Reduced susceptibility to carbapenems in *Klebsiella pneumoniae* clinical isolates associated with plasmid-mediated β -lactamase production and OmpK36 porin deficiency

Xuan Ding Wang, Jia Chang Cai, Hong Wei Zhou, Rong Zhang and Gong-Xiang Chen

Correspondence
Gong-Xiang Chen
chengong218@163.com

2nd Affiliated Hospital of Zhejiang University, Zhejiang University, 88 Jiefang Road, Hangzhou 310009, PR China

Two carbapenem-non-susceptible *Klebsiella pneumoniae* isolates, Z2554 and Z2110, were collected from a hospital in China and analysed by PFGE. *K. pneumoniae* Z2554 and Z2110 were genetically unrelated and showed resistance to ertapenem, and reduced susceptibility to imipenem and meropenem. Analysis of their β -lactamases indicated that *K. pneumoniae* Z2554 produced TEM-1 and CTX-M-14 β -lactamases, whilst Z2110 produced a plasmid-mediated AmpC β -lactamase, DHA-1, in addition to TEM-1 and CTX-M-14. SDS-PAGE analysis of the outer-membrane proteins (OMPs) revealed that both isolates lacked an OMP of ~39 kDa (OmpK36), whilst Z2110 had an additional protein with an approximate molecular mass of 26 kDa. Analysis of the OMP-encoding genes demonstrated that the *ompK35* sequence of *K. pneumoniae* Z2554 and Z2110 contained a number of silent mutations. In *ompK36*, several insertions and deletions of short DNA fragments (1–6 bp) were detected in both isolates. The N-terminal sequence of the ~26 kDa protein band identified in Z2110 had no similarity to the sequence of OmpK36. Instead, it shared high similarity with hypothetical protein KPN_03267 originating from *K. pneumoniae* subsp. *pneumoniae* MGH 78578. It was concluded that β -lactamase production combined with OmpK36 deficiency results in ertapenem resistance, and reduced imipenem and meropenem susceptibility, in *K. pneumoniae* Z2554 and Z2110. OmpK36 may play an important role in the resistance or reduced susceptibility to carbapenems in *K. pneumoniae* producing AmpC, extended-spectrum β -lactamase or broad-spectrum β -lactamase.

Received 17 November 2008

Accepted 12 May 2009

INTRODUCTION

Klebsiella pneumoniae is one of the most important pathogens that causes nosocomial infections. Due to the extensive use of extended-spectrum cephalosporins, extended-spectrum β -lactamase (ESBL)-producing *K. pneumoniae* has become an increasingly serious problem. Carbapenems are commonly used to treat serious infections caused by multidrug-resistant Gram-negative bacteria, especially strains producing high levels of AmpC cephalosporinases or ESBLs. Although carbapenem-resistant *K. pneumoniae* is uncommon, a number of *K. pneumoniae* isolates have been reported to be resistant to carbapenems mediated by metallo- β -lactamases in Singapore, Brazil, Turkey and Taiwan, including IMP-1 and IMP-8 (Aktas *et al.*, 2006; Koh *et al.*, 1999; Lincopan *et al.*, 2005; Yan *et al.*, 2001). Recently, the number of *K. pneumoniae* isolates producing KPC-type carbapenemases

has increased dramatically (Smith Moland *et al.*, 2003; Wei *et al.*, 2007; Woodford *et al.*, 2004; Yigit *et al.*, 2001).

A combination of high-level production of AmpC β -lactamases (e.g. ACT-1, CMY-4 and DHA-1) or SHV-2 together with porin loss can result in resistance or reduced susceptibility to carbapenems in *K. pneumoniae* (Bradford *et al.*, 1997; Cao *et al.*, 2000; Crowley *et al.*, 2002; Lee *et al.*, 2007a). Recently, an outbreak of imipenem-non-susceptible *K. pneumoniae*, caused by the production of CMY-2 and DHA-1 together with OmpK35 and OmpK36 porin loss, was reported in Korea (Lee *et al.*, 2007b).

Studies have been conducted to elucidate the mechanism of resistance to carbapenems in *K. pneumoniae* as a result of a deficiency of porins in combination with the production of β -lactamases. Lee *et al.* (2007a) reported that strains that are deficient in both OmpK35 and OmpK36 have resistance to carbapenems in the presence of β -lactamases. However, in a strain where only OmpK35 was deficient and OmpK36 was expressed, the strain maintained its suscept-

Abbreviations: ESBL, extended-spectrum β -lactamase; i.v., intravenously; OMP, outer-membrane protein; pI, isoelectric point.

ibility regardless of the introduction of other β -lactamases such as DHA-1. In a separate study, Mena *et al.* (2006) reported that, in the presence of CTX-M-1, loss of both OmpK35 and OmpK36 resulted in resistance, and expression of OmpK36 alone resulted in the recovery of susceptibility. These findings suggest that OmpK36 plays an important role in resistance. However, all studies to date have been carried out in strains in which OmpK35 is also deficient.

