OmpK26, a Novel Porin Associated with Carbapenem Resistance in Klebsiella pneumoniae

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Clinical isolates of Klebsiella pneumoniae resistant to carbapenems are being isolated with increasing frequency. Loss of the expression of the major nonspecific porins OmpK35/36 is a frequent feature in these isolates. In this study, we looked for porins that could compensate for the loss of the major porins in carbapenem-resistant organisms. Comparison of the outer membrane proteins from two K. pneumoniae clinical isogenic isolates that are susceptible (KpCS-1) and resistant (KpCR-1) to carbapenems revealed the absence of OmpK35/36 and the presence of a new 26-kDa protein in the resistant isolate. An identical result was obtained when another pair of isogenic isolates that are homoresistant (KpN-3) and heteroresistant (KpN-17) to carbapenems were compared. Mass spectrometry and DNA sequencing analysis demonstrated that this new protein, designated OmpK26, is a small monomeric oligogalacturonate-specific porin that belongs to the KdgM family of porins. Insertion-duplication mutagenesis of the OmpK26 coding gene, yjhA, in the carbapenem-resistant, porin-deficient isole KpCR-1 caused the expression of OmpK26 and the reversion to the carbapenem-susceptible phenotype, suggesting that OmpK26 is indispensable for KpCR-1 to lose OmpK36 and become resistant to these antibiotics. Moreover, loss of the major porin and expression of OmpK26 reduced in vitro fitness and attenuated virulence in a murine model of acute systemic infection. Altogether, these results indicate that expression of the oligogalacturonate-specific porin OmpK26 compensates for the absence of OmpK35/36 and allows carbapenem resistance in K. pneumoniae but cannot restore the fitness of the microorganism.

Klebsiella pneumoniae is one of the most prevalent nosocomial enterobacterial pathogens, causing infections that range from mild urinary infections to severe bacteremia and pneumonia with a high rate of mortality and morbidity (19). An increasing worldwide emergence of multidrug resistance among K. pneumoniae nosocomial isolates has limited the therapeutic options for treatment of these infections (4, 6, 13, 17, 22). The multidrug resistance phenotype results from the progressive accumulation of different mechanisms of resistance in the same microorganism, including high-level production of extended-spectrum β-lactamases (ESBLs), overproduction of efflux pumps, and porin deficiency.

K. pneumoniae encodes two major nonspecific porins called OmpK35 and OmpK36 through which nutrients and other hydrophilic molecules, such as β-lactams, penetrate into the bacteria (11). ESBL-producing strains usually are deficient in one of these porins, which, in turn, makes them good candidates for developing a major increase in antimicrobial resistance by losing the expression of the remaining porin (11). Clinical and experimental evidence indicates that a loss of porins in K. pneumoniae strains producing ESBLs causes resistance to cefoxitin, increased resistance to oximino cephalosporins and monobactams, and decreased susceptibility to fluoroquinolones (7, 8, 12, 13, 16, 17). Additionally, in strains producing some CTX-M type β-lactamases or AmpC β-lactamases, porin loss causes reduced susceptibility to carbapenems (6, 17). Besides the major porins, K. pneumoniae may express other porins, such as PhoE, LamB, and OmpK37, which might be crucial in the absence of OmpK35/36. Kaczmarek et al. and García-Sureda et al. reported that PhoE and LamB, respectively, represented alternative porins that could compensate for the function of the major porins in OmpK36/K35-deficient K. pneumoniae clinical isolates, avoiding the penetration of certain antibiotics (10, 13). In contrast, OmpK37, a quiescent porin, played a minimal role in antimicrobial resistance (7).

