An Ertapenem-Resistant Extended-Spectrum- β -Lactamase-Producing *Klebsiella pneumoniae* Clone Carries a Novel OmpK36 Porin Variant[▽]

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Received 11 September 2009/Returned for modification 11 December 2009/Accepted 16 July 2010

Carbapenem-resistant *Klebsiella pneumoniae* caused an outbreak in a hospital in Rome, Italy. The clinical isolates were tested by antimicrobial susceptibility testing, pulsed-field gel electrophoresis, multilocus sequence typing, plasmid typing, and β -lactamase identification. The OmpK35 and OmpK36 porins were analyzed by SDS-PAGE, and their genes were amplified and sequenced. Complementation experiments were performed using a recombinant unrelated *ompK36* gene. An ertapenem-resistant and imipenem- and meropenem-susceptible clone was identified and assigned to the sequence type 37 lineage by MLST; it carried SHV-12 and CTX-M-15 ESBLs, did not produce the OmpK35 due to a nonsense mutation, and expressed a novel OmpK36 variant (OmpK36V). This variant showed two additional amino acids located within the L3 internal loop, one of the highly conserved domains of the protein. Two isolates of the same clone also exhibited resistance to imipenem and meropenem, due to the loss of OmpK36 expression by a nonsense mutation occurring in the *ompK36V* variant gene. These were the first carbapenem-resistant *K. pneumoniae* isolates identified within the hospital. Screening for the *ompK36V* gene of unrelated *K. pneumoniae* isolates derived from patients from 2006 to 2009 demonstrated the high frequency of this gene variant as well as its association with ertapenem resistance, reduced susceptibility to meropenem, and susceptibility to imipenem.

The susceptibility to carbapenems in the *Enterobacteriaceae* family is no longer guaranteed since the increasing occurrence of carbapenem-resistant isolates (26, 38). Carbapenem resistance can arise by acquisition of resistance genes encoding metallo- β -lactamases (MBLs) and non-metallo-carbapenemases (of the KPC, GES, or OXA type) but has also been associated with the AmpC cephalosporinase or extended-spectrum β -lactamase (ESBL) production and alterations of the expression of the major nonspecific porins. The latter mechanism has been reported worldwide in clinical isolates of *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter* spp., and others (11, 12, 14–18, 23, 24, 31, 35). The alteration of porin expression may be caused by several different events, including the disruption of the gene by insertion sequences, the termination of translation by nonsense mutations, and the down-regulation of transcription by mutations occurring within the promoter (12, 18, 29). In particular, the effect of the alteration of OmpK35 and OmpK36 expression in ESBL-producing *K. pneumoniae* isolates has been demonstrated to confer a high level of ertapenem resistance, with the MICs of imipenem and meropenem being raised (11, 12, 14, 16, 17, 24, 39).

Sequence analysis of the *ompK36* genes of clinical isolates of *Enterobacter aerogenes*, *E. coli*, and *K. pneumoniae* demonstrated the importance of transmembrane β -strand loop 3 (L3) in the specific uptake of β -lactams (3, 8, 36). The L3 loop

constitutes the channel eyelet of enterobacterial porins, extends inside the barrel, and constricts the pore (1). The mutations occurring in L3 drastically change the channel properties, reducing the conductance of the channel and the uptake of carbapenems and cephalosporins (3, 8, 36). In particular, the ertapenem has a net negative charge, its two carboxylic groups are able to form hydrogen bonds with the basic residues of the channel, and OmpK36 and OmpK35 represent the main pathway for the penetration of this drug into the bacterial periplasmic space (17, 19).

In the present study, two carbapenem-resistant *K. pneumoniae* strains producing ESBL and lacking the OmpK35 and OmpK36 porins were derived from a colonized patient at the University Hospital Policlinico Umberto I in Rome, Italy. *K. pneumoniae* clonal isolates showing ertapenem resistance and increased MICs of meropenem but susceptibility to imipenem were also isolated from the same patient. These strains lacked the OmpK35 porin but produced normal levels of the OmpK36 porin and were further characterized, revealing a particular OmpK36 porin variant. This porin variant was found to be widely distributed in unrelated *K. pneumoniae* strains within the hospital, and in association with ESBL production and the OmpK35 depletion, the variation conferred a characteristic ertapenem-resistant (ETP^r) phenotype which is different from that observed for carbapenem-resistant strains lacking both the OmpK35 and OmpK36 porins.

