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Emergence of Oxacillinase-Mediated Resistance to Imipenem in *Klebsiella pneumoniae*

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***Klebsiella pneumoniae* strain 11978 was isolated in Turkey in 2001 and was found to be resistant to all β-lactams, including carbapenems. Cloning and expression in *Escherichia coli* identified five β-lactamases, including two novel oxacillinases. The β-lactamase OXA-48 hydrolyzed imipenem at a high level and was remotely related (less than 46% amino acid identity) to the other oxacillinases. It hydrolyzed penicillins and imipenem but not expanded-spectrum cephalosporins. The bla_{OXA-48} gene was plasmid encoded and not associated with an integron, in contrast to most of the oxacillinase genes. An insertion sequence, IS*1999*, was found immediately upstream of bla_{OXA-48}. Another plasmid that encoded a second oxacillinase gene, bla_{OXA-47}, located inside a class 1 integron was identified in *K. pneumoniae* 11978. OXA-47 had a narrow spectrum of hydrolysis activity and did not hydrolyze ceftazidime or imipenem, as is found for the β-lactamase (OXA-1) to which it is related. In addition, β-lactamases TEM-1 and SHV-2a were expressed from the same *K. pneumoniae* isolate. Analysis of the outer membrane proteins of this isolate revealed that it lacked a porin of ca. 36 kDa. Thus, the high-level resistance to β-lactams of this clinical isolate resulted from peculiar β-lactamases and modification of outer membrane proteins.**

Acquired resistance to expanded-spectrum cephalosporins has been reported in *Klebsiella pneumoniae* and is mainly related to the production of Ambler class A extended-spectrum β-lactamases (ESBLs) (20) and plasmid-encoded AmpC cephalosporinases (28). A few reports have described β-lactamase-mediated resistance to carbapenems in *K. pneumoniae* related to Ambler class B metalloenzymes, i.e., IMP-1 (15) and IMP-8 (38) from Southeast Asia. The plasmid-mediated Ambler class A β-lactamase KPC-1 has also been reported to be a source of carbapenem resistance in a *K. pneumoniae* isolate from North Carolina (39). In this case, modification of outer membrane proteins (OMPs) was shown to play an additional role in carbapenem resistance, as is found with AmpC-type enzymes (2, 7, 18, 19).

Among the Ambler class D enzymes, rare β-lactamases with weak carbapenem-hydrolyzing activities have been characterized. These are OXA-24, OXA-25, OXA-26, OXA-27, and OXA-40 from *Acinetobacter baumannii* isolates (1, 6, 12), whereas OXA-23 was found in *A. baumannii* and *Proteus mirabilis* (5, 10, 26). The OXA-23 and OXA-27 β-lactamases share 99% amino acid identity, whereas they share 60% identity with the amino acid sequences of a second group of these oxacillinases, consisting of OXA-24, OXA-25, OXA-26, and OXA-40, which differ by a few amino acid substitutions (12, 34).

In the present study we have characterized the β-lactamase content of a *K. pneumoniae* isolate that was resistant to all available β-lactams. Thus, we have identified a novel carbapenem-hydrolyzing oxacillinase with strong carbapenem-hydrolyzing activity.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *K. pneumoniae* 11978 was a clinical isolate from the Istanbul Faculty Hospital, Istanbul, Turkey. It was identified with the API 32GN system (bioMérieux, Marcy l'Etoile, France). *Escherichia coli* reference strain DH10B and plasmid pBK-CMV (Stratagene, Amsterdam, The Netherlands) were used for cloning experiments. *K. pneumoniae* CIP53153 and *E. coli* K-12 (a gift from P. Plésiat) were used as reference strains in endonuclease I-*Ceu*I digestion experiments. *K. pneumoniae* CIP53153 was also used in transformation experiments.

Antimicrobial agents and MIC determinations. The antimicrobial agents and their sources have been described elsewhere (29). Antibiotic-containing disks were used for detection of antibiotic susceptibility with Mueller-Hinton agar plates by a disk diffusion assay (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). MICs were determined by an agar dilution technique, as reported previously (31), and the results were interpreted in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (24).

Cloning experiments. Whole-cell DNA of *K. pneumoniae* 11978 was extracted as described previously (29). Cloning experiments were performed with *Bam*HI- or *Bss*HII-digested DNA of *K. pneumoniae* 11978 and *Bam*HI- or *Bss*HII-restricted plasmid pBK-CMV, followed by expression of recombinant plasmids in *E. coli* DH10B, as described previously (31). Antibiograms were performed with *E. coli* DH10B harboring recombinant plasmids, and the sizes of the plasmid inserts were determined by restriction analysis (35). Recombinant plasmids pVT-1 to pVT-5 were retained for further analysis. In addition, since part of the IS*1999* element was present in pVT-1, it was amplified from whole-cell DNA of *K. pneumoniae* 11978 by PCR with primers IS1999B and OXA-TKext (Table 1).