To identify new porins that could compensate for the loss of the major porins in carbapenem-resistant isolates, we conducted a comparative study of the outer membrane proteins from a pair of isogenic clinical isolates, one susceptible and one resistant. Interestingly, we detected a novel porin, which was exclusively present in the porin-deficient resistant isolate. This work describes the identification of this porin and its contribution to K. pneumoniae antimicrobial resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. K. pneumoniae carbapenem-susceptible clinical strain KpCS-1 and its isogenic carbapenem-resistant derivative
KpCR-1 were isolated from the same patient before and after receiving carbapenem treatment, respectively (17). The pulsed-field gel electrophoresis macrorestriction pattern of KpCR-1 was found to be identical to that of KpCS-1. The carbapenem-heteroresistant clinical strain Kpn-3 and the carbapenem-homoresistant strain Kpn-17 are isogenic epidemic clones isolated at Hospital Ramón y Cajal in Madrid (25). *Escherichia coli* strains used in the cloning experiments were DH5α and strain S17-1 λpir, which encodes protein π from the pir gene essential for replication of plasmid pFS100 (20). Plasmid pGEM-T Easy (Invitrogen) is the TA cloning vector used for cloning PCR products. Plasmids pFS100 (19) and pDT1558 (25) were used to create insertion-duplication mutations by homologous recombination and as a source of the tellurite resistance cassette kil4-Ter, respectively. Plasmid pSH2A, coding the OmpK36 porin, was previously described (16). Bacterial cells were grown in cation-supplemented Mueller-Hinton (MH) or Luria-Bertani (LB) broth at 37°C with shaking or were solidified with 1.5% agar. When necessary, media were supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or tellurite (10 μg/ml).

**Susceptibility testing.** MICs of several antibiotics were determined by using Etest strips (bioMérieux, Marcy l’Etoile, France), following the manufacturer's recommendations.

**Isolation, analysis, and identification of outer membrane proteins.** Isolation of outer membrane proteins (OMP) were performed as previously described (11). Cell envelopes were isolated from *K. pneumoniae* strains by centrifugation at 100,000 × g for 1 h at 4°C after French press cell lysis. OMP were isolated as sodium lauryl sarcosinate-insoluble material. Electrophoretic analysis of OMP by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 11% acrylamide-0.35% bisacrylamide-0.1% SDS by using Laemmli's buffer and Coomassie blue staining. Samples were boiled for 5 min in sample buffer before electrophoresis. Western blot analysis of SDS-PAGE-separated OMP was carried out with the buffers and conditions described by Hernández-Allés et al. (11). Immobilon P membranes (Millipore), rabbit anti-serum raised against purified OmpK36 porin, and alkaline phosphatase-labeled anti-rabbit immunoglobulin G (Sigma). Enzyme was detected with 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium. Selected protein spots were excised from the gels, trypsin digested, and identified by microcapillary liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (MS-MS) by the Unidad de Proteómica at the Universidad Complutense of Madrid, as described elsewhere (5). The search for filtered peptides was performed using GPS Explorer v3.5 software with a licensed version 1.9 of MASCOT.

**DNA procedures.** Plasmid DNA was isolated using the Wizard Miniprep kit (Promega) according to the manufacturer's instructions. Isolation of genomic DNA, transformation, and conjugations were carried out by standard techniques (1). T4 DNA ligase and restriction endonucleases were used following the manufacturer's recommendations (GE Healthcare). DNA fragments prepared by restriction enzyme digestion were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. PCRs were performed according to standard techniques (1). For Southern blot analysis and probe labeling and detection, we used the ECL kit (GE Healthcare) according to the manufacturer's protocol.

The internal fragment of the OmpK26 coding gene, yhJ4, used to generate a knockout mutant and as a probe in the Southern blot experiments, was obtained by PCR amplification of the *K. pneumoniae* KpCR-1 genomic DNA with primers YhJaF3 (5'-CTGGTTTCCTCGGCCCTCTGCGG-3') and YhJaR2 (5'-CCATA CGGGGACGAGTATCC-3').

DNA sequencing was performed using the BigDye Terminator kit (PE-Applied Biosystems), and sequences were analyzed with the ABI Prism 3100 DNA sequencer (PE-Applied Biosystems). Alignments were generated by ClustalW and subsequently rectified to position insertions and deletions in loop regions.

**Construction of an OmpK26 knockout mutant from strain KpCR-1.** To generate an OmpK26 knockout mutant from KpCR-1, an internal fragment of the gene yhJ4, obtained by PCR as described above, was first cloned into pGEM-T Easy and then transferred into the *π* protein-dependent shuttle vector pFS100 digested with EcoRI to generate plasmid pFSOMP26, which was endowed with the tellurite resistance from pDT1558 and introduced in *K. pneumoniae* by conjugation. One integrant of plasmid pFSOMP26-Te into the *K. pneumoniae* chromosome, which disrupted expression of yhJ4, was further investigated.