MATERIALS AND METHODS

Strains and antimicrobial susceptibility testing. The *K. pneumoniae* strains analyzed in this study were isolated and identified at the Microbiology Laboratory of the Policlinico Umberto I Hospital in Rome by routine procedures.

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[▽] Published ahead of print on 26 July 2010.

Antimicrobial susceptibility was determined by the Vitek 2 system with the AST-N089 card (bioMérieux, Marcy l'Etoile, France). The MICs of piperacillin-tazobactam (TZP), aztreonam (ATM), cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IPM), and meropenem (MEM) were determined by Etest on Mueller-Hinton agar II (bioMérieux, Marcy l'Etoile, France). The MICs of ETP were determined by a microdilution technique in Mueller-Hinton broth (7). ESBL production was confirmed by cefotaxime/cefotaxime-clavulanic acid (CT/CTL) ESBL strips (AB bioMérieux, Solna, Sweden) on Mueller-Hinton agar II. Breakpoints and MICs were interpreted in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

Screening tests for β -lactamases and ESBL gene identification. β -Lactamase extracts were obtained by ultrasonic treatment of bacterial cells suspended in phosphate buffer (0.1 M, pH 7.0) and clarified by centrifugation. Analytical isoelectric focusing (IEF) was performed in polyacrylamide gels containing ampholytes (pH range, 3.5 to 9.5; APBiotec, Piscataway, NJ). β -Lactamases were detected with nitrocefin (0.2 mg/ml; Oxoid Ltd., Basingstoke, United Kingdom). Detection of carbapenemase activity in the cell-free β -lactamase extracts was performed by a bioassay, as described previously (42), by using *E. coli* DH5 α (Invitrogen, Milan, Italy) as a susceptible indicator strain and imipenem (10- μ g) and meropenem (10- μ g) discs (Oxoid Ltd.).

PCR amplification for detection of the *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}, *bla*_{OXA} (including *bla*_{OXA-48}), *bla*_{KPC}, *bla*_{GES}, and *ampC* genes was carried out as described previously (2, 6, 28, 32–34, 40, 41). The PCR products were purified by a Wizard PCR preps DNA purification system (Promega, Madison, WI), sequenced, and analyzed at the <http://www.ncbi.nlm.nih.gov> website by the Basic Local Alignment Search Tool (BLAST) program.

Plasmid localization of ESBL genes. Plasmid DNA was purified with a plasmid midikit (Qiagen Inc., Milan, Italy), separated by 0.8% agarose gel electrophoresis, and blotted onto a positively charged nylon membrane (Roche Diagnostics, Monza, Italy) by standard procedures. Filters were hybridized with digoxigenin-labeled (PCR DIG probe synthesis kit; Roche Diagnostics GmbH, Mannheim, Germany) *repF*, *bla*_{CTX-M-15}, and *bla*_{SHV} amplicons, obtained with primers described previously (2, 5, 6).

Plasmids were transferred by transformation (MAX Efficiency DH5 α chemically competent *E. coli* cells; Invitrogen) and conjugation (the rifampin-resistant *E. coli* CSH26 was used as the recipient strain). Selection of the transformants and transconjugants was performed on LB agar plates containing ampicillin (100 μ g/ml) and ampicillin plus rifampin (100 μ g/ml) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), respectively. Plasmids were typed by the PCR-based replicon typing method (PBRT), as described previously (5).

Clone genotyping by PFGE and MLST. The genetic relatedness of the isolates was assessed by pulsed-field gel electrophoresis (PFGE) analysis of XbaI-digested genomic DNA, as described previously (37). Electrophoresis was for 30 h at 14°C with pulse times ranging from 5 to 35 s at 6 V/cm. Multilocus sequence typing (MLST) was carried out with the *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB* genes, as described previously (10). Sequence types (STs) were assigned at the <http://www.pasteur.fr/recherche/genopole/PF8/mlst/> website.

