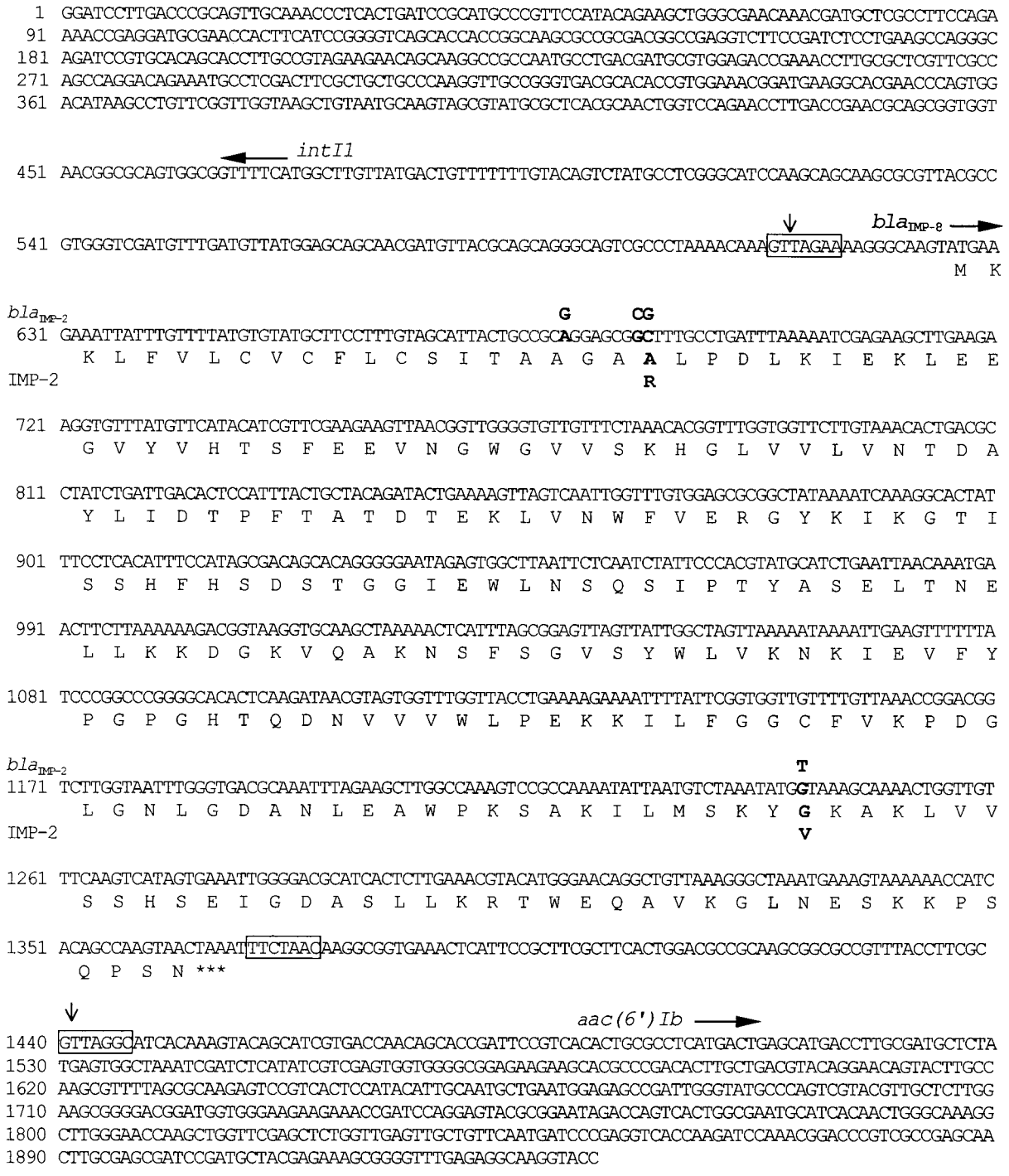

FIG. 1. Nucleotide sequence of the bla_{IMP-8} gene and flanking regions. The deduced amino acid sequence of bla_{IMP-8} is indicated in the single-letter code below the nucleotide sequence of bla_{IMP-8}. The start codons of *intII*, bla_{IMP-8}, and *aac(6′)Ib* are indicated by horizontal arrows, and the stop codon of bla_{IMP-8} is indicated by three asterisks. The nucleotides and amino acids that differ between bla_{IMP-8} and bla_{IMP-2} are marked by bold letters, and the nucleotides and amino acids for bla_{IMP-2} and IMP-2 that differ from the sequences for bla_{IMP-8} and IMP-8 are shown above and below the sequences. The bla_{IMP-8} cassette boundaries are indicated by vertical arrows. The conserved core and inverse core sites located at the bla_{IMP-8} cassette boundaries are boxed.

