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Klebsiella pneumoniae Major Porins OmpK35 and OmpK36 Allow More Efficient Diffusion of β-Lactams than Their *Escherichia coli* Homologs OmpF and OmpC

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ABSTRACT

***Klebsiella pneumoniae*, one of the most important nosocomial pathogens, is becoming a major problem in health care because of its resistance to multiple antibiotics, including cephalosporins of the latest generation and, more recently, even carbapenems. This is largely due to the spread of plasmid-encoded extended-spectrum β-lactamases. However, antimicrobial agents must first penetrate the outer membrane barrier in order to reach their targets, and hydrophilic and charged β-lactams presumably diffuse through the porin channels. Unfortunately, the properties of *K. pneumoniae* porin channels are largely unknown. In this study, we made clean deletions of *K. pneumoniae* porin genes *ompK35* and *ompK36* and examined the antibiotic susceptibilities and diffusion rates of β-lactams. The results showed that OmpK35 and OmpK36 produced larger more permeable channels than their *Escherichia coli* homologs OmpF and OmpC; OmpK35 especially produced a diffusion channel of remarkably high permeability toward lipophilic (benzylpenicillin) and large (cefepime) compounds. These results were also confirmed by expressing various porins in an *E. coli* strain lacking major porins and the major multidrug efflux pump AcrAB. Our data explain why the development of drug resistance in *K. pneumoniae* is so often accompanied by the mutational loss of its porins, especially OmpK35, in addition to the various plasmid-carried genes of antibiotic resistance, because even hydrolysis by β-lactamases becomes inefficient in producing high levels of resistance if the bacterium continues to allow a rapid influx of β-lactams through its wide porin channels.**

IMPORTANCE

In Gram-negative bacteria, drugs must first enter the outer membrane, usually through porin channels. Thus, the quantitative examination of influx rates is essential for the assessment of resistance mechanisms, yet no such studies exist for a very important nosocomial pathogen, *Klebsiella pneumoniae*. We found that the larger channel porin of this organism, OmpK35, produces a significantly larger channel than its *Escherichia coli* homolog, OmpF. This makes unmodified *K. pneumoniae* strains more susceptible to relatively large antibiotics, such as the third- and fourth-generation cephalosporins. Also, even the acquisition of powerful β-lactamases is not likely to make them fully resistant in the presence of such an effective influx process, explaining why so many clinical isolates of this organism lack porins.

Klebsiella pneumoniae is currently one of the most important Gram-negative nosocomial pathogens, which are often carbapenem resistant (1), and its wide dissemination is helped by its capacity to survive in the hospital environment (2), presumably aided by the production of thick capsules. A remarkable feature of multidrug-resistant isolates of this species is the absence of porin(s) (for an early report, see reference 3, and for reviews, see references 4 and 5). *K. pneumoniae* produces two classical trimeric porins, OmpK35 and OmpK36, which are homologs of OmpF and OmpC of *Escherichia coli*, respectively (6, 7). Although OmpK35 loss seems to be more often reported than that of OmpK36, the separation and identification of these two porin species are often difficult (8).

Antimicrobial drugs must first penetrate the outer membrane to reach the periplasm, and with β-lactams that are generally hydrophilic and charged, porin channels appear to be the principal route of penetration (4). Thus, it is essential to understand the properties of the porin channels in order to elucidate the mechanism of resistance, and those in *E. coli* have been studied extensively (9–13). However, in other organisms of *Enterobacteriaceae*, including *K. pneumoniae*, hardly any study exists except a few earlier scattered studies on *Enterobacter cloacae* (14), *Serratia marcescens* (15), and *Proteus*, *Morganella*, and *Providencia* (16), and the permeation of first-generation cephalosporins was recently examined in *Enterobacter aerogenes* (E. Sugawara and H. Nikaido, unpublished data). We therefore examined carefully the permeability properties of *K. pneumoniae* porin channels, with the hope of understanding why porin-deficient strains are so frequent among resistant clinical isolates of this species.

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MATERIALS AND METHODS

Bacterial strains. *E. coli* strain LA51AFC (Δ*acrAB* Δ*ompF*::Tn*5* Δ*ompC*) was constructed previously (13). JW4111-2A is an Δ*acrAB* derivative of JW4111-2 [Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ⁻ *rph-1* Δ(*rhaD-rhaB*)*568* Δ*ampC777*::*kan hsdR514*] (obtained from Coli Genetic Stock Center, Yale University, CT). First, the *kan* gene was removed by using FLP recombinase from pCP20 (17). JW4111-2A was then constructed by transducing the Δ*acrAB*::*kan* gene from strain RAM1337 (18), and finally, the *kan* marker was removed as described above. JW4111-2AF is an *ompF*::Tn*5* derivative of JW4112-2A and was constructed by transducing the *ompF*::Tn*5* gene from strain LA51A Δ*ompF* (12). JW4111-2AFC is a derivative lacking both OmpF and OmpC and was made by transducing the Δ*ompC* mutant from CS1253 (19), selecting for the linked *zei298*::Tn*10* marker with 10 μg/ml tetracycline. Transduction was done using P1*cml*,*clr100* phage, according to standard protocols (20). *E. coli* strain *Ec CC118* λ*pir* and vector pJTOOL3 were gifts from K. Rajakumar, University of Leicester.

