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KHM-1, a Novel Plasmid-Mediated Metallo-β-Lactamase from a *Citrobacter freundii* Clinical Isolate▽

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A novel gene, bla_{KHM-1}, encoding a metallo-β-lactamase, KHM-1, was cloned from a clinical isolate of *Citrobacter freundii* resistant to most β-lactam antibiotics. *Escherichia coli* expressing bla_{KHM-1} was resistant to all broad-spectrum β-lactams except for monobactams and showed reduced susceptibility to carbapenems. Recombinant KHM-1 exhibited EDTA-inhibitable hydrolytic activity against most β-lactams, with an overall preference for cephalosporins.

Acquired metallo-β-lactamases (MBLs) produced by gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter* spp., and several enterobacteria, confer resistance to all β-lactams except the monobactams (2). Acquired MBLs are categorized on the basis of amino acid sequences into various types (2, 23). The IMP- and VIM-type enzymes are the most common and are found worldwide (2, 8, 23). Recently, four additional types, SPM, GIM, SIM, and AIM, have been found in Brazil (21), Germany (3), Korea (11), and Australia (24), respectively. We report here on the detection of a novel acquired MBL in a clinical isolate of *Citrobacter freundii* identified in Japan.

C. freundii strain KHM243 was isolated in 1997 from a patient with catheter-associated urinary tract infection at Kyorin University Hospital (Tokyo, Japan). *Escherichia coli* K-12 strain W1895 was used as the recipient in conjugation experiments. *E. coli* JM109 (Takara Bio, Shiga, Japan) was used as the host for recombinant plasmids. Plasmid pHSG396 (Takara Bio) was used for the cloning of bla_{KHM-1} fragments.

Susceptibility to β-lactams was determined by the microdilution method (4). The production of MBL was detected by a double-disk synergy test with disks containing sodium mercaptoacetic acid (MBL production test; Eiken Chemical Co. Ltd., Tokyo, Japan), as described by Arakawa et al. (1).

The transfer of resistance by conjugation was analyzed as described previously (7). *E. coli* transconjugants were selected on Penassay broth agar (antibiotic medium no. 3; Becton Dickinson, Franklin Lakes, NJ) containing rifampin (200 μg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and moxalactam (16 μg/ml; Shionogi & Co., Ltd., Osaka, Japan). Plasmid DNA was extracted by an alkaline lysis procedure (9). Plasmid R100 (94.5 kb) (13) from *E. coli* CSH2, plasmid R478 (275 kb) (6) from *E. coli* J53, and three cryptic plasmids (200, 60, and 2.4 kb) from *Salmonella enterica* serovar Enteritidis L119 (15) were used as molecular size markers.

PCR analysis specific for class 1 integrons was performed as described previously (12). DNA sequences flanking bla_{KHM-1} were determined by inverse PCR (16). Briefly, plasmid DNA extracted from *E. coli* transconjugant W1895(pCF243) was digested with EcoRV or XspI (Takara Bio). Self-ligated digests were used as the template for an inverse PCR. The upstream and downstream flanking regions of bla_{KHM-1} were amplified by inverse PCR with two sets of primers: primers 5′-CGATA TAACAAGAGCTATTTTCAT-3′ and 5′-GGTATGCGCTG ACGATTC-3′ for the upstream region and primers 5′-GGTG TACAGATAAACGCCG-3′ and 5′-TTTATTTGGTGGCTG TTTTGTC-3′ for the downstream region.

The KHM-1 MBL from *E. coli* JM109(pKHM-1) was purified with HiTrap Q HP and Superdex 200 columns (GE Healthcare Bio-Sciences KK, Tokyo, Japan), as described by Franceschini et al. (5). During the purification procedure, the presence of β-lactamase activity was monitored with 100 μM nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). The protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Kinetic analysis was carried out in 50 mM phosphate buffer (pH 7.0) at 25°C with a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m and k_{cat} values and the k_{cat}/K_m ratio were determined by analyzing β-lactam hydrolysis under initial-rate conditions by use of the Lineweaver-Burk plot.