DNA sequencing and protein analysis. Both strands of the cloned DNA fragments were sequenced with an Applied Biosystems sequencer (ABI 373). The nucleotide and deduced protein sequences were analyzed with software available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Plasmid analysis and hybridizations. Extraction of plasmid DNA from *K. pneumoniae* 11978 and the electroporants was performed by the method of Kieser (14). Analysis was performed on a 0.7% agarose gel with ethidium bromide staining after migration. Southern transfer was performed on a nylon membrane (Hybond N^+; Amersham Pharmacia Biotech, Orsay, France) as described previously (33). The plasmid extract of *K. pneumoniae* 11978 was also

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TABLE 1. Sequences of primers designed for this study

Primer	Sequence (5′→3′)[a]	Location
KPC-1A	CGTTCTTGTCTCTCATGGCC	bla_{KPC-1}
KPC-1B	CCTCGCTGTGCTTGTCATCC	bla_{KPC-1}, reverse primer
OXA-48A	TTGGTGGCATCGATTATCGG	bla_{OXA-48}
OXA-48B	GAGCACTTCTTTTGTGATGGC	bla_{OXA-48}, reverse primer
OXA-TKext1	AATACACGCATAACGTCCCC	bla_{OXA-48}, 5′ reverse primer
OXA-1A	TCAACTTTCAAGATCGCA	bla_{OXA-47}
OXA-1B	GTGTGTTTAGAATGGTGA	bla_{OXA-47}, reverse primer
OXA-9A	TTCGTTTCCGCCACTCTCCC	bla_{OXA-9}
OXA-9B	ACGAGAATATCCTCTCGTGC	bla_{OXA-9}, reverse primer
SWSHV-A	AAGATCCACTATCGCCAGCAG	bla_{SHV}
SWSHV-B	ATTCAGTTCCGTTTCCCAGCGG	bla_{SHV}, reverse primer
TEMalpha	GAGTATTCAACATTTCCGTGTC	bla_{TEM}
TEMbeta	TAATCAGTGAGGCACCTATCTC	bla_{TEM} reverse primer
IS1999A	CAGCAATTCTTTCTCCGTG	IS1999 *tnpA*
IS1999B	CAAGCACAACATCAAGCGC	IS1999 *tnpA*, reverse primer
A	AGAGTTTGATCHTGGYTYAGA	16S RNA gene
B	ACGGYTACCTTGTTACGACTTC	16S RNA gene
5′CS	GGCATCCAAGCAGCAAG	*attI* recombination site
3′CS	AAGCAGACTTGACCTGA	*qacEΔ1*, 5′ end, reverse primer

[a] H stands for A, C, or T; Y stands for C or T.

used for transformation experiments with a Gene Pulser II electroporator (Bio-Rad, Ivry-sur-Seine, France) and selection on amoxicillin (100 μg/ml)-containing plates.

The endonuclease I-*Ceu*I (New England Biolabs, Saint-Quentin-en-Yvelines, France), which digests a 26-bp sequence in *rrn* genes for 23S large-subunit rRNA (17), was used to identify the genetic locations of the β-lactamase genes. After digestion, the DNA fragments were separated by pulsed-field gel electrophoresis (PFGE) with a CHEF-DRII apparatus (Bio-Rad) (11). The sizes of the endonuclease I-*Ceu*I-generated fragments were determined by comparison with those generated from *E. coli* K-12 (17). The I-*Ceu*I-generated DNA fragments of *K. pneumoniae* 11978 and CIP53513 and plasmid DNA of *K. pneumoniae* 11978 and the transformants analyzed by PFGE were transferred onto a nylon membrane by the Southern technique and subsequently UV cross-linked (Stratalinker; Stratagene) for 2 min. The membranes were successively hybridized with several probes, including a 743-bp PCR-generated probe specific for the bla_{OXA-48} gene (primers OXA-48A and OXA-48B [Table 1]), a 609-bp PCR-generated probe specific for the bla_{OXA-47} gene (primers OXA-1A and OXA-1B [Table 1]), a 300-bp PCR-generated probe specific for bla_{SHV} genes (primers SWSHV-A and SWSHV-B [Table 1]), a 850-bp PCR-generated probe specific for the bla_{TEM-1} gene (primers TEMalpha and TEMbeta [Table 1]), a 985-bp PCR-generated probe specific for the IS*1999* transposase gene (primers IS1999A and IS1999B [Table 1]), and a 1,504-bp PCR-generated probe specific for 16S and 23S rRNA genes (primers A and B [Table 1]). Southern hybridizations were performed with the ECL nonradioactive labeling and detection kit (Amersham Pharmacia Biotech) as described by the manufacturer.

IEF analysis. Isoelectric focusing (IEF) analysis was performed with an ampholine polyacrylamide gel (pH 3.5 to 9.5) as described previously (31) with culture extracts of *K. pneumoniae* 11978 and *E. coli* DH10B harboring recombinant plasmids.