We collected two carbapenem-non-susceptible *K. pneumoniae* isolates, Z2554 and Z2110, that did not produce any type of carbapenemase, although carbapenem-resistant *Enterobacteriaceae* isolated in our hospital are mainly due to the production of KPC-2 (Cai *et al.*, 2008; Zhang *et al.*, 2007, 2008), indicating that other carbapenem resistance mechanisms, such as changes in outer-membrane permeability combined with β -lactamase production or an efflux pump, might be involved. Further studies indicated that the two strains produced a number of β -lactamases. Examination of their outer-membrane proteins (OMPs) showed that both strains expressed OmpK35 but failed to express OmpK36. In addition, an unidentified protein of ~26 kDa was found in one of the strains. To the best of our knowledge, this is the first report of *K. pneumoniae* clinical isolates with reduced carbapenem susceptibility that produce OmpK35 but have no OmpK36 expression.

METHODS

Bacterial strains and antimicrobial susceptibility testing. Two carbapenem-non-susceptible *K. pneumoniae* strains, Z2554 and Z2110, were isolated from the sputum of two patients in November 2006 and identified using a Vitek system (bioMérieux). One patient was hospitalized in the oncology ward due to extensive abdominal cavity metastasis after a radical operation for gastric cancer. Cefoperazone/sulbactam and ciprofloxacin [2.0 g, intravenously (i.v.), twice a day] and ciprofloxacin (0.4 g, i.v., twice a day) were administered for pulmonary infection for 2 weeks and then replaced with cefminox (2.0 g, i.v., twice a day) and gatifloxacin (0.4 g, i.v., once a day). Two days later, *K. pneumoniae* Z2554 was isolated. The other patient was hospitalized in the respiratory ward for severe pneumonia and treated with multiple antimicrobials, including ceftriaxone (2.0 g, i.v., once a day) and ciprofloxacin (0.5 g, i.v., once a day) for 5 days, panipenem (1.0 g, i.v., every 12 h) and mycomycin (500 000 U, orally, three times a day) for 2 weeks, and cefminox (2.0 g, i.v., twice a day) and gatifloxacin (0.4 g, i.v., once a day) for 5 days, after which *K. pneumoniae* Z2110 was isolated.

The MICs of 16 antimicrobial agents were determined using an agar dilution method according to Clinical and Laboratory Standards Institute recommendations (CLSI, 2006). The antimicrobial agents were: piperacillin, cefotaxime, gentamicin, tetracycline, sulfonamide (Sigma), imipenem, ertapenem, ceftazidime (Merck), meropenem (Dainippon Sumitomo Pharma), piperacillin/tazobactam (Wyeth Holdings), cefoperazone/sulbactam (Pfizer), ceftazidime (GlaxoSmithKline), ceftriaxone (Roche), aztreonam, cefepime (Bristol-Meyers Squibb) and ciprofloxacin (Bayer).

PFGE. PFGE of the two *K. pneumoniae* isolates was performed according to the protocols of PulseNet (<http://www.cdc.gov/pulsenet/protocols.htm>) in a Rotaphor system 6.0 instrument (Whatman

Biometra). The *Xba*I restriction patterns of their genomic DNA were analysed and interpreted according to the criteria of Tenover *et al.* (1995).

Conjugation experiment. A conjugal transfer experiment was carried out in mixed broth cultures. Rifampicin-resistant *Escherichia coli* EC600 (LacZ⁻, Nal^R, Rif^R) was used as the recipient. *E. coli* transconjugants were selected on Mueller–Hinton agar containing rifampicin (700 μ g ml⁻¹) and imipenem (0.5 μ g ml⁻¹), or rifampicin (700 μ g ml⁻¹) and ceftazidime (16 μ g ml⁻¹) (all from Sigma). The selected colonies were picked and identified using a Vitek system. Plasmid DNA of the *E. coli* transconjugant, *K. pneumoniae* clinical isolates and *E. coli* V517 (Macrina *et al.*, 1978) were obtained using an AxyPrep plasmid miniprep kit (Axygen Scientific).

IEF of β -lactamase. Crude β -lactamase preparations were obtained by ultrasonic treatment of bacterial cells. IEF was carried out on a PhastGel polyacrylamide gel (pH 3–9; Amersham Biosciences) using a PhastSystem (Pharmacia Biotech) and the method of Mathew *et al.* (1975). β -Lactamase activity was visualized by staining the gel with nitrocefin (Oxoid). Isoelectric points (pIs) were determined after comparison with the known β -lactamases TEM-28 (pI 6.1), SHV-7 (pI 7.6) and ACT-1 (pI 9.0).

PCR amplification and DNA sequence analysis of *bla* genes. Plasmids from *K. pneumoniae* Z2554 and Z2110 and the *E. coli* transconjugant were used as templates. The primers used to amplify *bla*_{TEM}, *bla*_{CTX-M} and *bla*_{DHA} were as described by Yan *et al.* (2002) and Yu *et al.* (2007). The reaction was conducted in a GeneAmp PCR system 9600 thermal cycler (Applied Biosystems). PCR products were purified and sequenced directly. DNA sequences were compared with reported nucleotide sequences in GenBank.

Analysis of OMPs. OMPs were obtained as described by Hernández-Allés *et al.* (1999). Strains were grown in Mueller–Hinton broth overnight at 37 °C with shaking. OMP profiles were determined by SDS-PAGE using 11.6 % acrylamide/0.4 % bisacrylamide/0.1 % SDS gels (0.75 mm thick). These mini-gels were run at a constant current of 20 mA for 85 min with a Mini Protein 3 slab electrophoresis cell (Bio-Rad). Bands were visualized by staining with Coomassie brilliant blue.