Construction of *ompK35*- or *ompK36*-null mutant of *K. pneumoniae*. *K. pneumoniae* strains ATCC 11296 Δ*ompK35* and ATCC 11296 Δ*ompK36* were constructed by using long primers and a suicide vector (21). The *ompK35* or *ompK36* gene was replaced by a gentamicin resistance (Gm^r) gene by using a gene replacement plasmid, pJTOOL-3. Two regions located upstream (860 bp) and downstream (400 bp) from the *ompK35* gene were amplified by using primers (K35Up-F, 5′-ATAAGAATGCGGCCGCCGGCAATGGCAGTTCTGGCGCCGGGCA-3′; K35Up-R, 5′-GCTCTAGATATTTATTACCCTCATTAATATTTTTATATGAACACGTGCC-3′; K35Dn-F, 5′-GCTCTAGACAAGAACATGAACGTCTAGCCTGCGTATAAATT-3′; and K35Dn-R, 5′-ACGCGTCGACAATACCATCGATGCCCAGATAGTTTTTAGTCGT-3′). These PCR products, the *ompK35* upstream region, and *ompK35* downstream region were digested and ligated between the NotI and XbaI sites and the XbaI and SalI sites, respectively, of pJTOOL-3, creating pJTOOL-3_ompK35Up-Down. In order to create a gentamicin resistance gene with the FLP recombination target (FRT) site at both ends, it was amplified by using primers containing FRT site (Gm^R-F, 5′-GCTCTAGAGAAGTTCCTATTCTCAAGAAAGTATAGGAACTTCCTAGCGCGTCGACATAAGCCTGTTCGGTTCGTA-3′, and Gm^R-R, 5′-GCTCTAGAGAAGTTCCTATACTTTCTTGAGAATAGGAACTTCCTAGCGCGTCGTCGGCCGGGAAGCCGATC-3′), by using the vector pUCGm-lox as the template. The PCR product was digested and ligated into the XbaI site of pJTOOL-3_ompK35Up-Down, creating an *ompK35* deletion plasmid, pJTOOL-3_*ompK35-Gm^R*. The *ompK36* deletion plasmid, pJTOOL-3_*ompK36-Gm^R* was constructed in a similar manner. (The primer sequences used are available from the authors.)

The gene replacement plasmid, pJTOOL-3_*ompK35-Gm^R*, or pJTOOL-3_*ompK36-Gm^R*, was introduced into *K. pneumoniae* strain ATCC 11296 by conjugation, because *K. pneumoniae* strains are covered with thick capsules that makes electroporation difficult. We used the protocol described by van Aartsen and Rajakumar (21). Overnight cultures of the recipient *K. pneumoniae* strain ATCC 11296 and donor *E. coli* S17-1 λ⁻ *pir*/pJTOOL-3_*ompK35-Gm^R*, or *E. coli* S17-1 λ⁻ *pir*/pJTOOL-3_*ompK36-Gm^R*, were diluted 1:100 (recipient) and 1:50 (donor) into fresh LB and grown until the optical density at 600 nm (OD_{600}) reached around 0.8. One milliliter of each culture was mixed, pelleted, and resuspended into 20 μl of 10 mM $MgSO_4$. The mating mixture was spotted onto LB agar plates and incubated overnight at 37°C. The next day, the mating mixtures were resuspended into 1 ml of 10 mM $MgSO_4$, and a 100-μl portion of the suspension (10^9 to 10^{10} cells) was plated onto M9 minimal medium containing gentamicin (9 μg/ml) and incubated at 37°C for 2 days. Integrants of the Gm^r plasmid were then plated on LB agar without NaCl, supplemented with 6% (wt/vol) sucrose, and incubated overnight at room temperature in order to select for strains that had lost the plasmid sequence. Sucrose-resistant colonies were ascertained to have the chloramphenicol-sensitive phenotype as the result of plasmid eviction. The absence of the *ompK35* or *ompK36* gene sequence was monitored by PCR, and the identity of the remaining porin OmpK36 or OmpK35 was confirmed by mass spectrometry (Proteomics/Mass Spectrometry Laboratory at UC Berkeley) of the tryptic lysates of the protein band in the urea-SDS-PAGE. The programs SEQUEST (22) and DTASELECT (23) were used to identify peptides and proteins from a custom database consisting of OmpK35 and OmpK36 sequences and common contaminants.

Cloning of the *ompK35*, *ompK36*, *ompF*, and *ompC* genes. For constructing the OmpK36 expression vector, the *ompK36* gene of *K. pneumoniae*, together with 170 and 27 nucleotides of upstream and downstream sequences, was amplified from chromosomal DNA of ATCC 11296 and cloned into pHSG575 (24). For OmpK35, the *ompK35* coding sequence was amplified from the same source and fused by overlapping PCR with the upstream region of the *E. coli ompF* gene (base positions −213 to −1) containing its promoter sequence. This construct was then inserted into pHSG575. The construction of pHSG575-based expression plasmids for *E. coli ompF* and *ompC* genes has been described earlier (13).

Cloning of *oxa163* gene. The *oxa163* gene (808 bp) (25), with a PstI site at both ends, was synthesized by GenScript (Piscataway, NJ). This gene was digested and ligated into the PstI site of pBAD33, creating pBAD33_*oxa163*. In order to create coexpression system in *E. coli* JW4111-2AFC, we had to remove the chloramphenicol resistance (Cm^r) gene in the pBAD33_*oxa163*, because the porin expression plasmid pHSG575 contains the same selective marker. This gene was deleted by digestion of the plasmid with Eco47III and StyI and religation after the T4 DNA polymerase treatment (26), creating pBAD33Δ*Cm^r*_*oxa163*. The *oxa163* gene was expressed under the arabinose promoter in this plasmid, and cephalosporin resistance could be used as the selective marker for the presence of plasmid.