β-Lactamase purification. A culture of *E. coli* DH10B harboring recombinant plasmid pVT-1 that produced OXA-48 was grown overnight at 37°C in 4 liters of tryptic soy broth containing 100 μg of amoxicillin per ml and 30 μg of kanamycin per ml. The protein extracts obtained were purified as described previously (32). Briefly, the extracts were subjected to purification steps, including ion-exchange chromatography with Q-Sepharose and 20 mM Tris-HCl buffer (pH 9.0), followed by chromatography with S-Sepharose columns and 100 mM sodium phosphate buffer (pH 5.8). Elution of the β-lactamase was performed with a K_2SO_4 gradient in order to prevent any potential inhibition by NaCl. Peaks of β-lactamase activity were concentrated by using Centrisart-C30 spin columns (Sartorius, Göttingen, Germany) and were dialyzed with 100 mM phosphate buffer (pH 7.0).

Kinetic studies. Purified β-lactamases were used for kinetic measurements, which were determined at 30°C in 100 mM sodium phosphate (pH 7.0) (34). The k_{cat} and K_m values were determined by analyzing β-lactam hydrolysis under initial-rate conditions with a UV spectrophotometer, as described previously (31). The 50% inhibitory concentrations (IC_{50}s) of clavulanic acid, tazobactam, sulbactam, and NaCl were determined as described previously (31). The specific activities of the protein extracts and purified β-lactamase from the culture of *E. coli* DH10B(pVT-1) were determined as described previously (3). The protein purification rate was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The protein content was measured by the Bio-Rad DC protein assay. The specific activity of the β-lactamase for imipenem was also determined by UV spectrophotometry (wavelength, 297 nm) with a culture extract of *K. pneumoniae* 11978 from 10 ml of tryptic soy broth with 100 μM imipenem, as described previously (3). One unit of enzyme activity was defined as the activity that hydrolyzed 1 μmol of imipenem per min per mg of protein.

OMP analysis. OMPs were isolated by sarcosyl extraction of total membrane preparations, as described previously (13). Briefly, cell cultures were harvested in logarithmic phase and lysed by sonication. OMPs were obtained after treatment of the cell membranes with sodium lauryl sarcosylate (Sigma, Saint-Quentin-Fallavier, France) and subsequent ultracentrifugation. The proteins were examined by SDS-PAGE on 10% polyacrylamide gels.

Nucleotide sequence accession numbers. The nucleotide sequences of bla_{OXA-48} and bla_{OXA-47} reported in this paper have been submitted to the EMBL/GenBank nucleotide sequence database and have been given accession nos. AY236073 and AY237830, respectively.

RESULTS

Origin of *K. pneumoniae* isolate and preliminary antibiotic susceptibility testing. *K. pneumoniae* 11978 was isolated in September 2001 at the Istanbul Faculty Hospital from the urinary tract of a 54-year-old man with a urinary tract infection and skin burns. An antibiotic regimen consisting of vancomycin and meropenem had been given for 1 month before strain isolation. Antibiotic susceptibility testing suggested an uncommon mechanism of resistance since the isolate was resistant to all β-lactams tested (Table 2). Addition of clavulanic acid and tazobactam did not significantly decrease the MICs of ticarcillin, piperacillin, and ceftazidime (Table 2). *K. pneumoniae* 11978 was also resistant to aminoglycosides, chloramphenicol, nalidixic acid, rifampin, sulfonamides, and tetracycline and of intermediate susceptibility to ciprofloxacin (data not shown). Culture extracts of *K. pneumoniae* 11978 analyzed by IEF gave five β-lactamases with pIs of 5.4, 7.2, 7.4, 7.6, and 7.7 (data not shown). To determine whether resistance to carbapenems was due to at least one of these β-lactamases, preliminary hydro-

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TABLE 2. MICs of β-lactams for *K. pneumoniae* 11978; *K. pneumoniae* CIP53153 harboring natural plasmid pA-1; *E. coli* DH10B harboring recombinant plasmids pVT-1, pVT-2, and pVT-3; *E. coli* DH10B harboring natural plasmid pA-1 alone or with pA-2; and *E. coli* DH10B and *K. pneumoniae* CIP53153 reference strains

β-Lactam(s)[a]	MIC (μg/ml)								
	K. pneumoniae 11978	*K. pneumoniae* CIP53153 (pA-1) (OXA-48)	*K. pneumoniae* CIP53153	*E. coli* DH10B (pA-1) (OXA-48)	*E. coli* DH10B (pA-1, pA-2) (TEM-1, SHV-2a, OXA-47, OXA-48)	*E. coli* DH10B (pVT-1) (OXA-48)	*E. coli* DH10B (pVT-2) (OXA-47)	*E. coli* DH10B (pVT-3) (SHV-2a)	*E. coli* DH10B
Amoxicillin	>512	>512	>512	>512	>512	>512	>512	>512	4
Amoxicillin + CLA	>512	128	4	>512	>512	>512	128	128	4
Ticarcillin	>512	>512	>512	>512	>512	>512	>512	>512	4
Ticarcillin + CLA	>512	128	2	>512	>512	>512	128	256	4
Piperacillin	>512	8	2	64	256	128	32	>512	2
Piperacillin + TZB	512	4	0.5	64	128	128	16	128	2
Cephalothin	>512	0.5	0.5	8	512	64	2	>512	4
Cefuroxime	256	0.12	0.12	8	8	8	4	512	4
Cefoxitin	128	2	2	4	8	8	1	32	4
Ceftazidime	512	0.12	0.12	0.12	64	0.12	0.06	>512	0.06
Ceftazidime + CLA	256	0.12	0.12	0.12	2	0.12	0.06	2	0.06
Cefotaxime	64	0.06	0.06	0.25	2	0.25	0.06	128	0.06
Cefepime	32	0.06	0.06	0.25	0.25	0.06	0.06	32	0.06
Cefpirome	128	0.12	0.06	0.5	0.5	0.25	0.12	32	0.06
Moxalactam	64	0.06	0.06	4	4	2	0.06	1	0.06
Aztreonam	512	0.06	0.06	0.06	64	0.06	0.06	>512	0.12
Imipenem	64	2	0.06	2	2	2	0.06	0.25	0.06
Meropenem	64	0.25	0.06	0.25	0.25	0.25	0.06	0.06	0.06