Examination of porins. Outer membrane protein (OMP) preparations were obtained after sonication of bacterial cells grown in Mueller-Hinton broth, followed by selective solubilization of the cytoplasmic material with sodium *N*-lauroyl sarcosinate (2% wt/vol) and ultracentrifugation (30). The preparations were run on discontinuous SDS-polyacrylamide gels (12.0%) and stained with Coomassie blue. The coding sequences of the *ompK36* and *ompK35* genes from *K. pneumoniae* were amplified and sequenced using previously described primers (21, 31).

Restoration of OmpK36. Plasmid pSHA2, encoding a wild-type OmpK36 protein (30), was introduced into the *K. pneumoniae* cells by electroporation, and transformants were selected on LB agar plates containing ampicillin (50 μ g/ml) and potassium tellurite (30 μ g/ml) (Sigma-Aldrich Chemie GmbH). Expression of the cloned porin was analyzed by SDS-PAGE. The MICs for the transformants were measured as described above.

Amplification of the *ompK36V* gene variant. Primers *ompK36V* Fw (5'-ATG AAAGTTAAAGTACTGTC-3') and *ompK36V* Rv (5'-CGTAGGTGTGCGGTG TC-3') were designed to detect strains carrying the *ompK36V* gene.

DNA sequencing. Amplicons were sequenced by the fluorescent dye-labeled dideoxynucleotide method with an ABI 3730 automatic DNA sequencer (Perkin-Elmer, Foster City, CA). The sequences from multiple PCR products were determined in both directions.

Nucleotide sequence accession numbers. The nucleotide sequences of the *ompK35* and *ompK36* genes were released under EMBL accession nos. GU460159, GU460160, GU460161, and GU460162 and nos. GQ433376 and GQ433377, respectively.

RESULTS AND DISCUSSION

Case description. On 13 May 2008, a 74-year-old man (patient A) was admitted to the University Hospital Policlinico Umberto I in Rome for ischemic heart disease. On day 10 after hospital admission, he underwent placement of an aortic-coronary bypass. During the postsurgery period, he required tracheostomy and prolonged mechanical ventilator support. His clinical course was also complicated by acute renal insufficiency requiring continuous renal replacement therapy. On 9 June, he underwent reconstructive surgical closure of a dehiscence sternal wound with pectoral muscle flaps. On 15 July, a second dehiscence on the sternal wound with coinfection by *Staphylococcus epidermidis* and *Enterococcus faecalis* required reconstructive surgery and treatment with piperacillin-tazobactam (2.25 g intravenously every 6 h for 3 days) plus linezolid (600 mg intravenously every 12 h for 21 days). On 3 September, the patient developed a sepsis syndrome with acute renal insufficiency due to *Pseudomonas aeruginosa* mediastinitis. After surgical debridement, a 4-week course of treatment with meropenem (1 g intravenously every 8 h) was administered and cured the infection. On the following days, the patient required additional antibiotic treatment with meropenem (1 g intravenously every 8 h) due to recurrent ventilator-associated respiratory tract infections caused by a multiple-antibiotic-resistant *P. aeruginosa* strain and sternal wound infections from which *P. aeruginosa* and ESBL-producing and carbapenem-susceptible strains of *E. coli* and *K. pneumoniae* (strain 16855) were isolated on 30 October. On 3 November an ETP^r and meropenem- and imipenem-susceptible *K. pneumoniae* strain (strain 17049) was isolated from a sacral ulcer wound swab. On 13 and 19 November, two carbapenem-resistant (CBP^r) *K. pneumoniae* strains (strains 17829 and 18260) were isolated from the sacral ulcer and rectal swab, respectively. Additional ETP^r *K. pneumoniae* strains were isolated from tracheal/bronchial aspirates (strains 18567 and 19039) and rectal swabs (strain 18918), until the patient's death on 10 December 2008 due to central venous catheter bacteremia caused by *P. aeruginosa* infection. Except for strain 16855, none of the other *K. pneumoniae* isolates were considered pathogens. *K. pneumoniae* did not appear to be involved in the final fatal outcome in this patient.

Identification of outbreak clone. The seven *K. pneumoniae* strains isolated from patient A were genotyped along with two additional ETP^r strains (strains 17830 and 17834) isolated on the same day that strain 17829 was but from patients B and C, respectively; and these were probably the three patients involved in an outbreak that occurred within the hospital (Table 1).