Influx assay for penicillins. An influx assay of penicillins in *E. coli* was carried out using LA51AFC, which lacks both OmpF and OmpC, as well as the main efflux pump AcrAB, as the host by combining the drug influx with their subsequent hydrolysis in the periplasm in intact cells (27). We relied on the chromosomal AmpC enzyme for hydrolysis (12). For the expression of each porin in LA51AFC, pHSG575_*ompK35*, pHSG575_*ompK36*, pHSG575_*ompF*, or pHSG575_*ompC* was transformed into LA51AFC. Fresh transformants selected on LB agar plates containing 10 μg/ml chloramphenicol were grown in LB medium containing 5 mM $MgCl_2$ at 37°C with shaking and harvested at late-exponential phase (OD_{600}, 1). Cells were then suspended in KP buffer (50 mM potassium phosphate buffer [pH 7.0] containing 5 mM $MgCl_2$). The assay was started by mixing the cell suspension with substrates (ampicillin or benzylpenicillin) in a glass test tube at room temperature. After the incubation period of 0, 5, 10, and 15 min, the reaction was stopped by heating the test tube in a boiling water bath for 1 min with constant shaking. The hydrolysis product was quantified by microiodometry (28). The hydrolysis rate of the substrate is equal to the influx rate (V_{in}) of the substrate, because active efflux was negligible in this system thanks to the deletion of the major efflux system genes *acrAB*. Calculation of the permeability coefficients was done as described earlier (12).

The diffusion rates of penicillins through the outer membrane of *K. pneumoniae* were determined by measuring the rates of their hydrolysis by the endogenous periplasmic β-lactamase in intact cells. The assay was performed in the same way as described above except for the addition of 40 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in the reaction mixture to inhibit the efflux pump(s). Calculation of the permeability coefficient was done as described above, by assuming that the cell surface area per unit weight of *K. pneumoniae* is the same as that in *E. coli*.

Influx assay for cephalosporins. The permeability of cephalosporins was determined by using a coexpression system in *E. coli* strain JW4111-2AFC [Δ(*araD-araB*) Δ*ampC* Δ*acrAB ompF*::Tn*5* Δ*ompC*], because the introduction of OXA-163 β-lactamase (25), besides the expression of porin, was necessary for the hydrolysis of extended-spectrum cephalosporins. Although a copy of the *ampC* gene was indeed replaced by a kanamycin cassette in the parent strain JW4111-2, an intact *ampC* gene(s) was

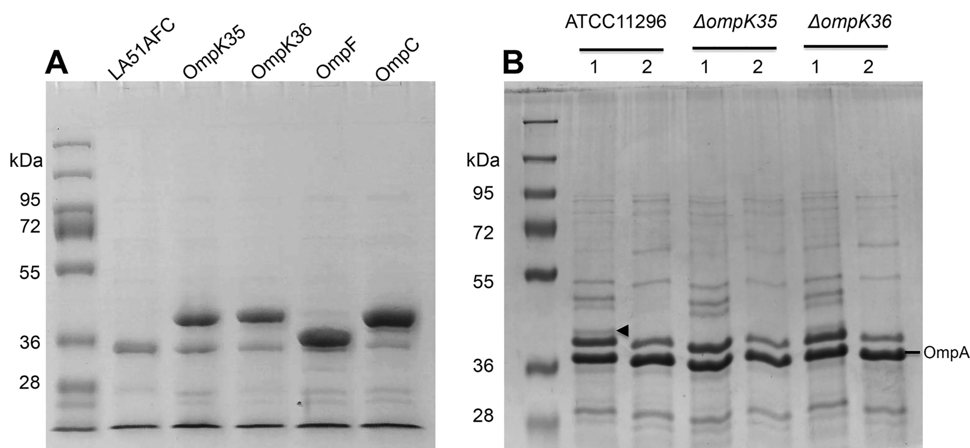

FIG 1 SDS-PAGE of the outer membrane proteins from *K. pneumoniae* ATCC 11296 and its mutants, Δ*ompK35* and Δ*ompK36*. (A) Samples containing 10 μg of total outer membrane proteins from *E. coli* LA51AFC expressing each porin were applied to 10% SDS-PAGE containing 6 M urea after heating at 100°C for 5 min in the sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 5% β-mercaptoethanol, 2% SDS). (B) The total outer membrane proteins from *K. pneumoniae* ATCC 11296 and its mutants, Δ*ompK35* and Δ*ompK36*, grown in nutrient broth (lane 1) or nutrient broth plus 20% sorbitol (lane 2) at 37 C overnight were heated at 100°C for 5 min in the sample buffer. Samples containing 10 μg of total outer membrane proteins were applied to SDS-PAGE (separating gel contained 11% acrylamide, 0.21% bisacrylamide, 0.2% SDS, 0.375 M Tris-HCl [pH 8.8], 0.025% ammonium persulfate, and 0.2% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine [TEMED]) (8). The band identified as OmpK35 is marked by an arrowhead, and the position of OmpA band is also indicated.

detected by PCR with primers in this strain, and AmpC activity was still detected in this strain (results not shown). However, because the AmpC enzyme hydrolyzes extended-spectrum cephalosporins with very low V_{max} values (14), we believe that it contributes little to the much more rapid hydrolysis of these compounds caused by OXA-163. The plasmid pBAD33Δ*Cm*ʳ*_oxa163* was transformed into JW4111-2AFC by electroporation, and transformants were selected in an LB agar plate containing 10 mM L-arabinose and 10 μg/ml oxacillin. The next day, 0.6 ml of an overnight culture of JW4111-2AFC/pBAD33Δ*Cm*ʳ*_oxa163* was diluted into 60 ml of LB containing 10 mM L-arabinose and 10 μg/ml oxacillin, and the cells were grown with shaking at 37°C until the OD_{600} reached about 0.6. These cells were washed three times with ice-cold 10% glycerol and were resuspended in 0.5 ml of 10% glycerol. The porin expression plasmid was transformed into these cells by electroporation, and the transformants were selected on LB agar plates containing 10 μg/ml chloramphenicol, 10 mM L-arabinose, and 10 μg/ml oxacillin. Fresh transformants were then used for the influx assay. Cells were grown in LB medium containing 5 mM $MgCl_2$ at 37°C with shaking and harvested at late-exponential phase (OD_{600}, 1). Cells were washed once and were resuspended in NaP buffer (10 mM sodium phosphate buffer [pH 6.0] containing 5 mM $MgCl_2$). Intact cells (0.15 mg [dry weight]) or sonicated extract were added to an assay medium containing 1 mM cephalosporin in the same buffer (final volume, 0.5 ml). The suspension was mixed and rapidly transferred to a cuvette with a 1-mm light path, and the decrease in optical density at 260 nm was monitored with a Uvikon 860 spectrophotometer (11). The permeability coefficients were calculated as described above and corrected for the expression levels of each porin, as indicated by the analysis with urea-SDS-PAGE.