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

lysis experiments were performed with culture extracts of *K. pneumoniae* 11978, which showed significant imipenem-hydrolyzing activity (22 mU/mg of protein).

Cloning and sequencing of β-lactamase content. Cloning of *Bss*HII- or *Sau*3AI-restricted DNA of *K. pneumoniae* 11978 into pBK-CMV and expression in *E. coli* DH10B gave recombinant plasmids expressing different β-lactam resistance phenotypes. *E. coli* DH10B with recombinant plasmid pVT-1 had an oxacillinase (pI 7.2) phenotype, consistent with a carbapenem-hydrolyzing activity, which confers resistance to most penicillins and reduced susceptibility to imipenem (Table 2).

DNA sequence analysis of the 1.5-kb insert of pVT-1 identified a 798-bp open reading frame (ORF) for bla_{OXA-48} that encoded a 265-amino-acid protein (Fig. 1). A serine-threonine-phenylalanine-lysine tetrad (S-T-F-K) was found at positions 70 to 73 in the deduced protein encoded by this ORF. All four structural elements characteristic of Ambler class D β-lactamases (DBLs) were found: W-X-X-X-X-X-I-X at DBL positions 164 to 172, Q-X-X-X-L at DBL positions 176 to 180, K-T-G at positions 216 to 218, and YGN at DBL positions 144 to 146 (8, 16, 21). A K-T-G element (positions 216 to 218) was found, as in the carbapenem-hydrolyzing OXA-23 and OXA-27 β-lactamases, whereas it was replaced by a K-S-G motif in the second group of carbapenem-hydrolyzing oxacillinases, consisting of OXA-24, OXA-25, OXA-26, and OXA-40. The fourth structural element of oxacillinases, YGN, was not replaced by an FGN motif in OXA-48 as it was in the other carbapenem-hydrolyzing oxacillinases (34). OXA-48 was weakly related to other oxacillinases and shared 46, 36, 32, and 21% amino acid identities with OXA-10; OXA-23 and OXA-27; a cluster consisting of OXA-24, OXA-25, OXA-26, and OXA-40; and OXA-1, respectively (Fig. 2).

Part of insertion sequence element IS*1999* was found upstream of the bla_{OXA-48} gene in pVT-1 (Fig. 1). Since only 96 bp upstream of the bla_{OXA-48} was cloned in pVT-1, PCR experiments were performed with combinations of primers specific for the IS*1999* transposase gene and for the 5′ part of bla_{OXA-48} and with whole-cell DNA of *K. pneumoniae* 11978. An entire IS*1999* element was found. This element is also found upstream of the class A ESBL bla_{VEB-1} gene of *Pseudomonas aeruginosa* isolates from Thailand (23). The IS*1999* element that was located 26 bp upstream of the start codon of bla_{OXA-48} likely provided promoter sequences. Indeed, DNA sequences typical of −35 and −10 enterobacterial promoter regions were found inside the IS*1999* transposase-coding sequence (Fig. 1; P_{out}). No transposon or integron structure was found downstream of the bla_{OXA-48} gene. Thus, unlike most of the oxacillinase genes (21, 34), bla_{OXA-48} was not in the form of a gene cassette.

A second recombinant strain, *E. coli* DH10B(pVT-2), expressed another oxacillinase (pI 7.4) phenotype and was resistant to penicillins and β-lactamase inhibitors and susceptible to most cephalosporins and carbapenems (Table 2). Analysis of part of the 8-kb cloned fragment of pVT-2 identified the bla_{OXA-47} gene encoding a novel oxacillinase that had seven amino acid substitutions compared to the sequence of OXA-1, i.e., Pro34Gln, Thr65Ala, Thr98Ile, Lys191Arg, Glu200Asp, Asp208Glu, and Asn254Ser (DBL numbering [8]). None of these changes was located inside the conserved motifs of DBLs (16, 21). The bla_{OXA-47} gene was in the form of a gene cassette