All but one of the isolates showed indistinguishable PFGE patterns, suggesting the presence of an epidemic clone circulating within the cardiac surgery intensive care unit and the general surgery ward (data not shown). Carbapenem-susceptible strain 16855, the first *K. pneumoniae* strain isolated from patient A, showed a different PFGE pattern.

MLST identified the outbreak clone as being of ST37. The only CBP^r *K. pneumoniae* strain belonging to the ST37 lineage previously detected was isolated in the United States in 1996 and carries the KPC-2 β -lactamase (22).

Characterization of ESBL plasmids. β -Lactamases were characterized for strains 17829 (patient A), 17830 (patient B),

TABLE 1. *K. pneumoniae* strains analyzed in this study^a

Strain	Date (mo/day/yr)	PT	Sample	Ward	ESBL production	MIC (μg/ml)									SDS-PAGE result (gene lesion) ^b	
						ETP	IPM	MEM	TZP	ATM	FOX	CTX	FEP	CAZ	OmpK36	OmpK35
16855	10/30/08	A	WOU	CSI	POS	0.094	0.25	0.064	32	96	24	>32	64	>32	POS	POS
17049	11/03/08	A	ULC	CSI	POS	8	0.25	2	>256	>256	>256	>32	>256	>32	POS (36V)	NEG (*36)
17829	11/13/08	A	ULC	CSI	POS	128	12	12	>256	>256	>256	>32	>256	>32	NEG (*125)	NEG (*36)
17830	11/13/08	B	ULC	CSI	POS	8	0.19	1.5	>256	>256	96	>32	>256	>32	POS (36V)	NEG (*36)
17834	11/13/08	C	INF	SUR	POS	6	0.19	0.75	>256	>256	96	>32	>256	>32	POS (36V)	NEG (*36)
18260	11/19/08	A	REC	CSI	POS	128	12	12	>256	>256	>256	>32	>256	>32	NEG (*125)	NEG (*36)
18567	11/24/08	A	TAF	CSI	POS	6	0.19	1	>256	>256	>256	>32	>256	>32	POS (36V)	NEG (*36)
18918	11/28/08	A	REC	CSI	POS	8	0.19	1	>256	>256	>256	>32	>256	>32	POS (36V)	NEG (*36)
19039	12/01/08	A	TAF	CSI	POS	8	0.19	1	>256	>256	>256	>32	>256	>32	POS (36V)	NEG (*36)
17829(pSHA2)						16	1	1	>256	>256	256	>32	>256	>32	POS (pSHA2)	NEG (*36)
18260(pSHA2)						16	1	1	>256	>256	256	>32	>256	>32	POS (pSHA2)	NEG (*36)
50748	09/07/06	E	URI	AMB	POS	0.25	0.25	0.094	12	>256	6	>32	48	>32	POS	NEG (*36)
69036	11/19/07	F	VAG	MED	POS	6	0.19	1	>256	>256	128	>32	>256	>32	POS (36V)	NEG (*36)
69928	12/04/07	G	WOU	SUR	POS	6	0.50	1.5	>256	>256	48	>32	>256	>32	POS (36V)	NEG (*36)
71697	01/10/08	D	WOU	SUR	POS	8	0.19	1.5	>256	>256	128	>32	>256	>32	POS (36V)	NEG (*36)
6267	05/17/08	H	WOU	ICU	POS	4	0.50	1	12	96	32	>32	>256	>32	POS (36V)	NEG (*36)
11932	08/12/08	I	TAF	EME	POS	0.19	0.19	0.064	>256	>256	6	>32	48	>32	POS	NEG (*63)
16830	10/30/08	L	URI	MED	POS	0.125	0.125	0.047	16	>256	3	>32	24	>32	POS	NEG (*75)
25008	03/10/09	M	BLO	ICU	POS	16	1	4	>256	>256	64	>32	>256	>32	NEG (ND)	NEG (*63)
26077	03/25/09	N	URI	CSI	POS	6	0.19	1	>256	>256	128	>32	>256	>32	POS (36V)	NEG (*36)
287942	05/08/09	O	WOU	SUR	POS	0.19	0.19	0.064	>256	>256	4	>32	64	>32	POS	NEG (*63)
29459	05/18/09	P	TAF	ICU	POS	0.25	0.19	0.094	>256	>256	6	>32	96	>32	POS	NEG (*63)
29830	05/22/09	Q	BAL	ICU	POS	6	0.19	1	>256	>256	128	>32	>256	>32	POS (36V)	NEG (*36)
299332	05/25/09	S	CVC	ICU	NEG	0.016	0.25	0.032	6	0.25	4	0.125	0.094	<0.5	POS	NEG (*63)
30018	05/25/09	R	ULC	SUR	NEG	0.023	0.25	0.032	3	0.064	3	0.094	0.047	<0.5	NEG ^c	POS
31750	06/22/09	T	WOU	AMB	POS	0.125	0.19	0.064	8	32	3	>32	16	>32	POS	NEG ^c