The *ompK35* and *ompK36* genes of *K. pneumoniae* Z2554 and Z2110 were amplified by PCR (Kaczmarek *et al.*, 2006). The sequences were analysed by comparison with reported nucleotide sequences in GenBank.

N-terminal sequence analysis. The N-terminal sequence of the protein identified by SDS-PAGE of the OMPs from Z2110 was determined. The protein was transferred to a PVDF membrane after SDS-PAGE in a CAPS buffer system. The membrane was stained with Coomassie brilliant blue and the band corresponding to a protein of ~26 kDa was excised and used for N-terminal amino acid sequence analysis. The amino acid sequence of the transferred protein was determined using the Edman method using an Applied Biosystems protein sequencer (Procise 492 cLC). The sequence of 15 aa was determined. The sequence was used to search and compare with sequences in GenBank.

RESULTS AND DISCUSSION

Antimicrobial agent susceptibility and PFGE profiles

The MICs of 16 antimicrobial agents are presented in Table 1. *K. pneumoniae* Z2554 and Z2110 showed

Table 1. Antimicrobial susceptibility patterns of the *K. pneumoniae* isolates and the *E. coli* transconjugant

Antimicrobial agent*	MIC ($\mu\text{g ml}^{-1}$)			
	<i>K. pneumoniae</i> Z2554	<i>K. pneumoniae</i> Z2110	<i>K. pneumoniae</i> Z2110 <i>E. coli</i> transconjugant	<i>E. coli</i> EC600
Imipenem	0.5	4	≤ 0.125	≤ 0.125
Imipenem + CLA	0.25	4	NT	NT
Imipenem + CLO	0.5	0.25	NT	NT
Imipenem + CLA + CLO	≤ 0.125	≤ 0.125	NT	NT
Meropenem	8	2	≤ 0.125	≤ 0.125
Meropenem + CLA	2	1	NT	NT
Meropenem + CLO	8	0.5	NT	NT
Meropenem + CLA + CLO	1	0.25	NT	NT
Ertapenem	16	32	≤ 0.125	≤ 0.125
Ertapenem + CLA	4	16	NT	NT
Ertapenem + CLO	8	4	NT	NT
Ertapenem + CLA + CLO	1	0.5	NT	NT
Piperacillin	>256	>256	4	1
Piperacillin/tazobactam	256	64	4	1
Cefoxitin	32	>512	32	8
Ceftazidime	256	16	1	0.25
Cefotaxime	>256	128	0.25	≤ 0.125
Ceftriaxone	>256	256	≤ 0.125	≤ 0.125
Cefoperazone/sulbactam	256	64	≤ 0.125	≤ 0.125
Cefepime	>256	128	≤ 0.125	≤ 0.125
Aztreonam	>256	32	≤ 0.125	≤ 0.125
Ciprofloxacin	>32	>32	0.5	≤ 0.125
Gentamicin	1	>256	>256	0.25
Tetracycline	8	32	NT	NT
Sulfamethoxazole	16	8	NT	NT

NT, Not tested.

*CLA, Clavulanic acid (concentration fixed at $4 \mu\text{g ml}^{-1}$); CLO, cloxacillin (concentration fixed at $200 \mu\text{g ml}^{-1}$).

resistance to ertapenem and various levels of reduced susceptibilities to imipenem and meropenem. *K. pneumoniae* Z2554 was highly resistant to penicillins, cephalosporins, aztreonam and quinolones, but remained susceptible to aminoglycosides. *K. pneumoniae* Z2110 was resistant to all of the tested antimicrobial agents. The MIC of cefoxitin for *K. pneumoniae* Z2110 was much higher than that for Z2554, although the MICs of the β -lactamase inhibitors, cephalosporins and aztreonam were lower. The MICs against ertapenem, imipenem and meropenem were also determined in the presence of β -lactamase inhibitors. As shown in Table 1, the MICs were significantly reduced by these inhibitors.

K. pneumoniae Z2554 and Z2110 had different PFGE patterns (Fig. 1), indicating that they were genetically unrelated.

Transfer of antimicrobial agent resistance and plasmid analysis

For *K. pneumoniae* Z2554 and Z2110, no colonies grew on Mueller–Hinton agar containing rifampicin and imipenem.

Fig. 1. PFGE fingerprinting of *K. pneumoniae* Z2554 and Z2110 after *Xba*I digestion. Lanes: 1, Z2554; 2, Z2110.

Transfer of cefoxitin resistance from *K. pneumoniae* Z2110 to *E. coli* EC600 was successful. The transfer frequency was approximately 10^{-5} – 10^{-6} (60–100 transconjugants per 10^7 recipients). The *E. coli* transconjugant was resistant to cefoxitin and gentamicin, and moderately resistant to penicillins, but was susceptible to cephalosporins, aztreonam, quinolones and carbapenems (Table 1).

The plasmid profiles (Fig. 2) indicated that *K. pneumoniae* Z2554 and Z2110 harboured several plasmids. The *E. coli* transconjugant acquired a plasmid of ~50 kb from *K. pneumoniae* Z2110. This transfer into *E. coli* EC600 by conjugation resulted in resistance to cefoxitin and gentamicin, indicating that *bla*_{DHA-1} and the aminoglycoside-resistant determinant were located on the same plasmid.