Antibiotic susceptibility testing showed that *C. freundii* KHM243 was resistant to most β-lactams and showed reduced susceptibility to carbapenems (Table 1). However, KHM243 was susceptible to monobactams (carumonam and aztreonam). The isolate was positive by the MBL production test (data not shown).

C. freundii KHM243 has two plasmids, one of approximately 70 kb and one of approximately 200 kb. A conjugation experiment was done with KHM243 and *E. coli* W1895. W1895 transconjugants that were resistant to β-lactams and that contained a 200-kb plasmid, designated pCF243, were obtained. The transconjugant exhibited a profile of susceptibility to β-lactams similar to that of KHM243, although the MICs for

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TABLE 1. MICs of β-lactams for *C. freundii* KHM243, *E. coli* W1895(pCF243) transconjugant, *E. coli* JM109(pKHM-1) expressing the KHM-1 MBL, and *E. coli* host strains

Antibiotic(s)[a]	MIC (μg/ml)				
	C. freundii KHM243	*E. coli* W1895 (pCF243)[b]	*E. coli* W1895	*E. coli* JM109 (pKHM-1)[c]	*E. coli* JM109
Ampicillin	256	64	8	16	1
Ampicillin-sulbactam	64	64	1	16	0.5
Ticarcillin	>512	>512	2	512	2
Ticarcillin-clavulanic acid	512	>512	4	512	2
Piperacillin	4	16	2	4	0.25
Cephaloridine	512	128	2	64	1
Cefuroxime	>512	>512	2	>512	8
Ceftazidime	>512	>512	0.125	>512	0.063
Cefotaxime	64	>512	0.008	128	0.004
Cefepime	32	>512	0.002	64	0.004
Cefozopran	16	256	0.016	64	0.008
Imipenem	2	4	0.063	0.5	0.063
Meropenem	4	4	0.004	4	0.004
Aztreonam	0.25	0.063	0.031	0.063	0.031
Carumonam	0.25	0.125	0.031	0.063	0.031
Cefoxitin	512	>512	8	>512	8
Cefmetazole	512	512	0.5	>512	0.25
Cefotetan	128	512	0.125	>512	0.031
Cefbuperazone	128	256	0.063	512	0.031
Cefminox	512	>512	0.125	512	0.25
Moxalactam	256	>512	0.063	>512	0.031
Flomoxef	64	256	0.031	128	0.031

[a] The ratio of the ampicillin to sulbactam was 2:1. The ratio of ticarcillin to clavulanic acid was 15:1.
[b] Natural plasmid carrying the bla_{KHM-1} gene.
[c] Recombinant plasmid constructed by insertion of DNA fragment containing the bla_{KHM-1} gene into the cloning vector pHSG396.

some cephalosporins, including cefotaxime, cefepime, and cefozopran, were significantly higher in the transconjugant than in KHM243 (Table 1).

EcoRI-digested fragments of pCF243 were subcloned into pHSG396 and were transformed into *E. coli* JM109 cells, and transformants were selected on agar medium containing moxalactam (1 μg/ml). Strain JM109 carrying the plasmid that conferred resistance to moxalactam, named pKHM-1, exhibited a profile of susceptibility to β-lactams similar to the susceptibility profiles of KHM243 and the *E. coli* W1895 transconjugant carrying pCF243 (Table 1). However, the MICs of some antibiotics, including cefotaxime and cefepime, were lower for the transformant carrying pKHM-1 than the transconjugant. This might be explained by insufficient expression of the gene due to insertion of the DNA fragment with a small 5′-flanking region.