MIC determination. MICs were determined with 96-well microtiter plates using a standard 2-fold broth microdilution method with LB broth containing 5 mM $MgCl_2$. Approximately 10^3 cells were inoculated, and the results were read after incubation at 37°C for 16 h.

Preparation of crude outer membrane fractions. The crude envelope fraction, prepared with a French pressure cell disruption followed by centrifugation, was extracted with 0.5% Sarkosyl (29) to remove inner membrane proteins. The Sarkosyl-insoluble fraction containing outer membrane fragments was collected by high-speed centrifugation for 45 min at 60,000 rpm with a Beckman TLA100.2 ultracentrifuge. The pellet was washed with 1 ml of 20 mM HEPES-NaOH buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and finally resuspended in 50 μl of the same buffer.

Liposome swelling assay. The pore-forming activity of each porin was assayed by determining the osmotic swelling rates of proteoliposomes reconstituted with crude *E. coli* LA51AFC outer membranes obtained from the cells expressing only OmpK35, OmpK36, OmpF, or OmpC in an isotonic solution of sugars, as described earlier (10).

Outer membrane protein analysis on SDS-PAGE. All samples were dissolved in lysis buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue in 0.125 M Tris-HCl [pH 6.8]). After heating for 100°C for 5 min, samples corresponding 10 μg of outer membrane protein were separated in SDS-PAGE, as described in the legend for Fig. 1. Staining was performed with 0.05% Coomassie brilliant blue R250 in 25% 2-propanol and 10% acetic acid for 1 h. Image Studio Lite (Li-Cor Biotechnology) was used for the quantification of protein bands.

Identification of endogenous β-lactamase in *K. pneumoniae*. The standard isoelectric focusing (IEF) procedure was used with a precast gel (Novex pH 3 to 10; Invitrogen), followed by nitrocefin staining to evaluate the expression of β-lactamase genes in *K. pneumoniae*. We used a pI 4.45 to 9.6 IEF standards from Bio-Rad as well as the OXA-7 enzyme with pI 7.6 as a reference.

RESULTS

Drug diffusion through OmpK35 and OmpK36 in *K. pneumoniae*. *K. pneumoniae* produces three major proteins, OmpK35, OmpK36, and OmpA, in its outer membrane. Based on the sequence similarities of the *ompK35* and *ompK36* genes, OmpK35 is a homolog of OmpF (6) and OmpK36 is a homolog of OmpC (7). Since both OmpF and OmpC proteins are known to be nonspecific diffusion channels with high permeability in the outer membrane of *E. coli*, we assume that OmpK35 and OmpK36 have a similar function in the outer membrane of *K. pneumoniae*, although there are no quantitative data on the permeability of these diffusion channels. In order to evaluate the role of these proteins in *K. pneumoniae*, we constructed clean single-deletion mutants, the Δ*ompK35* or Δ*ompK36* mutant, in the strain ATCC 11296 using the method of van Aartsen and Rajakumar (21) (see Materials and Methods).

When the outer membrane proteins from each mutant were separated on SDS-PAGE, porin bands were found above the band of OmpA (Fig. 1B). The identification of OmpK35 and OmpK36,

TABLE 1 MICs of antibiotics for *K. pneumoniae* ATCC 11296 and its derivatives

Antibiotic	Hydrophobicity of uncharged form[b]	MIC[a] (μg/ml) for: ATCC 11296	ATCC 11296 Δ*ompK35*	ATCC 11296 Δ*ompK36*
Ampicillin	−1.1	50	**100**	50
Benzylpenicillin	1.8	100	**200**	100
Oxacillin	2.4	1,000	1,000	1,000
Cloxacillin	2.4	1,000	1,000	1,000
Cephalothin	−0.4	8	**32**	**16**
Cephaloridine	1.9	8	8	8
Cefoxitin	0	8	**32**	8
Cefamandole	−0.9	4	**8**	4
Cefotaxime	−1.4	0.25	**1**	0.25
Ceftazidime	0.4	1	**4**	1
Ceftriaxone	−1.3	0.06	**0.50**	**0.25**
Cefepime	−0.1	0.12	**0.50**	0.12
Imipenem	−0.7	0.12	**0.25**	0.12
Ertapenem	−1.5	0.015	**0.06**	0.015
Novobiocin	3.3	640	640	640
Erythromycin	2.7	640	640	640

[a] Numbers in bold indicate MICs that differ significantly from that for the wild type.
[b] Values are from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) and were obtained using XLogP3.