^a Abbreviations: PT, patient; POS, positive; NEG, negative; TAF, tracheo/bronchial aspirate fluid; BAL, bronchoalveolar lavage fluid; CVC, central venous catheter; INF, intraperitoneal fluid; REC, rectal swab; WOU, wound swab; BLO, hemoculture; ULC, sacral ulcer swab; VAG, vaginal swab; URI, urine; AMB, ambulatory ward; MED, clinical medicine ward; SUR, surgery ward; ICU, intensive care unit; EME, emergency unit; CSI, cardiac surgery intensive care unit.

^b Symbols indicating the different porin lesion types: *, the stop codon position in the deduced protein sequences; 36V, 6-nucleotide duplication in the *ompK36V* gene variant; ND, not determined (the *ompK36* or *ompK35* gene did not amplify).

^c The gene did not show any lesion, but porin expression was not detected by SDS-PAGE.

and 17834 (patient C). These strains were negative for carbapenemase activity (data not shown); but all of them produced TEM-1, SHV-11, and CTX-M-15 (pIs 5.4, 7.6, and 8.9, respectively). Strains 17829 and 17830 also produced the SHV-12 β-lactamase (pI 8.2), and strains 17830 and 17834 produced the OXA-1 β-lactamase (pI 7.4) (Table 2).

All the *bla* genes except *bla*_{SHV-11}, which is likely the naturally occurring *bla*_{SHV} gene located within the chromosome of *K. pneumoniae* (25), were identified on plasmids, as shown by transformation and conjugation experiments. The strains were positive for IncFII plasmids carrying different combinations of β-lactamase genes (Table 2). For instance, strain 17829 trans-

ferred the *bla*_{TEM-1}, *bla*_{SHV-12}, and *bla*_{CTX-M-15} genes on the same plasmid, while two different plasmids were obtained from strain 17830, one self-conjugative plasmid carrying the *bla*_{OXA-1} and *bla*_{CTX-M-15} genes and one plasmid untypeable by PBRT carrying the *bla*_{TEM-1} and *bla*_{SHV-12} genes. These data may indicate a plasmid fusion/resolution event within strain 17829 generating a single plasmid of the IncFII group carrying the two ESBL genes and *bla*_{TEM-1} but lacking the *bla*_{OXA-1} gene. The colocalization of the *bla*_{SHV-12} and *bla*_{CTX-M-15} genes within a large EcoRI band (>20 kb) was confirmed by hybridizations performed with the 17829 donor and transformant strains; this band was also positive for the repFII replicon (Fig. 1). The emergence and spread of the CTX-M-15 enzyme are described worldwide (27). The *bla*_{CTX-M-15} gene has been located on IncFII plasmids, often in association with *bla*_{TEM-1} and *bla*_{OXA-1} resistance genes; but this is the first report of the colocalization of the *bla*_{TEM-1}, *bla*_{SHV-12}, and *bla*_{CTX-M-15} genes on this plasmid type (4). This plasmid may favor the simultaneous spread of these resistance genes.