pKHM-1 contained an 837-bp insert with a complete open reading frame (ORF) (data not shown). The 726-bp ORF encoded a putative protein of 241 amino acids. The protein was similar to MBLs, such as Uvs123 from an uncultured bacterium (82% identity) (22), IMP-1 (59% identity) (17), and SIM-1 (59% identity) (11) (Fig. 1). The protein was somewhat less similar to VIM-1 (38% identity) (10), GIM-1 (50% identity) (3), and SPM-1 (46% identity) (21) (Fig. 1). We named the ORF encoding the protein bla_{KHM-1} and designated the protein KHM-1 (Kyorin Health *S*cience MBL 1). bla_{KHM-1} was different from the *Citrobacter freundii* genome in its GC contents (GC contents, 50.27% and 44.63%, respectively) and codon usage (data not shown). KHM-1 contained amino acid motifs conserved in MBL enzymes, including a zinc-binding motif (HXHXD, residues 97 to 101) and three other residues involved in zinc binding (residues 159, 178, and 217) (Fig. 2) (18, 23).

The DNA sequences flanking bla_{KHM-1} were determined from 774 bp upstream to 806 bp downstream of it. No sequence homologies for site-specific cointegration events, ORFs, or transmissible elements was detected within the 774-bp upstream of bla_{KHM-1}. A 360-bp ORF encoding a putative protein of 119 amino acids with 77% identity to hypothetical protein VP1798 of *Vibrio parahaemolyticus* (14) was located in the fragment 21 to 380 bp downstream of bla_{KHM-1}. Strain KHM243 carried a class 1 integron with an array of two gene cassettes, which carried the *aadA2* (20) and *aac(6′)-Iae* (19) aminoglycoside resistance determinants; however, bla_{KHM-1} was not detected in this integron.

Analysis of the purified KHM-1 protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a single 25-kDa band. The activity of KHM-1 against various β-lactams was analyzed with the purified protein. It showed hydrolytic activity against all β-lactams tested except aztreonam (Table 2). Enzymatic activity against aztreonam was undetectable un-