TABLE 2 Permeability coefficients of penicillins in porin mutants of *K. pneumoniae* ATCC 11296

Substrate (concn [μM])	Strain	Permeability coefficient[a] (nm/s) (no. of expts)
Ampicillin (60)	Parent	67 ± 18 (9)
	Δ*ompK35* mutant	37 ± 14 (10)
	Δ*ompK36* mutant	47 ± 14 (4)
Benzylpenicillin (150)	Parent	74 ± 24 (5)
	Δ*ompK35* mutant	46 ± 16 (5)
	Δ*ompK36* mutant	71 ± 24 (3)

[a] Values are averages ± standard deviations.

which appear as closely spaced double bands, was difficult, as has been reported by others (6, 8). In gels containing 6 M urea, OmpK35 produced in our *E. coli* host appears to migrate very slightly ahead of OmpK36 (Fig. 1A). However, in SDS gels not containing urea, the upper thinner band totally disappeared when *K. pneumoniae* was grown in high-osmolality medium containing 20% sorbitol (Fig. 1B, lane 2 of ATCC 11296). Since the production of OmpK35 is known to be repressed in high-osmolality media (6), we identified OmpK35 with the upper band in this gel; it has been also reported by others that OmpK35 migrates more slowly than OmpK36 in SDS-PAGE not containing urea (8, 30). In our Δ*ompK35* mutant, this band was indeed absent (Fig. 1B). In our Δ*ompK36* mutant, a porin band was seen, but confusingly, the pattern seemed similar to that of the parent strain ATCC 11296 (Fig. 1B). Nevertheless, mass spectrometry of the tryptic digest of this protein band unambiguously confirmed it to be OmpK35, and we suspect that its position of migration was altered as a consequence of the absence of the normally predominant OmpK36. As a control, similar mass spectrometry analysis of the porin band in the Δ*ompK35* mutant identified it as OmpK36.

The growth rates of these mutants in LB containing 50 mM NaCl and 5 mM $MgCl_2$ at 37°C were similar to that of parent strain (doubling times of the parent, Δ*ompK35* mutant, and Δ*ompK36* mutant strains were 27, 24, and 28 min, respectively).

MICs were determined for β-lactams and other antibiotics (Table 1). The parent strain ATCC 11296 showed high MICs for penicillins, including ampicillin, benzylpenicillin, oxacillin, and cloxacillin, but was relatively susceptible to third- and fourth-generation cephalosporins. Most *K. pneumoniae* strains are known to produce chromosomally encoded SHV-1-like β-lactamase (31, 32), which is likely to contribute to this pattern of susceptibility. (The pI of the endogenous β-lactamase extracted from the parent strain by sonication was similar to the OXA-7 β-lactamase used as a reference [pI = 7.6] on IEF gel [pH 3 to 10 IEF gel; Invitrogen], consistent with the SHV-1-like β-lactamase [pI = 7.6] [data not shown].)

Deletion of the *ompK35* gene caused a 2-fold increase in the MICs of ampicillin and benzylpenicillin and larger (4- to 8-fold) increases for third- and fourth-generation cephalosporins. Especially noteworthy is the strong (8-fold) increase in MIC of a large compound with two net negative charges, ceftriaxone. The deletion of the *ompK36* gene, in contrast, had almost no effect on MICs, except for a 4-fold increase for the ceftriaxone MIC. These results suggest that OmpK35, in spite of its lower level of expression (Fig. 1), plays a more important role as an entry channel for drugs, which are usually relatively large and very often negatively charged compounds, both factors decreasing the diffusion through nonspecific porin channels (9, 11). The MICs for large hydrophobic drugs, such as novobiocin (molecular weight [MW], 613) and erythromycin (MW, 734), were not altered detectably by the deletion of either OmpK35 or OmpK36, and this was expected, as the large size as well as the hydrophobicity appears to hinder the diffusion through porin channels (11); thus, these compounds are assumed to penetrate the lipid bilayer region of the outer membrane (4), although it is also possible that a strong efflux overwhelms differences in entry rates (see reference 33). Presumably, a similar explanation holds for the high (and unchanged) MICs of the hydrophobic penicillins oxacillin and cloxacillin, which, although not readily hydrolyzed, are pumped out very efficiently by the multidrug efflux pump AcrB-AcrA-TolC complex in *E. coli*, as indicated by a huge (128- to 512-fold) decrease in MICs caused by the deletion of the *acrAB* genes (60).

Influx of penicillins in *K. pneumoniae*. In order to analyze the diffusion through the porin channels more directly, we measured the influx rates of penicillins in intact cells of *K. pneumoniae* by combining their influx with their subsequent hydrolysis by the endogenous β-lactamase, as was done earlier with *E. coli* (11). In order to prevent the active efflux of the penicillins by the AcrAB-TolC complex, the assay was performed in the presence of 40 μM CCCP, which is expected to dissipate the proton motive force and thus inactivate the efflux. The chromosomal SHV-1-like β-lactamase hydrolyzes penicillins very well, with the maximum rates of hydrolysis (V_{max}) of 0.99 nmol/s/mg for ampicillin and 0.83 nmol/s/mg for benzylpenicillin. The K_m values for ampicillin and benzylpenicillin were 53 μM, and 22 μM, respectively. The permeability coefficient of ampicillin in the parent *K. pneumoniae* ATCC 11296 strain was 67 nm/s (Table 2), which is >3-fold higher than that in *E. coli* LA51A (20 nm/s) (12). We found that the permeability coefficient of benzylpenicillin in the *K. pneumoniae* was 74 nm/s (Table 2); this value was remarkably (10-fold) higher than

TABLE 3 Permeability coefficients of β-lactams through porins expressed in porinless *E. coli*[a]

Porin	Permeability coefficient[b] (nm/s) (no. of expts) for:				
	Ampicillin (20 μM)	Benzylpenicillin (150 μM)	Cephalothin (1.0 mM)	Cefotaxime (1.0 mM)	Cefepime (1.0 mM)
OmpK35	51 ± 21 (5)	34 ± 2 (4)	383 ± 19 (8)	31 ± 9 (8)	493 ± 18 (8)
OmpK36	14 ± 3 (5)	4 ± 2 (4)	44 ± 4 (8)	9 ± 2 (8)	27 ± 4 (8)
OmpF	42 ± 3 (4)	10 ± 2 (4)	268 ± 9 (8)	17 ± 2 (8)	237 ± 7 (8)
OmpC	15 ± 2 (4)	2 ± 1 (4)	36 ± 2 (8)	11 ± 3 (8)	16 ± 6 (8)

[a] Since each porin was expressed at different levels in the host, corrections were made. In the system used for penicillin permeation rates, urea-SDS-PAGE showed that the expression levels of OmpK35, OmpK36, and OmpF were 53.3%, 51.6%, and 92.8% of that of OmpC, respectively, and the permeability coefficient for each porin was normalized to correct for the expression level. In the system used for the examination of cephalosporin influx rates, OmpK35, OmpK36, and OmpF were expressed at 71.5%, 73.7%, and 94.9% of the OmpC expression level, respectively, and the results were corrected for these differences.