pUC19 (pEKPO787-1). The insert was further digested with *Bam*HI and *Kpn*I, and a 1.9-kb fragment was subcloned into vector pUC19 (pEKPO787D). Since there is no *Kpn*I site on pHP13, a 1.9-kb *Bam*HI-*Eco*RI fragment, which included the 1.9-kb *Bam*HI-*Kpn*I insert and the *Eco*RI site of pUC19, from pEKPO787D, was further ligated to pHP13, giving rise to pEKPO787D1. The insert was sequenced. Nucleotide and derived amino acid sequences were analyzed with the GCG pro-

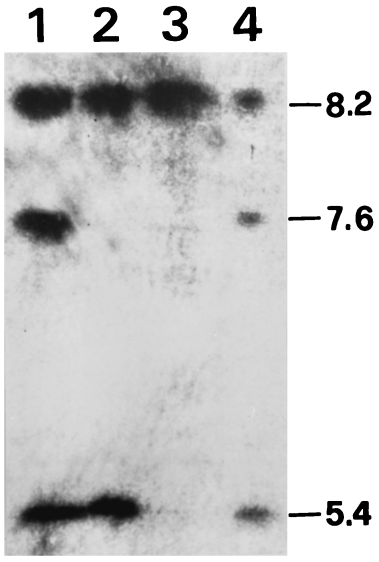

FIG. 2. Results of isoelectric focusing analysis. Lane 1, *K. pneumoniae* KPO787; lane 2, *E. coli* C600(pEKPO787); lane 3, *E. coli* HB101(pEKPO787D1); lane 4, *K. pneumoniae* KPT986, a clinical isolate known to produce TEM-1 (pI 5.4), SHV-1 (pI 7.6), and SHV-12 (pI 8.2) (18). The numbers on the right are pIs.

gram (Genetics Computer Group, Inc., Madison, Wis.). Related β-lactamases were identified by comparison with the GenBank and SWISS-PROT databases.

Isoelectric focusing of β-lactamases. Crude homogenates of β-lactamases were prepared as described previously (3). Isoelectric focusing analysis was performed by the method of Matthew et al. (10) as described previously (20).

An approximately 150-kb plasmid was successfully transferred from *K. pneumoniae* KPO787 to *E. coli* C600. PCR and sequence analyses showed that both the clinical isolate and its transconjugant carried SHV-12 and TEM-1.

The nucleotide sequence of a 1.9-kb fragment cloned into vector pUC19 and pHP13 contained an open reading frame of 741 nucleotides which corresponded to a putative protein of 246 amino acids (Fig. 1). The sequence of the gene is identical to that of bla_{IMP-2}, except for four nucleotide substitutions which resulted in two amino acid changes. Substitutions of a G for a C and a C for a G at nucleotide positions 61 and 62, respectively, and substitution of a G for a T at nucleotide position 617 resulted in the replacements of an Arg by an Ala and a Val by a Gly at amino acid positions 21 and 206, respectively, in the mature metallo-β-lactamase. The bla_{IMP-2}-related gene, now designated bla_{IMP-8}, was found to be flanked by nucleotide sequences identical to partial sequences of the *intII* and *aac(6')Ib* genes (Fig. 1), indicating that bla_{IMP-8} is also carried on an integron-borne gene cassette.

Isoelectric focusing revealed production of β-lactamases with pIs of 5.4, 7.6, and 8.2 by *K. pneumoniae* KPO787 (Fig. 2). The β-lactamases with pIs of 5.4 and 8.2 were transferred to *E. coli* C600. Thus, the pI 7.6 band probably represented the chromosomal SHV-1 β-lactamase of the *K. pneumoniae* strain (9). The *E. coli* HB101 strain transformed by pEKPO787D1 had a pI 8.2 band only, indicating that the pI of IMP-8 is 8.2, as is that of SHV-12.