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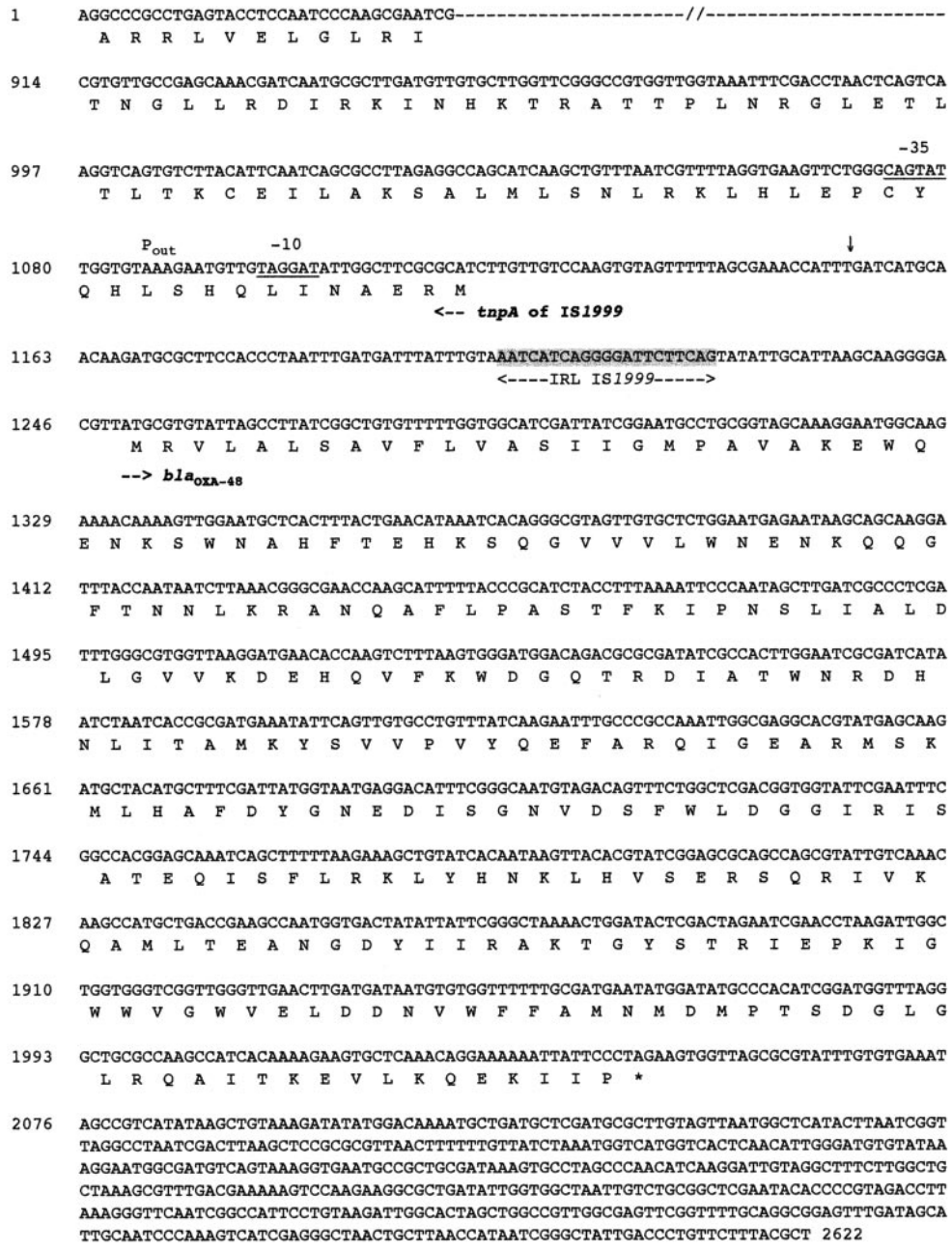


FIG. 1. Nucleotide sequence of a 2,622-bp fragment of recombinant plasmid pA-1 containing the bla_{OXA-48} gene. The deduced amino acid sequence is designated in single-letter code below the nucleotide sequence. The start codons of the ORFs are indicated by horizontal arrows. The left inverted repeat (IRL) of IS*1999* is shaded in gray, and the putative −35 and −10 promoter sequences (P_{out}) provided by the insertion sequence element are underlined. The *tnpA* transposase gene is indicated. The asterisk indicates the stop codon, and the vertical arrow indicates the *Sau*3AI cloning insertion site in pVT-1.

in a class 1 integron structure. The 59-bp-element sequence associated with this β-lactamase gene was 108 bp long and had a 1-bp change compared to the sequence of the *oxa-1* cassette (25). The *oxa-47* gene cassette was located downstream of an *aadA*-like gene cassette that was truncated in recombinant plasmid pVT-2 and downstream of a *qacF* gene cassette identical to that previously reported from *Enterobacter aerogenes* (30).