[b] Values are averages ± standard deviations.

that in *E. coli* LA51A (7 nm/s) (12). When assays were performed in single-deletion Δ*ompK35* and Δ*ompK36* mutants, deletion of the *ompK36* gene caused little (5%) decrease in the permeability of benzylpenicillin, as expected, although ampicillin permeability decreased somewhat more (30%). In contrast, deletion of the *ompK35* gene decreased drastically (about 50%) the permeability coefficients of both ampicillin and benzylpenicillin. These data showed that penicillins permeate the outer membrane of *K. pneumoniae* faster than *E. coli*, and the OmpK35 porin is the predominant route of entry, especially for the hydrophobic and anionic benzylpenicillin.

Finally, we can translate the permeability coefficients shown in Table 2 into the influx rates of penicillins, by using Fick's first law of diffusion, calculated as $v = P \times A \times (Co - Cp)$, where v, P, A, Co, and Cp represent the influx rate, permeability coefficient, cell surface area per milligram (dry weight) of cells, and external and periplasmic concentrations of the drug, respectively. By assuming that the value of A (132 cm^2 per mg), determined for *Salmonella* (11), is valid also for *K. pneumoniae*, and that Cp is negligible in relation to Co, we can calculate the influx rate of 0.26 nmol/s/mg for benzylpenicillin when its external concentration equals its MIC (100 μg/ml [Table 1], or about 300 μM). Although the V_{max} of the chromosomal β-lactamase (0.83 nmol/s/mg) far exceeds this influx rate, it should function much more slowly because of its high K_m value (22 μM; see above), and we can see that the loss of OmpK35 will make *K. pneumoniae* significantly more resistant, as seen in Table 1.

Influx of β-lactams through *K. pneumoniae* porins expressed in *E. coli*. The results described in the previous section, however, were affected by the levels of expression of each porin in *K. pneumoniae*. Furthermore, there was no guarantee that the use of 40 μM CCCP was sufficient to completely inhibit the efflux of penicillins by endogenous efflux pumps, such as AcrB, which is known to exist in this organism (34). Thus, we examined the permeability of two major porins in *K. pneumoniae*, OmpK35 and OmpK36, in comparison with *E. coli* OmpF and OmpC, by expressing each of them in a K-12 host strain, LA51AFC, which lacks OmpF, OmpC, and the AcrAB system (13). Because the major active efflux pump was eliminated, the hydrolysis rates of the penicillins by intact cells equal the influx rates. The influx rates of ampicillin and benzylpenicillin in the intact cells of the host LA51AFC were very low (permeability coefficients for ampicillin and benzylpenicillin were both at an essentially unmeasurable level [2 nm/s]), indicating that any measurable influx in the presence of plasmid-encoded porins can be assumed to be due to these porins. First, we tested the influx rates of ampicillin and benzylpenicillin in the cells containing pHSG575-*ompK35* or pHSG575-*ompK36* at several concentration of substrates. The influx rates of ampicillin and benzylpenicillin were generally proportional to the concentration gradient of substrates in both cases (results not shown) across the outer membrane, suggesting that these porins, OmpK35 and OmpK36, behaved essentially as nonspecific diffusion channels, similar to *E. coli* porins OmpF and OmpC (12). We also found large differences in the influx rates of penicillins between OmpK35 and OmpK36. The permeability coefficient of ampicillin in OmpK35 was nearly 4-fold higher than that in OmpK36, and that of benzylpenicillin in OmpK35 was almost 9-fold higher than that in OmpK36 (Table 3). *E. coli* OmpF similarly showed higher permeability than OmpC for these compounds, confirming previous results (11, 12), although the difference in permeability to benzylpenicillin was less than that found between OmpK35 and OmpK36 (Table 3). Importantly, the permeability coefficient of benzylpenicillin in the cells producing OmpK35 (34 nm/s) was much higher than in those producing OmpF (10 nm/s). Although zwitterionic ampicillin permeates severalfold faster than monoanionic benzylpenicillin through OmpF (Table 3) (11, 12), OmpK35 allowed the influx of benzylpenicillin at a rate approaching that of the zwitterionic ampicillin (Table 3).

The permeation rates of cephalosporins were measured in a coexpression system of *E. coli* JW4111-2AFC containing pBAD33ΔCM^R_*oxa163* and pHSG575 containing a porin gene. The expression of *oxa163* gene on the plasmid was induced by 10 mM arabinose: the maximum rates of hydrolysis (V_{max}) for cephalothin, cefotaxime, and cefepime were 6.75, 8.79, and 9.66 nmol/s/mg, respectively, and the K_m values for cephalothin, cefotaxime, and cefepime, were 86, 146, and 277 μM, respectively. The K_m values for cephalothin and cefepime were close to the values found in the literature, 80 and 350 μM, respectively (25), but our K_m value for cefotaxime was different from those previously reported, 850 μM (25) and 45 μM (35). The permeability coefficients of cephalothin, cefotaxime, and cefepime in the host cells without the porin expression plasmids were very low, at 1, 3, and 12 nm/s, respectively. The leakage of OXA-163 β-lactamase into the external medium was about 1% of the total enzyme activity measured by using sonicated extract, and corrections were made for the contribution of this extracellular activity. To confirm the validity of our assay conditions, we used cephalothin, whose permeability coefficients through OmpF and OmpC were determined previously in our laboratory by using intact cells containing TEM β-lactamase (11). We found that the permeability coefficient of cephalothin in the cells producing OmpF or OmpC were 268 or 36 nm/s; these values