IEF analysis

IEF analysis of the two isolates and the *E. coli* transconjugant of Z2110 demonstrated that there were two β -lactamases in *K. pneumoniae* Z2554 with pIs of 5.4 and 7.9, whilst *K. pneumoniae* Z2110 produced three β -lactamases with pIs of 5.4, 7.8 and 7.9. The *E. coli* transconjugant produced a single β -lactamase of pI 7.8 (data not shown).

PCR and DNA sequence analysis

To confirm the genotypes of the β -lactamases detected by IEF, PCR was conducted for plasmids extracted from *K. pneumoniae* Z2554 and Z2110 and the *E. coli* transconjugant. As a result, *bla*_{TEM} and *bla*_{CTX-M-9} group genes were detected in both clinical isolates, whilst the *bla*_{DHA} gene was detected in *K. pneumoniae* Z2110 and the *E. coli* transconjugant. After comparing the DNA sequences with known genes in GenBank, we found that isolate Z2554 contained sequences encoding TEM-1 and CTX-M-14,

whilst isolate Z2110 encoded an extra AmpC β -lactamase, DHA-1, and the *E. coli* transconjugant of Z2110 encoded a single DHA-1.

SDS-PAGE and OMP-encoding gene analysis

K. pneumoniae Z2554 and Z2110 showed various levels of resistance to carbapenems but did not produce any type of carbapenemase, indicating that other carbapenem resistance mechanisms, such as alteration in outer-membrane permeability or an efflux pump, might be involved. However, as the efflux mechanism is unlikely to be involved in the resistance of enterobacteria, we focused on an alteration in the OMPs. SDS-PAGE analysis of the OMPs was performed, and the result revealed loss of an OMP of ~39 kDa in both *K. pneumoniae* Z2554 and Z2110 compared with *K. pneumoniae* ATCC 13883 (Fig. 3). It was uncertain whether the 39 kDa OMP was OmpK36 or OmpK35, as in some strains OmpK36 migrates faster than OmpK35 (Hernández-Allés *et al.*, 1999). The *ompK35* gene was amplified, sequenced and aligned with that of *K. pneumoniae* KT755 (GenBank accession no. AJ011501) (Doménech-Sánchez *et al.*, 2003), and point mutations were found at nt 420, 474, 537 and 786 on the nucleotide sequence in both *K. pneumoniae* Z2554 and Z2110, and an additional point mutation was present in Z2554 at nt 249. However, point mutations in the *ompK35* gene were all silent and did not lead to amino acid sequence changes. Comparing the *ompK36* gene of *K. pneumoniae* Z2554 and Z2110 with that of *K. pneumoniae* C3 (GenBank accession no. Z33506; Albertí *et al.*, 1995), several small DNA fragment insertions and a number of point mutations were observed. The sequence of the *ompK36* gene from Z2110 and Z2554 is shown in Fig. 4. These mutations resulted in alteration of the ORF and disruption of the *ompK36* gene. These results suggested that it was OmpK36 that was not expressed in *K. pneumoniae* Z2554 and Z2110.

Fig. 2. Plasmid profiles of *K. pneumoniae* Z2554 and Z2110, and the *E. coli* transconjugant of Z2110. Lanes: 1, *E. coli* V517; 2, *K. pneumoniae* Z2554; 3, *K. pneumoniae* Z2110; 4, *E. coli* transconjugant of Z2110; 5, *E. coli* EC600 (negative control).

Fig. 3. SDS-PAGE analysis of the OMPs of *K. pneumoniae* isolates Z2554 and Z2110, and *K. pneumoniae* ATCC 13883. Lanes: 1, molecular mass standards (MBI Fermentas); 2, *K. pneumoniae* ATCC 13883; 3, *K. pneumoniae* Z2554; 4, *K. pneumoniae* Z2110.