Another recombinant plasmid, pVT-3, gave an ESBL (pI 7.7) phenotype to *E. coli* DH10B due to the encoded β-lactamase SHV-2a. *E. coli* DH10B(pVT-3) was resistant to penicillins and expanded-spectrum cephalosporins but not to carbapenems (Table 2). Its activity was inhibited by clavulanic acid and tazobactam (Table 2). As is observed in *P. aeruginosa* RP-1 (22), the bla_{SHV-2a} gene was preceded by an IS*26* insertion sequence element that gave rise to a likely hybrid promoter

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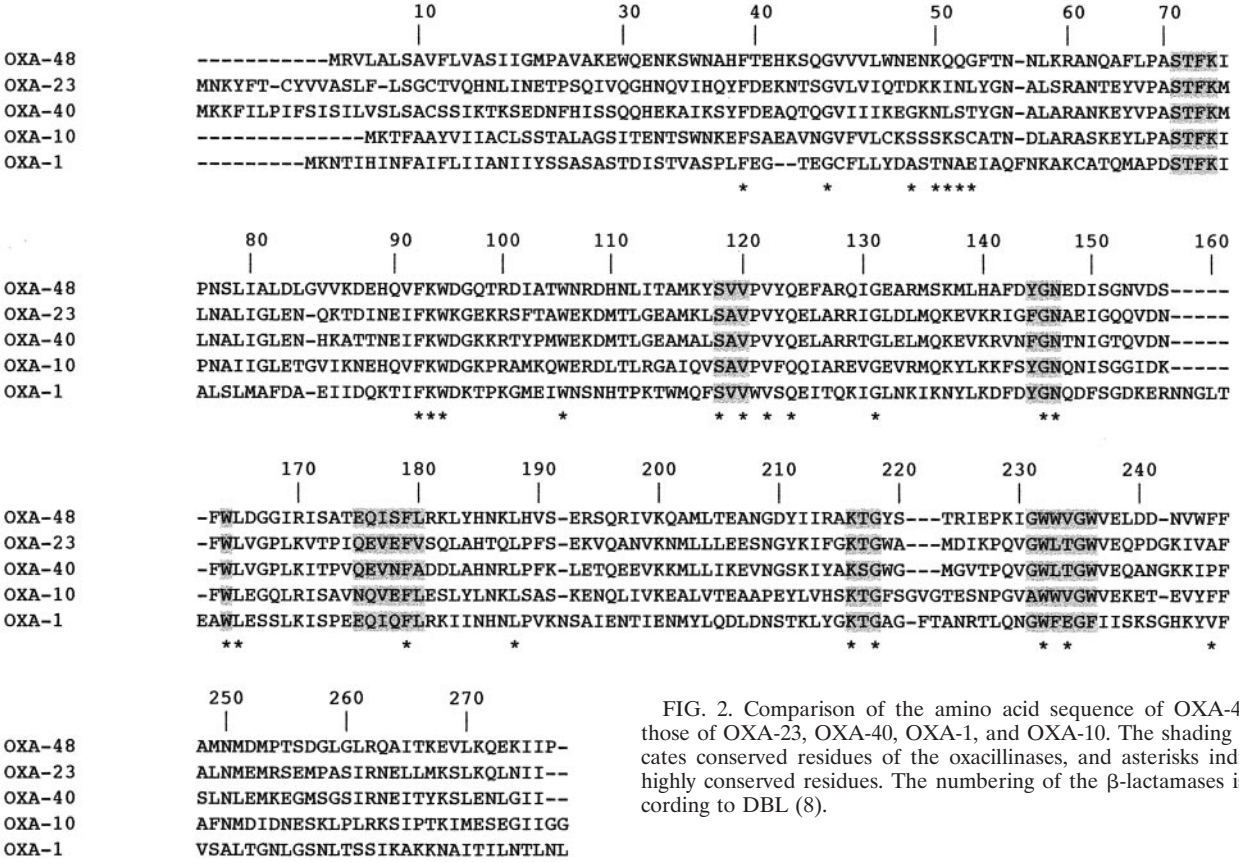


FIG. 2. Comparison of the amino acid sequence of OXA-48 to those of OXA-23, OXA-40, OXA-1, and OXA-10. The shading indicates conserved residues of the oxacillinases, and asterisks indicate highly conserved residues. The numbering of the β-lactamases is according to DBL (8).

with a −35 promoter sequence provided by the insertion sequence element and a −10 promoter sequence of the bla_{SHV-2a} gene (data not shown).

E. coli DH10B harboring recombinant plasmid pVT-4 had a penicillinase (pI 5.4) phenotype corresponding to TEM-1. The bla_{TEM-1} gene was preceded by a *tnpR* resolvase gene, which was preceded by the bla_{OXA-9} β-lactamase gene (data not shown). This structure was identical to that of Tn*1331*, a plasmid-located transposon that has also been identified in *K. pneumoniae* (36, 37). However, a single base substitution inside the coding sequence for bla_{OXA-9} resulted in a stop codon that truncated the last 163 carboxy-terminal amino acids. This observation was consistent with the β-lactam resistance phenotype of *E. coli* DH10B(pVT-4), which was susceptible to β-lactamase inhibitors (the lack of expression of oxacillinase OXA-9 likely had no effect on the β-lactam resistance phenotype).

The last recombinant plasmid, pVT-5, expressed a penicillinase (pI 7.6) phenotype in *E. coli* DH10B that corresponded to SHV-1. This β-lactamase gene corresponded to the naturally occurring SHV enzyme found in *K. pneumoniae* (4).