TABLE 4 Relative diffusion rates of sugars of various sizes determined by proteoliposome swelling

Solute	MW	Relative swelling rate (%) of proteoliposomes containing:			
		OmpK35	OmpF	OmpK36	OmpC
L-Arabinose	150	100	100	100	100
D-Glucose	180	69.8	68.4	67.5	62.3
D-Galactose	180	80.0	85.7	84.5	84.0
N-Acetyl-D-glucosamine	222	49.8	48.2	44.5	41.3
Phenyl-β-D-galactopyranoside	256	13.1	6.6	7.5	7.7
4-Aminophenyl-β-D-galactopyranoside	271	10.7	6.1	9.7	5.4
Sucrose	342	8.0	2.8	2.6	1.9
Lactose	342	9.3	3.9	3.8	2.3

were not very far from the values found earlier, at 100 and 16 nm/s, respectively (11), which are underestimates, because active efflux was not known at that time and its effect was not considered. We found that the permeability of cephalothin in the cells producing OmpK35 (383 nm/s) was slightly higher than that in the cells with OmpF. Cephalothin permeability in OmpK36-producing cells (44 nm/s) was almost one order of magnitude lower than that in OmpK35-producing cells. Cefotaxime (MW, 455) is significantly larger than cephalothin (MW, 396), but both are monoanionic. The permeability of cefotaxime in the cells producing OmpK35 was much slower, about 10-fold, than that of cephalothin (Table 3). A similar difference was also observed in the cells containing OmpF (Table 3). In contrast, cefepime, a large (MW, 480) but zwitterionic compound, penetrated OmpK35 even faster than cephalothin (Table 3). Remarkably, cefepime penetration of OmpK35 was nearly 20 times faster than that of OmpK36, suggesting the predominant role OmpK35 plays for this compound. We note also that the cefepime permeation through OmpK35 is more than two times faster than that through *E. coli* OmpF (Table 3).

Pore sizes of *K. pneumoniae* porins estimated by proteoliposome swelling assay. *K. pneumoniae* porins were used for osmotic swelling of proteoliposomes with sugars of different sizes (Table 4). For this assay, outer membrane fragments from LA51AFC containing each porin were reconstituted into liposomes. Because the molecular sizes of all compounds used for the influx assay are around 400, we were interested in diffusion rates of sugars with large sizes. Because the diffusion rates of disaccharides through OmpF were nearly two orders of magnitude lower than that of L-arabinose (10), we had to use larger amounts of protein in order to measure the swelling rates in solutions of large sugars accurately and then normalize the raw data on the basis of the amount of protein used for reconstitution.

No large differences were found in the relative diffusion rates of monosaccharides, such as glucose and galactose, through OmpK35 and OmpF or in the rates through OmpK36 and OmpC. However, differences in swelling rates between OmpK35 and OmpF became apparent with increasing sizes of the solutes, so that in phenyl-β-D-galactopyranoside, OmpK35 gave a rate about two times higher than OmpF, and similar or even larger differences were found with both disaccharides tested (Table 4). These data indicate that OmpK35 behaves as a larger channel than *E. coli* OmpF and are consistent with the faster penetration of large and/or lipophilic β-lactams through OmpK35 than through OmpF. Our data also confirm the earlier proteoliposome swelling data (36) showing that OmpK35 allows a more rapid diffusion of a disaccharide than OmpK36 does.

The *E. coli* OmpC channel behaves as a slightly narrower one than the OmpF channel (10, 11), and our data confirmed this conclusion, showing that in the solution of the larger sugars OmpC gives lower swelling rates (relative to that in L-arabinose) than OmpF (Table 4). The permeation rates of larger sugars through OmpK36 were indeed lower than that through OmpK35, yet they were often significantly higher than that through OmpC, as seen with 4-aminophenyl-β-D-galactoside (Table 4). However, differences in relative diffusion rates between OmpK36 and OmpC appeared to be less pronounced than those between OmpK35 and OmpF (Table 4).

DISCUSSION

When the third-generation cephalosporins, such as cefotaxime, ceftazidime, and ceftriaxone, were introduced into clinical use, *K. pneumoniae* isolates turned out to be even more susceptible than *E. coli* isolates (37). This is now understandable because of our finding that *K. pneumoniae* porins, especially OmpK35, allow a remarkably rapid influx of β-lactams, especially of larger compounds, such as the third-generation cephalosporins (see Results); this is also because the chromosomal SHV-1-like β-lactamase cannot hydrolyze these compounds efficiently (38), and its production is not induced by the presence of β-lactams (39), unlike the AmpC β-lactamases of *Enterobacter cloacae* or *Serratia marcescens* (40). In recent years, however, *K. pneumoniae* has become one of the most feared nosocomial pathogens, a member of the so-called ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (41). Indeed, a recent PubMed search for "resistant *Klebsiella pneumoniae*" yielded nearly 6,000 references. This is largely due to the rapid acquisition of extended-spectrum β-lactamases on plasmids (see reference 42), but many of the resistant isolates are also found to be lacking major porins (we cite here only early reports [6, 43–46]; in the review coauthored by one of us [4], we found that similar reports are so numerous in recent years that it was impossible to give exhaustive citations).