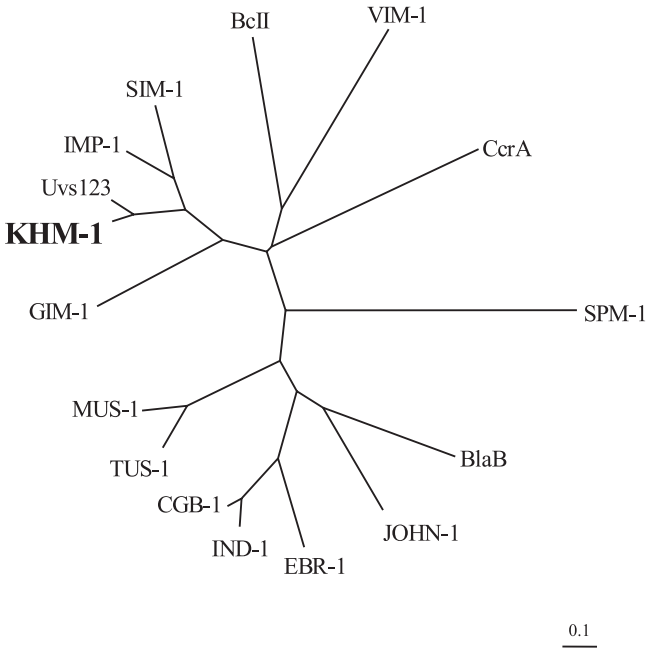

FIG. 1. Dendrogram showing the similarity of KHM-1 to other MBLs. KHM-1 and MBLs from a variety of organisms were tested. The dendrogram was created with the ClustalW program. Branch lengths correspond to the number of amino acid exchanges of the following MBL proteins (GenBank accession numbers, source organism) of BcII (P04190, from *Bacillus cereus*), BlaB (CAA65601, from *Elizabethkingia meningoseptica*), CcrA (P25910, from *Bacteroides fragilis*), CGB-1 (AAL55263, from *Chryseobacterium gleum*), EBR-1 (AAN32638, from *Empedobacter brevis*), GIM-1 (CAF05908, from *Pseudomonas aeruginosa*), IMP-1 (AAB30289, from *Serratia marcescens*), IND-1 (AAD20273, from *Chryseobacterium indologenes*), JOHN-1 (AAK38324, from *Flavobacterium johnsoniae*), MUS-1 (AAN63647, from *Myroides odoratimimus*), SIM-1 (AAX76774, from *Acinetobacter baumannii*), SPM-1 (CAD37801, from *P. aeruginosa*), TUS-1 (AAN63648, from *Myroides odoratus*), Uvs123 (AAP70377, from uncultured bacterium), and VIM-1 (CAB46686, from *P. aeruginosa*).