Carbapenem susceptibility and OmpK35 and OmpK36 porins. Of the eight outbreak strains from patients A, B, and C, six strains showed ertapenem MICs ranging from 6 to 8 μg/ml, imipenem MICs ranging from 0.19 to 0.25 μg/ml, and meropenem MICs ranging from 0.75 to 2 μg/ml. Strains 17829 and 18260 showed ertapenem MICs of 128 μg/ml, imipenem MICs of 12 μg/ml, and meropenem MICs of 12 μg/ml. The last three strains were the first two CBP^r *K. pneumoniae* strains identified at the hospital. Susceptible strain 16855 showed an ertapenem MIC of 0.094 μg/ml, an imipenem MIC of 0.25 μg/ml, and a meropenem MIC of 0.064 μg/ml (Table 1). The clonal

TABLE 2. β-Lactamases and plasmid types^a

Strain	pIs by IEF	β-Lactamases	Replicon
17829	5.4, 7.6, 8.2, 8.9	TEM-1, SHV-11, SHV-12, CTX-M-15	FII
17829TF	5.4, 8.2, 8.9	TEM-1, SHV-12, CTX-M-15	FII
17829TC	5.4, 8.2, 8.9	TEM-1, SHV-12, CTX-M-15	FII
17830	5.4, 7.4, 7.6, 8.2, 8.9	TEM-1, OXA-1, SHV-11, SHV-12, CTX-M-15	FII
17830TF1	7.4, 8.9	OXA-1, CTX-M-15	FII
17830TF2	5.4, 8.2	TEM-1, SHV-12	NT
17830TC	7.4, 8.9	OXA-1, CTX-M-15	FII
17834	5.4, 7.4, 7.6, 8.9	TEM-1, OXA-1, SHV-11, CTX-M-15	FII
17834TF	7.4, 8.9	OXA-1, CTX-M-15	FII
17834TC	7.4, 8.9	OXA-1, CTX-M-15	FII

^a Abbreviations: NT, not typeable; TF, transformant; TC, transconjugant.

FIG. 1. Restriction analysis of plasmids obtained from *K. pneumoniae* strain 17829 and its transformant, strain 17829TF, restricted by PstI and EcoRI and Southern blot hybridization with *bla*_{CTX-M-15}, *bla*_{SHV-12}, and *repFII* probes. Lanes MK, 1-kb DNA extension ladder.

strains also showed resistance to ciprofloxacin, tobramycin, trimethoprim, and sulfonamides (data not shown).

The six ETP^r strains did not express the OmpK35 protein but expressed OmpK36. The two CBP^r strains did not express either the OmpK35 or the OmpK36 porin (Fig. 2). The *ompK35* and *ompK36* genes were amplified and sequenced. In all these strains, the sequence of *ompK35* showed an A → T mutation at codon 36, generating a TAA nonsense mutation, resulting in the termination of translation (EMBL accession no. GU460159). Sequencing of the *ompK36* genes from the ETP^r strains revealed a novel OmpK36 protein variant (designated OmpK36V and indicated 36V in Table 1) showing 94% to 99% amino acid identities with other *K. pneumoniae* OmpK36 proteins (EMBL accession nos. Q48473, FJ577673, CAC50885, and ABR78053). In particular, in the deduced protein sequence we noted the insertion of two additional amino acids, Asp137 and Thr138, located in one of the most conserved sites of the protein among enterobacterial and non-enterobacterial porins, the so-called PEFXG motif (1, 13, 20). This alteration was generated by the insertion of six nucleotides (5'-GACACC-3') at nucleotide (nt) positions 454 to 459 (EMBL accession no. GQ433376), apparently due to the duplication of the adjacent region (nt positions 448 to 453) encoding the Asp135 and Thr136 codons. Subsequent to the duplication, the Asp135-Thr136-Asp137-Thr138 motif was generated. This motif flanked the PEFXG domain (codons 130 to 134) within the L3 loop (Fig. 3A). To our knowledge, this is the first description of a naturally occurring amino acid insertion within the L3 of OmpC-related enterobacterial proteins, and such a change has not been generated in functional studies

FIG. 2. OMP profiles of *K. pneumoniae* isolates on a 12.0% SDS-polyacrylamide gel. Lanes MK, protein mass marker.