Biochemical properties of OXA-48 β-lactamase. After purification from culture extracts of *E. coli* DH10B(pVT-1), the specific activity of the OXA-48 β-lactamase against cephalothin was 17 U/mg of protein, and its purification factor was 75-fold. Protein purity was estimated to be >90% by SDS-PAGE analysis (data not shown). The OXA-48 β-lactamase had a narrow-spectrum hydrolysis profile that included penicillins, cephalothin, and imipenem and, to a lesser extent, cefotaxime, ceftazidime, cefepime, and cefpirome (Table 3). The catalytic activity (k_{cat}/K_m) of OXA-48 for imipenem (Table 3) was 10- and 3-fold higher than those reported for OXA-40 and the class A carbapenemase KPC-1, respectively (39). Hydrolysis of meropenem was detectable at a very low level, as is the case for the carbapenem-hydrolyzing β-lactamases OXA-24, OXA-25, and OXA-26 (1, 6). OXA-48 had hydrolytic activity against ceftazidime, although at a lower level than that of OXA-40 (Table 3). Oxacillin was significantly hydrolyzed by OXA-48, whereas it was slightly hydrolyzed by the other carbapenem-hydrolyzing oxacillinases, including OXA-40 (Table 3) (12).

Studies of activity inhibition, as measured by determination of IC_{50}s, showed that OXA-48 was weakly inhibited by clavulanic acid (IC_{50}, 16 μM), tazobactam (IC_{50}, 1.7 μM), and sulbactam (IC_{50}, 50 μM), as has been found for most of the oxacillinases (24). Nevertheless, these IC_{50}s were lower than those reported for OXA-40 (12). OXA-48 activity was inhibited by NaCl (IC_{50}, 7 mM), which is a property commonly observed for oxacillinases except those possessing an FGN motif at DBL positions 144 to 146 (12).

Genetic support of the β-lactamase genes. Analysis of plasmid extracts of *K. pneumoniae* 11978 identified a 70-kb plasmid (pA-1) and a 140-kb plasmid (pA-2) (data not shown). After electroporation into *E. coli* DH10B, three β-lactam-resistant phenotypes were obtained. Transformants contained either pA-1 or pA-2, or both. *E. coli* DH10B(pA-1) expressed an oxacillinase phenotype with reduced susceptibility to imipenem

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TABLE 3. Kinetic parameters for purified β-lactamase OXA-48 from *K. pneumoniae* 11978 compared to those for OXA-40 from *A. baumannii* CLA-1[a]

Substrate	OXA-48			OXA-40[b]		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Benzylpenicillin	245	40	6,100	5	23	220
Ampicillin	340	5,200	65	5	220	20
Ticarcillin	45	55	820	1	60	20
Piperacillin	75	410	180	1	23	50
Cephalothin	3	20	150	3	72	50
Cephaloridine	2	27	75	5	1,000	5
Cefotaxime	10	190	60	ND[c]	—[d]	—
Ceftazidime	4	5,100	1	20	2,500	10
Cefepime	1	160	6	ND	—	—
Cefpirome	8	390	20	ND	—	—
Oxacillin	25	30	850	2	876	3
Aztreonam	ND	—	—	ND	—	—
Imipenem	2	14	145	0.1	6.5	15
Meropenem	0.1	200	0.5	ND	—	—

[a] Data are means of three independent experiments. Standard deviations were within 10% of the means.
[b] Data are from reference 12.
[c] ND, no detectable hydrolysis (<0.01 s^{-1}).
[d] —, not determinable.

(Table 2), since pA-1 had the bla_{OXA-48} gene. *E. coli* DH10B(pA-2) expressed an ESBL phenotype that corresponded to SHV-2a (data not shown). PCR experiments indicated that *E. coli* DH10B(pA-2) possessed the bla_{SHV-2a}, bla_{OXA-47}, and bla_{TEM-1} genes and a nonexpressed bla_{OXA-9} gene. Transformants harboring pA-1 and pA-2 expressed a broad-spectrum β-lactam resistance phenotype, including resistance to ceftazidime and aztreonam and reduced susceptibility to imipenem (Table 1).

Although most of the β-lactamase genes identified were located on plasmids, the I-*Ceu*I digestion technique was used to search the *K. pneumoniae* 11978 chromosome for an additional chromosomal location of these novel bla_{OXA-48} and bla_{OXA-47} genes. Whereas a DNA probe specific for rRNA genes hybridized with five of six fragments of whole-cell DNA of *K. pneumoniae* 11978 generated with I-*Ceu*I, the bla_{OXA-48}- and bla_{OXA-47}-specific probes did not hybridize with any of these fragments, indicating that these genes are located only on plasmids.