Obviously, β-lactams must first penetrate the outer membrane in order to reach their target, penicillin-binding proteins, and because they are relatively hydrophilic and carry charges, this penetration is likely to take place through the water-filled porin channels (47). The inactivation/removal of these drugs from the periplasm, via periplasmic β-lactamases and active efflux, will become more effective in increasing resistance if the influx is decreased through the loss of porins. However, porin deletion has its price, as the uptake of nutrients could also become affected. Thus, such a frequent observation of porin loss among clinical isolates of

K. pneumoniae but not in other species of *Enterobacteriaceae* is somewhat surprising. Perhaps one factor is that prior knowledge of porin-deficient isolates in this species compelled microbiologists to examine the porin pattern when they report resistant isolates of *K. pneumoniae*, thus increasing the chance of discovery of these mutations. However, when Charrel et al. examined a large number of β-lactam-resistant isolates of *Enterobacteriaceae* from French hospitals, 44% of the *Enterobacter aerogenes* strains had porin alterations, whereas such changes were found in only 6% of the *Enterobacter cloacae* strains (48), showing that porin alterations are more common in some species. Clearly, when a species produces a highly permeable porin, the resultant rapid influx of drugs into the periplasm can overload the resistance mechanisms located in the periplasm (β-lactamase and tripartite efflux pumps, such as AcrAB-TolC) and make these resistance mechanisms less effective, and the presence of drugs is expected to select for porin-deficient mutants (4). Indeed, *E. aerogenes*, mentioned above, has relatively permeable porins (Sugawara and Nikaido, unpublished); in contrast, *E. cloacae* porins show remarkably low (14-fold lower for cefazolin) permeability (14), and porin-deficient mutants are rare among resistant isolates of this species. In this view, a detailed examination of porin permeability is essential for our understanding of drug resistance in Gram-negative bacteria.

We found in this study that the major porins of *K. pneumoniae*, OmpK35 and OmpK36, indeed appear to produce larger channels than their *E. coli* homologs OmpF and OmpC (Tables 3 and 4) and that OmpK35 especially showed a remarkably high permeability to hydrophobic (benzylpenicillin) and to large (cefepime) compounds. Deletion of the *ompK35* gene but not the *ompK36* gene produced significant increases in the MICs of various β-lactams (Table 1), in spite of the usually low level of expression of OmpK35 (Fig. 1). We emphasize here that OmpK35 becomes an efficient conduit for large and/or lipophilic drugs because of its larger channel size. An alternative interpretation that it allows the permeation of these drugs, because the specific binding of drugs to the channel interior does not fit with our observation that simple sugars, such as disaccharides, also diffuse through the OmpK35 channel in the proteoliposome swelling assay (Table 4).

These results clarify the roles of each of these major *K. pneumoniae* porins in antibiotic permeation, roles that remained often unclear or confusing in previous studies. Thus, in most of the early reports of porin-deficient β-lactam-resistant *K. pneumoniae* strains, both of the porins appeared to be absent (3, 49–52), but confusingly, the group of Hernández-Allés, who have contributed much to this area, published a paper claiming that the loss of OmpK35 alone in an apparently accidental mutant did not change the β-lactam MIC at all (53). The same group, however, by introducing either the *ompK35* or *ompK36* gene into a strain deficient in both porins, showed that although both OmpK35 and OmpK36 made the cell more susceptible to various cephalosporins, OmpK35 was necessary for full susceptibility to large compounds, such as ceftazidime (MW, 547) or cefpirome (MW, 515) (6). The expression of porins from plasmids, however, cannot guarantee that they are expressed at physiologically correct levels, but porin-deficient mutants selected by antibiotics or by accident may obviously contain other unknown changes, as explained above. In this sense, our porin deletion mutants were isolated by the Datsenko-Wanner method (17); thus, we believe that their phenotypes (Table 1) are not affected by extraneous factors.

One recent development in the area of the roles of *K. pneumoniae* porins involves the putative role of OmpK36 in ertapenem penetration. Strains deficient in both OmpK35 and OmpK36 and containing carbapenem-hydrolyzing β-lactamase are obviously resistant to ertapenem (54). However, in 2009, clinical ertapenem-resistant strains lacking OmpK36 but not OmpK35 were reported in China (55). This is surprising, because OmpK35 should be able to allow an influx of this compound (MW, 475), although OmpK36 may also make significant contribution in view of its not-so-large size. In our hands, the deletion of OmpK36 in the presence of OmpK35 does not increase the ertapenem MIC (Table 1). When *ompK35* and *ompK36* genes were introduced on plasmids into porin-deficient *K. pneumoniae* isolates, their effect on ertapenem MIC was unpredictable, with the *ompK35* gene of some strains having a greater effect than the *ompK36* gene, while in other strains, an opposite effect was seen (56). In another study (57), ertapenem-resistant *K. pneumoniae* isolates were found to lack OmpK35 (as expected) and to produce a variant of OmpK36 with a two-residue addition to the L3 loop. A 2016 study reports that ertapenem-resistant isolates of *K. pneumoniae* produce similar OmpK36 variants, but they also contain an IS*1* insertion in front of the *ompK35* gene, likely diminishing the expression of OmpK35 (58).

Finally, we should note that our study was done by using ATCC 11296, which is a type strain of *K. pneumoniae* subsp. *ozaenae*, rather than *K. pneumoniae* subsp. *pneumoniae* that is presumably isolated more frequently from clinical sources than *K. pneumoniae* subsp. *ozaenae*. Although these two subspecies of *K. pneumoniae* share similar susceptibility to various antibiotics (59), we note that strains belonging to *K. pneumoniae* subsp. *ozaenae* are usually slightly more susceptible to various β-lactams than those belonging to *K. pneumoniae* subsp. *pneumoniae*, with their MICs being about 2-fold lower. Thus, we may have selected a strain with slightly larger porin channels than the average clinical isolates of *K. pneumoniae*, but we believe that our general conclusion on the size of *K. pneumoniae* porins is not materially affected by our choice of the *K. pneumoniae* strain.

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