The susceptibilities of *K. pneumoniae* KPO787, its transconjugant, and the transformant *E. coli* HB101 to various β-lactams are shown in Table 1. Compared with the quality control strain *E. coli* ATCC 25922 (11), the *K. pneumoniae* strain showed increased resistance to imipenem (MIC, 0.25 versus 8 μg/ml) and meropenem (MIC, 0.06 versus 16 μg/ml) and high-level resistance to all the other β-lactam agents tested. The resistance phenotype of the transconjugant is quite similar to that of the clinical isolate, except that the MICs of carbapen-

TABLE 1. MICs of β-lactams for strains tested[a]

Antibiotic(s)[b]	MIC (μg/ml)					
	KPO787	C600(pEKPO787)	HB101(pEKPO787D1)	C600(pEKPB657)	HB101(pUC19)	HB101
Amoxicillin	>256	>256	>256	>256	>256	4
Amoxicillin + CLA	>256	64	64	8	8	2
Piperacillin	>256	>256	16	64	256	1
Piperacillin + TZB	128	64	8	4	32	1
Cephalothin	>256	>256	>256	>256	256	2
Cefuroxime	>256	>256	>256	>256	8	0.5
Cefaclor	>256	>256	>256	>256	4	0.5
Cefotaxime	64	32	32	8	0.13	0.06
Cefotaxime + CLA	32	32	32	0.03	0.03	0.03
Ceftazidime	>256	>256	>256	128	0.25	0.13
Ceftazidime + CLA	>256	256	256	0.13	0.03	0.03
Ceftriaxone	64	32	32	16	0.13	0.13
Ceftriaxone + CLA	32	32	16	0.03	0.03	0.03
Cefoxitin	>256	>256	>256	4	4	2
Aztreonam	256	64	0.13	64	0.13	0.13
Aztreonam + CLA	0.13	0.03	0.03	0.03	0.03	0.03
Imipenem	8	2	2	0.13	0.13	0.13
Imipenem + CLA	2	2	2	0.03	0.03	0.03
Meropenem	16	2	1	0.13	0.13	0.06
Meropenem + CLA	2	1	0.5	0.03	0.03	0.03

[a] *K. pneumoniae* KPO787 and *E. coli* C600(pEKPO787) expressed IMP-7, SHV-12, and TEM-1. *E. coli* HB101(pEKPO787D1), C600(pEKPB657), and HB101(pUC19) expressed IMP-7, SHV-12, and TEM-1, respectively.

[b] CLA, clavulanic acid at a fixed concentration of 2 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.

ems were relatively low for the transconjugant, as was expected from previous findings (6). Unlike the clinical strain and its transconjugant, the transformant showed susceptibility to aztreonam.

IMP-2 was first reported on the chromosome of an *A. baumannii* strain in Italy (15). The bla_{IMP-2} gene shares an 88% nucleotide identity with bla_{IMP-1} and is carried by a gene cassette unrelated to the bla_{IMP-1} cassette. The bla_{IMP-8} gene found in this study is very closely related to bla_{IMP-2}, with only four nucleotide differences from bla_{IMP-2}. bla_{IMP-8} was also found to be carried by a gene cassette related to the bla_{IMP-2} cassette; however, in contrast to the bla_{IMP-2} cassette, the bla_{IMP-8} cassette was located on the plasmid, which would facilitate the spread of the resistance gene.

Production of IMP-8 in the *E. coli* transformant caused reduced susceptibilities to almost all β-lactams, including penicillins, cephalosporins, and carbapenems. Only aztreonam was unaffected, in agreement with the properties of other metallo-β-lactamases (6, 8, 12, 13, 15). Resistance to aztreonam in the *K. pneumoniae* isolate and its transconjugant should be due to the effects of SHV-12, the most prevalent type of extended-spectrum β-lactamase among clinical isolates of *K. pneumoniae* at the National Cheng Kung Medical Center (20). The bla_{IMP-8} containing integron was also found to carry an aminoglycoside resistance gene, *aac(6′)Ib*. Thus, antibiotics that can be used to treat infections with the microorganisms containing this multidrug-resistant plasmid are very limited. Since *K. pneumoniae* is a notorious host of resistance plasmids, the prevention of the spread of the multidrug-resistant plasmid and strain is critical and has become a big challenge to the hospital staff.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned accession no. AF322577.

This work was partially supported by grants NCKUH89-054 from National Cheng Kung University Hospital and NSC89-2320-B-006-149 from the National Science Council, Republic of China.

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