**In vitro competition experiments.** In *in vitro* competition experiments were performed as previously described (18). Briefly, exponentially growing cells of the corresponding strains in LB broth were mixed in a 1:1 ratio and diluted in Ringer solution. Approximately, 10^7 cells from each of the mixtures were inoculated into eight 10-ml LB broth tubes and grown at 37°C and 180 rpm for 9 h. Serial 10-fold dilutions were plated in duplicate onto LB agar alone and LB agar with the selective markers (meropenem [0.75 μg/ml] for KpCR-1 or tellurite [10 μg/ml] for KpCR-1ΔOompK26), in order to determine the total CFU and the CFU of the mutant, respectively, after overnight incubation at 37°C. Alternatively, when the difference in the numbers of colonies between the LB plates with agar alone and the LB plates with the selective marker was low (less than 2-fold), 100 randomly selected colonies from the LB plates were replicated in LB plates with the appropriate selection; mutant and wild-type colonies were recognized by the presence or absence of growth in these plates after overnight incubation. The competition index (CI) was defined as the mutant/wild-type ratio. CI values were calculated for each of the independent competitions, and the median values were recorded. Statistical analysis of the distribution of the CI values was performed using the Student’s t test. P values of <0.05 were considered to be statistically significant. During the standardization of the procedure, the absence of spontaneous loss of meropenem resistance was confirmed by plating KpCR-1 on LB agar plates with or without meropenem. Moreover, competition experiments between strains KpCR-1 and KpCR-1-pSH4A (harboring a vector with the tellurite resistance cassette) were performed; the CI values obtained (median, 0.94) ruled out any significant effect of the tellurite resistance cassette on bacterial fitness. To assess the growth rates under noncompetitive conditions, the doubling times of exponentially growing cells in LB broth at 37°C and 180 rpm were determined by plating serial 10-fold dilutions on LB at 1-h intervals. Three independent experiments were performed for each of the strains, and the results were compared using Student’s t test.

**Virulence and fitness in a murine model of systemic infection.** Virulence and fitness studies were performed with male ICR-CD1 mice, each weighing 20 to 30 g. For the mouse lethality studies, approximately 2 × 10^6 CFU of *K. pneumoniae* from an early-log-phase broth culture was administered by intraperitoneal injection to groups of 18 mice. Mortality was monitored daily for 7 days. In *in vivo* fitness was assessed by competition experiments in the mouse model of systemic infection. For this purpose, 1:1 mixtures of each of the pairs of tested strains containing a total of approximately 5 × 10^6 exponentially growing cells were inoculated intraperitoneally into at least 10 mice. Mice were sacrificed 24 h...
after inoculation, and their spleens were aseptically extracted and homogenized in 5 ml of 0.9% saline solution. The number of CFU of each strain and the CI values were determined as described for in vitro competition experiments.

RESULTS AND DISCUSSION

Identification of OmpK26. In order to seek for porins that could compensate for the loss of the major porins in carbapenem-resistant organisms, we compared the outer membrane proteins from a carbapenem-susceptible isolate and its derived carbapenem-resistant isogenic clinical isolate. As shown in Fig. 1A, outer membranes prepared from the susceptible isolate KpCS-1 analyzed by SDS-PAGE contained visible amounts of the major porin OmpK36 (indicated with white arrows in Fig. 1A), while membranes prepared from the resistant isolate KpCR-1 did not. The porin nature of the 36-kDa protein present in the susceptible isolate and absent in the resistant one was confirmed by Western blot analysis using specific antibodies against OmpK36 and MS analysis (Fig. 1B). PCR amplification and sequencing of the \textit{ompK36} gene from KpCR-1 revealed the presence of IS26 in the \textit{ompK36} coding region (17). In contrast, membranes from KpCR-1 contained a protein of about 26 kDa that was not present in the membranes of KpCS-1 (indicated with a black arrow in Fig. 1A). This result establishes that the resistance phenotype in this isolate correlates with the absence of OmpK36 porin and the presence of a novel protein of about 26 kDa in the outer membrane. A similar phenomenon was observed when we compared the outer membrane proteins of a pair of clinical isolates that are heteroresistant (Kpn-3) and homoresistant (Kpn-17) to carbapenems. The heteroresistant Kpn-3 isolate, which contains only a subpopulation of a few cells that grow in the presence of a high concentration of imipenem (32 μg/ml), expressed OmpK36, while the homoresistant isolate Kpn-17, which is highly resistant to imipenem (MIC, >128 μg/ml), failed to express OmpK36 and expressed a novel protein of about 26 kDa (Fig. 1, lanes 3 and 4, respectively). To identify the 26-kDa protein, the corresponding band was excised from the gel and the protein was subjected to MS analysis. In each resistant isolate, the band was found to correspond to a putative oligogalacturonate-specific porin homol-
nologous to KdgM, designated OmpK26. Interestingly, a protein with the same molecular weight appeared in two nonsusceptible carbapenem K. pneumoniae strains isolated in a Chinese hospital (26). The 15 determined N-terminal amino acids of this protein showed 100% similarity to the protein identified in our isolates, although its contribution to carbapenem resistance was not further investigated.