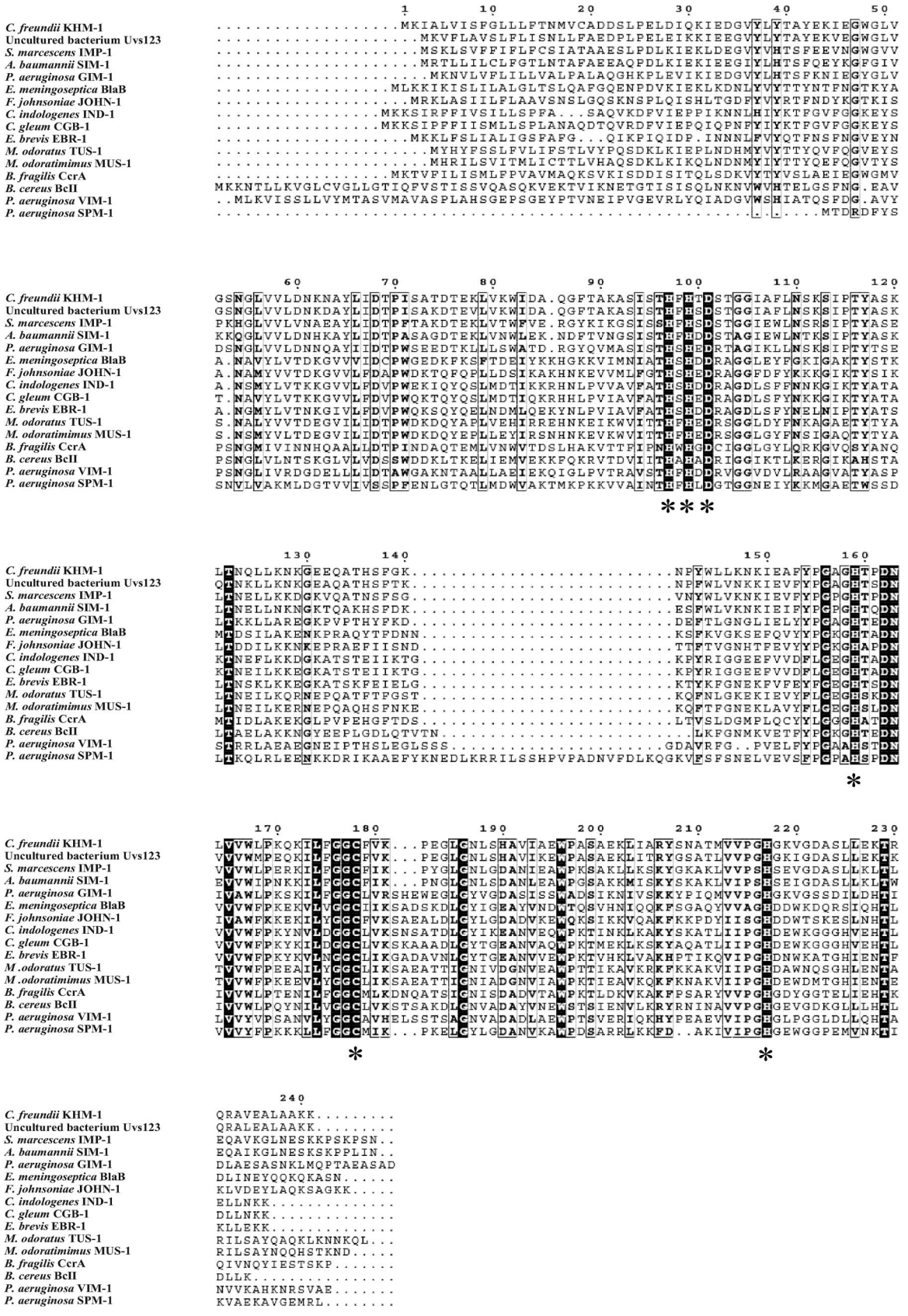

FIG. 2. Multiple-sequence alignments of the amino acid sequence of KHM-1 from *Citrobacter freundii* isolate KHM243 with those of other MBLs. The sequence sources are the same as those indicated in the legend to Fig. 1. Sequence comparison was performed by aligning the protein amino acid sequences by use of the ClustalW program (http://clustalw.ddbj.nig.ac.jp/top-e.html). The residues known to be involved in metal binding are indicated by asterisks. Identical residues are shaded.

TABLE 2. Kinetic parameters of β-lactamase KHM-1 with various substrates

Substrate	K_m (μM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Penicillin G	1,340 ± 56	23 ± 0.9	1.7×10^4
Ampicillin	978 ± 111	19 ± 2	1.9×10^4
Cephaloridine	4.4 ± 0.95	686 ± 12	1.6×10^8
Cefoxitin	81 ± 4	1,178 ± 164	1.4×10^7
Cefotaxime	13 ± 1.5	2,181 ± 208	1.7×10^8
Ceftazidime	8 ± 0.4	118 ± 3	1.5×10^7
Moxalactam	71 ± 8	2,794 ± 260	3.9×10^7
Aztreonam	—[b]	—	—
Meropenem	12 ± 3	0.4 ± 0.015	3.3×10^4
Imipenem	268 ± 53	15 ± 3	5.6×10^4

[a] The K_m and k_{cat} values represent the means of three independent experiments ± standard deviations.

[b] —, no hydrolysis was detected under conditions with a substrate concentration of up to 1 mM and an enzyme concentration of up to 840 nM.

der the experimental conditions adopted. This activity was inhibited by EDTA but was recovered by addition of Zn^{2+} (data not shown). The kinetic parameters, including K_m, k_{cat}, and the k_{cat}/K_m ratio, were determined for several different β-lactams (Table 2). Relatively higher values of the k_{cat}/K_m ratio ($>10^7$ $M^{-1} \cdot s^{-1}$), as a result of low values of K_m and high values of k_{cat}, were observed with the cephalosporins tested (cephaloridine, cefoxitin, cefotaxime, ceftazidime, and moxalactam); and lower values of the k_{cat}/K_m ratio ($<10^5$ $M^{-1} \cdot s^{-1}$) were observed with penicillin G, ampicillin, meropenem, and imipenem.

During 1997 and 1998, 104, 13, and 5 clinical isolates of *C. freundii*, *C. koseri*, and other *Citrobacter* spp., respectively, were collected in the hospital and were screened for imipenem resistance. Of these, four isolates of *C. freundii* showed reduced susceptibilities to imipenem (MICs, >8 μg/ml). However, bla_{KHM-1} was not detected in any of these isolates except the one from the patient infected with strain KHM243. A laboratory-based survey of other isolates of the family *Enterobacteriaceae* is in progress to detect bla_{KHM-1}.

Nucleotide sequence accession number. The nucleotide sequence data for bla_{KHM-1} and its flanking region from 774 bp upstream to 806 bp downstream reported here have been deposited in the EMBL/GenBank/DDBJ databases under accession number AB443628.

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