<i>K. pneumoniae</i> 2110/2554	1	ATGAAAGTTAAAGTACTGTCCCTCTGGTACCGGCTCTGCTGGTAGCAGGCGCAGCAAATGCGGCTGAAATTTAT
Protein	1	M K V K V L S L L V P A L L V A G A A N A A E I Y
<i>K. pneumoniae</i> 2110/2554	76	AACAAAGACGGCAACAAATAGACCTGTACGGTAAATTTGACGGTCTGCACATCTCTCTGACGACAAGACGGTC
Protein	26	N K D G N K L D L Y G K I D G L H Y F S D D K S V
<i>K. pneumoniae</i> 2110/2554	151	GACGGCGACACGACCTACATGCGTGTAGGCGTGAAAGGCGAAACCCAGATCAACGACGAGCTGACCGGTTACGGC
Protein	51	D G D Q T Y M R <u>V</u> G V K G E T Q I N D Q L T G Y G
<i>K. pneumoniae</i> 2110/2554	226	CAGTGGGAATACAACGTTACGGCGAACAACACTGAAAGCTCCAGCGATCAGGCATGGACTCGTCTGGCATTCGCA
Protein	76	Q W E Y N V Q A N N T E S S S D Q A W T R L A F A
<i>K. pneumoniae</i> 2110/2554	301	GGCTGAAATTTGGCGACGCGGCTCTTCGACTACGGTCGTAACACGGCGTATGATACGACGTAACTCTCTGG
Protein	101	G L K F G D A G S F D Y G R N Y G V V Y D V T S W
<i>K. pneumoniae</i> 2110	376	ACCGACGTTCTGCCGAATTCGGCGGCGACACCTACGG TTCTGACAACTTCCTGCAGTCCCGTGCTAACGGCGTT
<i>K. pneumoniae</i> 2554	376	ACCGACGTTCTGCCGAATTCGGCGGCGACACCTACGGTTTCTGACAACTTCCTGCAGTCCCGTGCTAACGGCGTT
Protein	126	T D V L P E F G G D T Y G S D N F L Q S R A N G V
	126	T D V L P E F G G D T Y G F L T T S C S P V L T A L
<i>K. pneumoniae</i> 2110	451	GCAACCTACCGTAACCTCTGATTTCTTCGGTCTGGTTGACGGCTGAACCTTGTCTGTCAGTATCAGGGTAAAAAC
<i>K. pneumoniae</i> 2554	453	GCAACCTACCGTAACCTCTGATTTCTTCGGTCTGGTTGACGGCTGACTTTGCTCTGTCAGTATCAGGGTAAAAAC
Protein	151	A T Y R N S D F F G L V D G L N F A L Q Y Q G K N
	152	Q P T V T L I S S V W L T A *
<i>K. pneumoniae</i> 2110	526	GGCAGCCCGAGCGCGAAGGCGCTCTCTCTCTACCAACAACGGTCGTACCGCTTGAAACAGAACGGCGACGGT
<i>K. pneumoniae</i> 2554	528	GGCAGCGTCAGCGCGAAGGTACT TCTCCGACCAACAACGGTCGTGGCGCTCTGAAACAGAACGGTGCAGGC
Protein	176	G S P S G E G A L S P T N N G R T A L K N C D G
<i>K. pneumoniae</i> 2110	601	TACGGTACTTCTCTGACCTATGACATCTATGATGGCATCAGCGCTGGTTTCGCTACTCTAACTCCAAACGTCCT
<i>K. pneumoniae</i> 2554	600	TTCCGTACCTCTCTGACCTATGACATCTATGATGGCATCAGCGCTGGTTTCGCGTACTCCGACTCCAAACGTAAC
Protein	201	Y G T S L T Y D I Y D G I S A G F A Y S N S K R L
<i>K. pneumoniae</i> 2110	676	GGCGACAGAACGCAAGCTGGCACTGGGTCGTGGCGACAACGCTGAAACCTACACCGCGCGTCTGAAATACGACG
<i>K. pneumoniae</i> 2554	675	GGCGATCAGAACTCGTT TGGATAAAGGCGGTGGCGACAACGCTGAAACCTACACCGGTGGTCTGAAATACGACG
Protein	226	G D Q N A S W H W V V A T T L K P T P A V *
<i>K. pneumoniae</i> 2110	751	CCAACAACATCTACCTGGCCACTCAGTACACCCAGACCTACAACGCGACCGCGCGGTTCCCTGGGC
<i>K. pneumoniae</i> 2554	748	CCAACAACATTTACCTGGCGACTCAGTACACCCAGACTTACAACGCAACTCGTTTCAGCGGCAACGGAGAATCTG
<i>K. pneumoniae</i> 2110	819	TTTGCTAACAAAGCGCAGAACTTCGAAGTGGTTGCTCAGTACCAAGTTCGACTTCGGTCTGC
<i>K. pneumoniae</i> 2554	823	ATTCTATTAGCGGTTTGTCTAACAAAGCAGAACTTCGAAGTGGTTGCTCAGTACCAAGTTCGACTTCGGTCTGC
<i>K. pneumoniae</i> 2110	880	GTCCGTCCGTGGCTTACCTGCAGTCTAAAGGTAAGGATCTGGAAGGCTACGGCGACCGGATCTGAAATATG
<i>K. pneumoniae</i> 2554	898	GTCCGTCCGTAGCTTACCTGCAGTCTAAAGGTAAGGATCTGGAAGGTTACGGCGACCGGATCTGAAATATG
<i>K. pneumoniae</i> 2110	955	TTGACGTTGGCGCAACCTACT
<i>K. pneumoniae</i> 2554	973	TTGACGTTGGCGGACCTACT

Fig. 4. Nucleotide sequences of the *ompK36* gene of Z2110 and Z2554, and the putative protein sequence. The nucleotide sequence of *ompK36* and its translated protein sequence were analysed by DNAMAN and compared with wild-type *K. pneumoniae* C3 (GenBank accession no. Z33506). The locations where Z2110 is different from the sequence of C3 are shown in bold and underlined. Asterisks indicate the location of the stop codons, which are shown in italic and underlined.

We realized that in strain Z2110, although OmpK36 was not expressed, there was an additional protein band of ~26 kDa seen by SDS-PAGE (Fig. 3). An early termination of translation might lead to a truncated protein of ~26 kDa – in other words, we suspected that the new ~26 kDa protein in Z2110 might be the truncated product of OmpK36. If that were the case, then it indicates that the truncated C-terminal portion of the OmpK36 protein plays a critical role in drug resistance. After SDS-PAGE and protein transfer, we analysed the N-terminal amino acid sequence of this protein. The 15 aa determined are shown in Fig. 5. Comparing the sequence of the determined N-terminal sequence with the putative sequence of the *ompK36* gene, we were unable to find any similarity, suggesting that the newly discovered ~26 kDa protein from Z2110 was not a truncated OmpK36 protein. To understand the nature of this protein, we searched GenBank to look for potential homologous sequences. We found a hypothetical protein called KPN_03267 originating from *K. pneumoniae* subsp. *pneumoniae* MGH 78578 (GenBank accession no. YP_001336895), which