The OmpK26 coding gene, yjhA, from KpCR-1 and Kpn-17 was sequenced and showed 100% homology with the gene that originated from K. pneumoniae subsp. pneumoniae MGH 78578 (GenBank accession no. YP_001336895).

The amino acid sequence of K. pneumoniae OmpK26 was aligned with the sequences of the oligogalacturonate-specific porins NanC from E. coli and KdgM from Dickeya dadantii, whose secondary structure has been predicted (21, 27), on the basis of the conservation of the β-strands and some key residues (highlighted in black in Fig. 2) that are highly conserved in this new family of small monomeric outer membrane channels. OmpK26 presents a typical β-strand structure with five short periplasmic turns and six extracellular loops of variable length. The two facing strings of arginines and lysines inside the pores of KdgM and NanC, which play an important role in ion conductance and selectivity properties, are also present in OmpK26 (indicated with black circles in Fig. 2), suggesting that, as in other KdgM family members, this porin is involved in the transport of acidic oligosaccharides. It is likely that the characteristics of the transmembrane channel of OmpK26 differ from those of OmpK36 as it occurs with NanC from E. coli, which, at the constriction zone, exhibits an electrostatic quadrupole instead of the dipolar arrangement described in OmpK36 (9, 27). This structural difference between OmpK36 and OmpK26 might lead to a lower penetration of carbapenems and cause a reduced susceptibility in OmpK26-deficient strains.

**Role of OmpK26 in antimicrobial resistance.** To investigate the role of this porin in antimicrobial resistance, we constructed a knockout mutant from K. pneumoniae KpCR-1 by insertion-duplication of the OmpK26 encoding gene, yjhA. A schematic representation of the resistant isolate KpCR-1 and the knockout KpCR-1ΔOmpK26 chromosomes is shown in Fig. 3A. Southern blot analysis of the genomic DNA of the knockout using a specific probe for yjhA confirmed that two incomplete copies of the gene were generated by the integration of the plasmid (Fig. 3B). SDS-PAGE analysis of the outer membrane proteins of KpCR-1ΔOmpK26 confirmed that the mutation of yjhA abolished the expression of OmpK26 but also revealed that the OmpK36-deficient phenotype, which is highly stable, reverted to the wild-type phenotype (Fig. 3C). Western blot analysis with specific anti-OmpK36 antibodies and MS-MS analysis confirmed the presence of OmpK36 in KpCR-1ΔOmpK26 (Fig. 3D).

Expression of OmpK36 in KpCR-1ΔOmpK26 reduced the MICs of ceftazidime, cefotaxim, and carbapenems to values similar to the MICs for the related clinical isolate KpCS-1 (Table 1). Identical results were obtained when KpCR-1 was transformed with plasmid pSHA2 carrying the complete gene.
coding for OmpK36 porin. Altogether, these results complement previous observations in which carbapenem resistance was associated with OmpK36 deficiency (14, 15, 17, 26) and strongly support the contribution of OmpK36 to the penetration of these antibiotics.