produce the OmpK35 porin due to nonsense mutations occurring in the *ompK35* gene: seven strains showed a stop codon at position 36, namely, the same sequence observed in the ST37 clonal strains (EMBL accession no. GU460159), and five strains and one strain showed a stop codon at positions 63 and 75, respectively (EMBL accession nos. GU460160 and GU460161, respectively; Fig. 3B). Two strains showed a wild-type *ompK35* gene, but strain 31750 did not produce the protein, possibly due to mutations occurring in the promoter (EMBL accession no. GU460162; Fig. 2). Strain 25008 did not produce either OmpK35 or OmpK36 and did not amplify the *ompK36* gene (Table 1; Fig. 2). The presence of the variant *ompK36V* in PCR-positive strains was confirmed by DNA sequencing of the *ompK36* gene. All these strains expressed OmpK36V but did not express the OmpK35 because of the nonsense mutation at codon 36 (Fig. 3B). The MLST analysis performed with these strains demonstrated that they belonged to the ST37 clone, while strains showing different *ompK36* and *ompK35* genes did not (data not shown). Interestingly, the earliest isolate of our collection, strain 50748, isolated in 2006, also belonged to ST37 and showed the nonsense mutation at codon 36 in OmpK35 but carried a wild-type OmpK36 porin. This strain produced the CTX-M-15, SHV-11, and TEM-1 β -lactamases (data not shown) and was susceptible to the three carbapenems tested (Table 1).

The results obtained suggested that the depletion of OmpK35 and the production of a wild-type OmpK36 did not associate with a significant increase in the MICs of carbapenems in ESBL-producing strains (strains 50748, 31750, 11932, 16830, 287942, and 29459 in Table 1). An additional mutational event leading to the production of OmpK36V correlated, in ESBL-producing strains, with ertapenem resistance and increased MICs of meropenem (strains 17049, 17830, 17834, 18567, 18918, 19039, 69036, 69928, 71697, 6267, 26077, and 29830 in Table 1). High levels of resistance to carbapenems were observed in the ESBL-producing strains that did not produce either the OmpK35 or the OmpK36 porin (strains 17829, 18260, and 25008 in Table 1).

Concluding remarks. A *K. pneumoniae* ST37 clone producing CTX-M-15 and wild-type OmpK36 but lacking OmpK35 because of a nonsense mutation has likely circulated in our hospital since 2006. This clone carrying the *bla*_{CTX-M-15} gene located on the IncFII plasmid might have evolved by a 6-nucleotide duplication in the *ompK36* gene, generating the OmpK36V variant, which was identified in strains from patients in the hospital since 2007. Strains carrying this variant showed increased MICs of several β -lactams, including cefoxitin, cefepime, and meropenem, and were resistant to ertapenem. More recently, in patient A, this clone evolved by acquisition of a nonsense mutation causing the termination of translation of the gene for OmpK36V, conferring resistance to all carbapenems. Patient A probably acted as a reservoir of this clone, since several strains were isolated from the sacral ulcer and rectal swabs and the carbapenem-resistant variants were likely positively selected by the treatment of the patient with meropenem. We can hypothesize that the presence of the OmpK36V conferring an increased MIC of meropenem could have favored the survival of the bacteria under meropenem treatment, allowing the emergence of the fully carbapenem-resistant strains.

With the increasing use of carbapenems that are specifically designed to resist degradation by bacterial enzymes, the pathogen is forced to adopt novel strategies to restrict the intracellular antibiotic concentration. The OmpK36V porin mutation detected in this study may represent just an example of bacterial selection under the pressure of antibiotic treatment. Ertapenem-resistant strains have been described to be hidden within populations of susceptible bacteria, appearing as a subpopulation at a frequency of 10^{-6} only under the positive selection exerted by the ertapenem administration or direct selection on culture media (24).

The presence of mutations altering the properties of the OmpK36 porin might be underestimated by standard analysis. It is also important to point out that loss or modification of the porins must be associated with the production of AmpC or ESBLs to confer carbapenem resistance. Consequently, multifactorial events are required to establish the carbapenem resistance phenotype in enterobacterial pathogens. However, once these events take place, the positive selection imposed by the extensive use of antimicrobials may favor the expansion of the resistant clones, promoting their diffusion within the hospital.

ACKNOWLEDGMENTS

We thank Stefania Mochi for skillful technical assistance, L. Martínez-Martínez for providing plasmid pSHA2, and Antonio Cassone for critical reading of the manuscript and helpful suggestions.

This work has been partly funded by the FIRB project Costruzione di un laboratorio nazionale per lo studio delle resistenze batteriche agli antibiotici as part of a contract with the Italian Ministry of Research and University, 2007 to 2009, Rome, Italy.

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