showed 100 % similarity to the 15 determined N-terminal amino acids. The sequence of this putative protein is shown in Fig. 5. This protein has 231 aa in total with an approximate molecular mass of 25.9 kDa, which is similar to the protein we observed in Z2110. However, transmembrane structure analysis using ConPre II software conducted for this protein revealed that there is no transmembrane topology in this sequence. Therefore, this protein is unable to fold into a transmembrane structure to form a channel, as does OmpK36. It is possible that the replacement of OmpK36 with a new protein of ~26 kDa resulted in the loss of the channel function, which eventually leads to resistance in the strain Z2110 in addition to the high-level expression of different types of β -lactamases. Currently, the contribution of this new protein to drug resistance in strain Z2110 is unclear and is worthy of further study.

K. pneumoniae contains two major porins, OmpK35 and OmpK36, which correspond to OmpF and OmpC, respectively, in *E. coli*, and OmpK37, which is not expressed or is expressed at very low levels under

Z2110:	1			<u>VTIDLRHEFI</u>	<u>DGGKS</u>				
KPN_03267:	1	MLKRSVLVLA	LCGMSFAATA	VTIDLRHEFI	DGGKSDKSNA	DRVSVSHRFA	NGLGFTVEAK		
	61	WRSGGDNGSQ	PYSDVVGNGH	EDTISWRWKA	TSNLFLLTPGF	TIESNDSRSI	YKPHLHAQYS		
	121	FDNGFYVAAR	YRYEYTRYPN	NAGKDDDKVN	RGDAWAGFVL	GDWRTELNYV	YARSSEGVS		
	181	NDNKPYSQEQ	NVKVAYKLDN	NWSPYGEIGN	VGVNDRSDRQ	TRFRVGVAYS	F		

Fig. 5. The N-terminal amino acid sequence of the ~26 kDa protein from Z2110 compared with the putative amino acid sequence of hypothetical protein KPN_03267 (*K. pneumoniae* subsp. *pneumoniae* MGH 78578). The underlined sequence shown at the top is the N-terminal sequence of the protein from Z2110.

laboratory conditions and is undetectable by SDS-PAGE (Doménech-Sánchez *et al.*, 1999). Loss of either OmpK35 or OmpK36 in *K. pneumoniae* leads to reduced susceptibility to cefoxitin and cefotaxime (MIC 2–4 µg ml⁻¹), but maintains sensitivity to carbapenems (MIC 0.06–0.125 µg ml⁻¹). However, loss of both porins can result in reduced susceptibility to imipenem and meropenem (MIC 0.25–2 µg ml⁻¹) (Doménech-Sánchez *et al.*, 1999). Loss of one of the porins combined with broad-spectrum β -lactamase and/or ESBL production in *K. pneumoniae* can cause resistance to cefoxitin and cephalosporin whilst maintaining susceptibility to imipenem (Martínez-Martínez *et al.*, 1996; Rice *et al.*, 1993), whilst combining with AmpC β -lactamase expression results in resistance or reduced susceptibility to carbapenems (Bradford *et al.*, 1997; Cao *et al.*, 2000; Lee *et al.*, 2007b). This view is supported by the observations in *K. pneumoniae* Z2110 in the current study. Loss of both porins combined with broad-spectrum β -lactamase and/or ESBL production would result in moderate resistance or reduced susceptibility to imipenem and meropenem, as well as resistance to ertapenem (Crowley *et al.*, 2002; Elliott *et al.*, 2006; Jacoby *et al.*, 2004). Lee *et al.* (2007a) reported that *K. pneumoniae* isolates Lkp11–13 that produced CTX-M-3 and SHV-5 but with OmpK35 deficiency and OmpK36 expression were susceptible to carbapenems. Genetically related *K. pneumoniae* Lkp14 producing an extra DHA-1 and failing to express OmpK36 showed resistance to ertapenem, and reduced susceptibility to imipenem and meropenem. The introduction of a *bla*_{DHA-1}-carrying plasmid into Lkp11 only resulted in a significant increase in the MICs of cephamycins and β -lactamase inhibitors, and a slight increase in the MIC of ertapenem, but not in those of imipenem and meropenem. Mena *et al.* (2006) also reported that CTX-M-1-producing *K. pneumoniae* that expressed one of the two major porins, OmpK36, was susceptible to carbapenems, whereas isolates that did not express either of the porins were resistant to carbapenems. In this study, two clinical isolates of genetically unrelated, OmpK36-deficient, OmpK35-expressing *K. pneumoniae* were investigated. *K. pneumoniae* Z2110 producing TEM-1, CTX-M-14 and plasmid-mediated DHA-1 β -lactamase was resistant to ertapenem, and had reduced susceptibility to imipenem and meropenem as expected. *K. pneumoniae* Z2554 producing TEM-1 and CTX-M-14 but not DHA-1, however, showed a similar resistance phenotype for

carbapenems. Thus, we believe that OmpK36 may play an important role in the resistance or reduced susceptibility to carbapenems in *K. pneumoniae* isolates producing AmpC, ESBL or broad-spectrum β -lactamase. To the best of our knowledge this is the first report of clinical isolates of *K. pneumoniae* with reduced carbapenem susceptibility that lacked OmpK36 but showed expression of OmpK35, and provides further evidence that OmpK36 may be more important than OmpK35 in resistance.