Several attempts to generate by insertion-duplication mutagenesis an OmpK26 knockout mutant derived from KpCR-1 without reversion of OmpK36 expression were unsuccessful, suggesting that expression of OmpK26 is an essential mechanism for K. pneumoniae to compensate for the loss of the major porin OmpK36. Two other porins, PhoE (13) and LamB (10), have been reported to compensate for the absence of the OmpK35/36 porins in K. pneumoniae. However, in both studies, reduced carbapenem susceptibility was correlated with the absence of PhoE or LamB while, in our study, resistance to these antibiotics correlates with the expression of OmpK26. Indeed, abolishment of LamB expression in a LamB-expressing K. pneumoniae strain caused expression of OmpK26 and reduced susceptibility to meropenem (10). To the best of our knowledge, this is the first porin of K. pneumoniae that may represent an alternative pore for the uptake of nutrients and allows the resistance to carbapenems in the absence of the major nonspecific porins.

**Biological cost of porin deficiency in K. pneumoniae expressing OmpK26.** KdgM plays a crucial role in the virulence of the plant pathogen D. dadantii, allowing the entrance of the oligogalacturonates produced by plant cell wall degradation (3). In contrast, the biological significance of the members of the KdgM family of porins in the Enterobacteriaceae remains poorly investigated. It is likely that the expression of these porins could allow the uptake and bacterial growth on sialic acid present in the host as the only carbon and nitrogen source. However, the monomers and oligomers of sialic acid are efficiently translocated through OmpF/OmpC and the involvement of the oligogalacturonate-specific porins may be minimal. This might not be the case in the porin-deficient multiresistant microorganisms, where OmpK26 expression could be critical to compensate for the absence of the major porins and maintain the fitness of the microorganism.

To investigate the biological cost of the absence of the major porins in K. pneumoniae expressing OmpK26, we conducted a set of in vitro competition experiments (Fig. 4). All of the strains used in the competition experiments showed similar growth rates with insignificant differences; doubling time in the mid-log phase ranged from 33.3 min to 36.1 min. The results of KpCR-1/KpCS-1 competition (median CI, 0.035; P = 0.0036) showed a marked decrease in fitness of KpCR-1. Similarly, the KpCR-1 ΔOmpK26 mutant expressing OmpK36 outcompeted KpCR-1 (median CI for the competition of KpCR-1 and KpCR-1 ΔOmpK26, 0.101; P = 0.0023). In contrast, KpCR-1 ΔOmpK26 retained full in vivo fitness relative to that of the wild-type strain, since it was not outcompeted by KpCS-1 (median CI, 0.679; P < 0.001). Overall, the results of the in vitro competition experiments show that OmpK36 deficiency has a significant effect on the fitness of the strain for which the expression of OmpK26 cannot compensate. Moreover, expression of OmpK36 in KpCR-1 ΔOmpK26 restored the fitness to the levels of the parental strain.

The fitness of these strains was also evaluated in a murine model of systemic infection (Fig. 5). KpCR-1 was strongly outcompeted by the parent strain KpCS-1, as shown by the median CI of 0.004 (P = 0.01) in bacteria recovered from the spleen 24 h after intraperitoneal inoculation. Similarly, KpCR-1 was also outcompeted by KpCR-1 ΔOmpK26 (median CI, 0.008; P = 0.0028). As was found in the in vitro experiments, KpCR-1 ΔOmpK26 was as competent as KpCS-1 (median CI, 0.7). To study the virulence, the mortality of groups of 18 mice inoculated with each of the strains studied was monitored daily for 7 days (Fig. 6). The mortality of mice infected with KpCS-1 (50%) or KpCR-1 ΔOmpK26 (83.3%) was significantly higher (P < 0.05) than that of mice infected with...
KpCR-1, which was unable to kill any mice. In contrast, there were no significant differences in the survival rates between the mice infected with KpCS-1 and those infected with KpCR-1ΔOmpK26.

These results indicate that OmpK35/36 deficiency reduces the virulence of K. pneumoniae in a mouse model of systemic infection, and they are consistent with those recently reported by Bialek et al. (2) and Tsai et al. (24) which, using a P. aeruginosa infection, and they are consistent with those recently reported which may help in explaining the attenuated phenotype.

Therefore, although bacteria with altered OmpK35/36 expression represent a threat because they exhibit increased levels of antibiotic selective pressure, they are less competitive than the wild-type strains.

In summary, we showed that the expression of the oligogalacturonate-specific porin OmpK26 compensated for the absence of the expression of OmpK35/36 in carbapenem-resistant K. pneumoniae but could not restore the fitness of the microorganism either in vitro or in vivo.

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