OMPs of *K. pneumoniae* 11978. Because natural plasmid pAT-1 conferred decreased susceptibility to imipenem once it was transformed into *E. coli* DH10B, this plasmid was also used to transform reference strain *K. pneumoniae* CIP53153 to evaluate its effect on the susceptibility of a wild-type *K. pneumoniae* strain. Similar β-lactam MICs were obtained for the reference strain, indicating that the type of species of the family *Enterobacteriaceae* by itself does not explain the high imipenem MIC for *K. pneumoniae* 11978 (Table 2). OMP analysis of *K. pneumoniae* 11978 was performed, and the results were compared to that for a *K. pneumoniae* reference strain. *K. pneumoniae* 11978 lacked a 36-kDa porin, whereas the 35- and 37 kDa porins were found in cell extracts of both strains in the form of a doublet (Fig. 3).

DISCUSSION

A novel carbapenem-hydrolyzing oxacillinase, OXA-48, that was weakly related to other oxacillinases, including those with carbapenem-hydrolyzing activities, was identified. The OXA-48 β-lactamase significantly hydrolyzed carbapenems at a level much higher than those of the other carbapenem-hydrolyzing oxacillinases and at a level similar to that of the class A carbapenem-hydrolyzing β-lactamase KPC-1 (39).

Unlike OXA-23 to OXA-27 and OXA-40, OXA-48 pos-

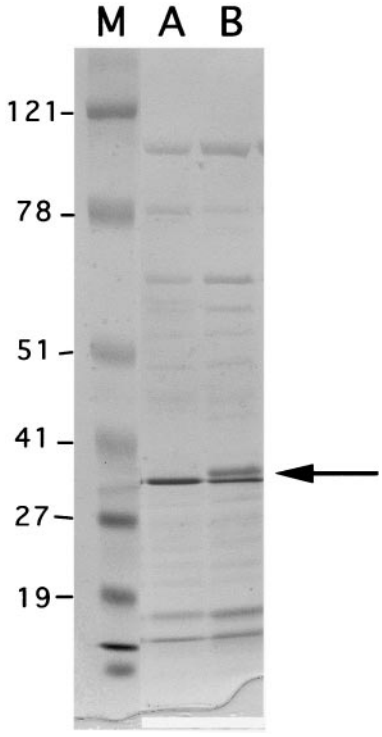


FIG. 3. OMP profiles of *K. pneumoniae* 11978 (lane A) and reference strain *K. pneumoniae* CIP53153 (lane B). Lane M, molecular size marker, with sizes (in kilodaltons) indicated on the left. The arrow indicates the position of the 36-kDa protein that was missing from *K. pneumoniae* 11978, whereas the 35- and 37-kDa proteins likely appeared in the form of a doublet.

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sesses the classical YGN motif of oxacillinases instead of an FGN motif at DBL positions 144 to 146, confirming that a Phe residue at DBL position 144 is not required by itself to provide carbapenem hydrolytic activity (12). The inhibition of OXA-48 activity by NaCl confirmed that this inhibition property is related to a Tyr residue at DBL position 144, whereas the other carbapenem-hydrolyzing oxacillinases, which have a Phe residue at this position, are resistant to inhibition by NaCl (12).

The plasmid-encoded $bla_{\text{OXA-48}}$ gene was located just downstream of IS*1999*, which likely provided promoter sequences and which may also have played a role in the process of mobilization of this β-lactamase gene. This is the first report of a tight association between an insertion sequence element and an oxacillinase gene. This observation raises the question of the origin of IS*1999*, which has so far been found in association with the class A ESBL $bla_{\text{VEB-1}}$ gene in *P. aeruginosa* in Thailand and *A. baumannii* in France (23; personal communication). Like the other carbapenem-hydrolyzing oxacillinase genes, $bla_{\text{OXA-48}}$ was not present in the form of a gene cassette in a class 1 integron, as opposed to most of the oxacillinase genes. This result again raises the question of the origin of these peculiar oxacillinase genes.

Five additional β-lactamase genes were identified in the same *K. pneumoniae* isolate, providing further confirmation that a single isolate can carry multiple β-lactamase genes. Two studies reported that expression of the SHV-2 ESBL in *K. pneumoniae*, which was associated with reduced porin production, may contribute to cephalosporin resistance and reduced susceptibility to imipenem (9, 18). On the other hand, Martinez-Martinez et al. (19) did not observe a significant increase in the imipenem MIC when the SHV-2, SHV-3, or SHV-5 ESBL was expressed in a *K. pneumoniae* mutant from which OmpK36 was deleted, whereas the imipenem MIC increased significantly for a mutant from which OmpK36 was deleted and which carried an AmpC β-lactamase. Although OXA-48 contributed to the carbapenem resistance in *K. pneumoniae* 11978, OMP deficiency may also have participated by further raising the level of carbapenem resistance.

This study is the second description of a carbapenem-hydrolyzing oxacillinase in a member of the family *Enterobacteriaceae*, after that of OXA-23 in *P. mirabilis* (5). Interestingly, the level of imipenem resistance conferred by OXA-48 in *E. coli* is similar to that reported for several class B enzymes. It would be interesting to estimate the prevalence of this novel β-lactamase gene in Turkey, where the OXA-48-positive isolate originated, since gram-negative bacteria in Turkey have a high level of resistance to β-lactams (27).

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