Jacoby *et al.* (2004) reported that most porin-deficient *K. pneumoniae* derivatives producing plasmid-mediated β -lactamases are resistant to ertapenem but have only slightly reduced susceptibility to imipenem and meropenem. A similar carbapenem efficacy for porin-deficient *K. pneumoniae* clinical isolates was observed in the current study. In summary, we have described two clinical isolates of *K. pneumoniae* deficient in OmpK36 with resistance to ertapenem and reduced susceptibility to imipenem and meropenem, and have provided a detailed molecular analysis of their antimicrobial drug resistance mechanisms. Expression of OmpK35 was not sufficient to recover resistance to ertapenem or the reduced susceptibility to imipenem and meropenem in these *K. pneumoniae* isolates, suggesting that OmpK36 plays an important role in the resistance or reduced susceptibility to carbapenems in *K. pneumoniae* isolates producing AmpC, ESBL or broad-spectrum β -lactamase.

REFERENCES

- Aktas, Z., Bal, C., Midilli, K., Poirel, L. & Nordmann, P. (2006). First IMP-1-producing *Klebsiella pneumoniae* isolate in Turkey. *Clin Microbiol Infect* 12, 695–696.
- Alberti, S., Rodríguez-Quinoñes, F., Schirmer, T., Rummel, G., Tomás, J. M., Rosenbusch, J. P. & Benedí, V. J. (1995). A porin from *Klebsiella pneumoniae*: sequence homology, three-dimensional model, and complement binding. *Infect Immun* 63, 903–910.
- Bradford, P. A., Urban, C., Mariano, N., Projan, S. L., Rahal, J. J. & Bush, K. (1997). Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β -lactamase, and the loss of an outer membrane protein. *Antimicrob Agents Chemother* 41, 563–569.
- Cai, J. C., Zhou, H. W., Zhang, R. & Chen, G. X. (2008). Emergence of *Serratia marcescens*, *Klebsiella pneumoniae* and *Escherichia coli* possessing the plasmid-mediated carbapenem-hydrolyzing β -lactamase KPC-2 in intensive care units of a Chinese hospital. *Antimicrob Agents Chemother* 52, 2014–2018.

- Cao, V. T., Arlet, G., Ericsson, B. M., Tammelin, A., Courvalin, P. & Lambert, T. (2000). Emergence of imipenem resistance in *Klebsiella pneumoniae* owing to combination of plasmid-mediated CMY-4 and permeability alteration. *J Antimicrob Chemother* **46**, 895–900.
- CLSI (2006). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, approved standard M7–A7. Wayne, PA: Clinical and Laboratory Standards Institute.
- Crowley, B., Benedi, V. J. & Doménech-Sánchez, A. (2002). Expression of SHV-2 β -lactamase and of reduced amounts of OmpK36 porin in *Klebsiella pneumoniae* results in increased resistance to cephalosporins and carbapenems. *Antimicrob Agents Chemother* **46**, 3679–3682.
- Doménech-Sánchez, A., Hernández-Allés, S., Martínez-Martínez, L., Benedi, V. J. & Alberti, S. (1999). Identification and characterization of a new porin gene of *Klebsiella pneumoniae*: its role in β -lactam antibiotic resistance. *J Bacteriol* **181**, 2726–2732.
- Doménech-Sánchez, A., Martínez-Martínez, L., Hernández-Allés, S., Conejo, M. C., Pascual, A., Tomás, J. M., Alberti, S. & Benedi, V. J. (2003). Role of *Klebsiella pneumoniae* OmpK35 porin in antimicrobial resistance. *Antimicrob Agents Chemother* **47**, 3332–3335.
- Elliott, E., Brink, A. J., van Greune, J., Els, Z., Woodford, N., Turton, J., Warner, M. & Livermore, D. M. (2006). In vivo development of ertapenem resistance in a patient with pneumonia caused by *Klebsiella pneumoniae* with an extended-spectrum β -lactamase. *Clin Infect Dis* **42**, e95–e98.
- Hernández-Allés, S., Alberti, S., Alvarez, D., Doménech-Sánchez, A., Martínez-Martínez, L., Gil, J., Tomás, J. M. & Benedi, V. J. (1999). Porin expression in clinical isolates of *Klebsiella pneumoniae*. *Microbiology* **145**, 673–679.
- Jacoby, G. A., Mills, D. M. & Chow, N. (2004). Role of β -lactamases and porins in resistance to ertapenem and other β -lactams in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **48**, 3203–3206.
- Kaczmarek, F. M., Dib-Hajj, F., Shang, W. & Gootz, T. D. (2006). High-level carbapenem resistance in a *Klebsiella pneumoniae* clinical isolate is due to the combination of bla_{ACT-1} β -lactamase production, porin OmpK35/36 insertional inactivation, and down-regulation of the phosphate transport porin PhoE. *Antimicrob Agents Chemother* **50**, 3396–3406.
- Koh, T. H., Babini, G. S., Woodford, N., Sng, L. H., Hall, L. M. & Livermore, D. M. (1999). Carbapenem-hydrolyzing IMP-1 β -lactamase in *Klebsiella pneumoniae* from Singapore. *Lancet* **353**, 2162.
- Lee, C. H., Chu, C., Liu, J. W., Chen, Y. S., Chiu, C. J. & Su, L. H. (2007a). Collateral damage of flomoxef therapy: *in vivo* development of porin deficiency and acquisition of bla_{DHA-1} leading to ertapenem resistance in a clinical isolate of *Klebsiella pneumoniae* producing CTX-M-3 and SHV-5 β -lactamases. *J Antimicrob Chemother* **60**, 410–413.
- Lee, K., Yong, D., Choi, Y. S., Yum, J. H., Kim, J. K., Woodford, N., Livermore, D. M. & Chong, Y. (2007b). Reduced imipenem susceptibility in *Klebsiella pneumoniae* clinical isolates with plasmid-mediated CMY-2 and DHA-1 β -lactamases co-mediated by porin loss. *Int J Antimicrob Agents* **29**, 201–206.
- Lincopan, N., McCulloch, J. A., Reinert, C., Cassettari, V. C., Gales, A. C. & Mamizuka, E. M. (2005). First isolation of metallo- β -lactamase-producing multiresistant *Klebsiella pneumoniae* from a patient in Brazil. *J Clin Microbiol* **43**, 516–519.
- Macrina, F. L., Kopecko, D. J., Jones, K. R., Ayers, D. J. & McCowen, S. M. (1978). A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* **1**, 417–420.
- Martínez-Martínez, L., Hernández-Allés, S., Alberti, S., Tomás, J. M., Benedi, V. J. & Jacoby, G. A. (1996). In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum cephalosporins. *Antimicrob Agents Chemother* **40**, 342–348.
- Mathew, A., Harris, A. M., Marshall, M. J. & Ross, G. W. (1975). The use of analytical isoelectric focusing for detection and identification of β -lactamase. *J Gen Microbiol* **88**, 169–178.
- Mena, A., Plasencia, V., García, L., Hidalgo, O., Ayestarán, J. I., Alberti, S., Borrell, N., Pérez, J. L. & Oliver, A. (2006). Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae* and mechanisms leading to in vivo carbapenem resistance development. *J Clin Microbiol* **44**, 2831–2837.
- Rice, L. B., Carias, L. L., Etter, L. & Shlase, D. M. (1993). Resistance to cefoperazone-sulbactam in *Klebsiella pneumoniae*: evidence for enhanced resistance resulting from the coexistence of two different resistance mechanisms. *Antimicrob Agents Chemother* **37**, 1061–1064.
- Smith Moland, E., Hanson, N. D., Herrera, V. L., Black, J. A., Lockhart, T. J., Hossain, A., Johnson, J. A., Goering, R. V. & Thomson, K. S. (2003). Plasmid mediated, carbapenem-hydrolysing β -lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. *J Antimicrob Chemother* **51**, 711–714.
- Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.
- Wei, Z. Q., Du, X. X., Yu, Y. S., Shen, P., Chen, Y. G. & Li, L. J. (2007). Plasmid-mediated KPC-2 in a *Klebsiella pneumoniae* isolate from China. *Antimicrob Agents Chemother* **51**, 763–765.
- Woodford, N., Tierno, P. M., Young, K., Jr, Tysall, L., Palepou, M.-F. I., Ward, E., Painter, R. E., Suber, D. F., Shungu, D. & other authors (2004). Outbreak of *Klebsiella pneumoniae* producing a new carbapenem-hydrolyzing class A β -lactamase, KPC-3, in a New York medical center. *Antimicrob Agents Chemother* **48**, 4793–4799.
- Yan, J. J., Ko, W. C., Tsai, S. H., Wu, H. M. & Wu, J. J. (2001). Outbreak of infection with multidrug-resistant *Klebsiella pneumoniae* carrying bla_{IMP-8} in a university medical center in Taiwan. *J Clin Microbiol* **39**, 4433–4439.
- Yan, J. J., Ko, W. C., Jung, Y. C., Chuang, C. L. & Wu, J. J. (2002). Emergence of *Klebsiella pneumoniae* isolates producing inducible DHA-1 β -lactamase in a university hospital in Taiwan. *J Clin Microbiol* **40**, 3121–3126.
- Yigit, H., Queenan, A. M., Anderson, G. J., Doménech-Sánchez, A., Biddle, J. W., Steward, C. D., Alberti, S., Bush, K. & Tenover, F. C. (2001). Novel carbapenem-hydrolyzing β -lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **45**, 1151–1161.
- Yu, Y., Ji, S., Chen, Y., Zhou, W., Wei, Z., Li, L. & Ma, Y. (2007). Resistance of strains producing extended-spectrum β -lactamases and genotype distribution in China. *J Infect* **54**, 53–57.
- Zhang, R., Zhou, H. W., Cai, J. C. & Chen, G. X. (2007). Plasmid-mediated carbapenem-hydrolysing β -lactamase KPC-2 in carbapenem-resistant *Serratia marcescens* isolates from Hangzhou, China. *J Antimicrob Chemother* **59**, 574–576.
- Zhang, R., Yang, L., Cai, J. C., Zhou, H. W. & Chen, G. X. (2008). High-level carbapenem resistance in a *Citrobacter freundii* clinical isolate is due to a combination of KPC-2 production and decreased porin expression. *J Med Microbiol* **57**, 332–337.