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Role of *Klebsiella pneumoniae* OmpK35 Porin in Antimicrobial Resistance

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OmpK35 from *Klebsiella pneumoniae* is the homologue of *Escherichia coli* OmpF porin. Expression of OmpK35 in *K. pneumoniae* strain CSUB10R (lacking both OmpK35 and OmpK36) decreased the MICs of cephalosporins and meropenem ≥128-fold and decreased the MICs of imipenem, ciprofloxacin, and chloramphenicol ≥8-fold. MIC reductions by OmpK35 were 4 times (cefepime), 8 times (cefotetan, cefotaxime, and cefpirome), or 128 times (ceftazidime) higher than those caused by OmpK36, but the MICs were similar or 1 dilution lower for other evaluated agents.

Klebsiella pneumoniae produces two major porins (OmpK35 and OmpK36) and the quiescent porin OmpK37. Details on OmpK36 and OmpK37 have been previously reported (1, 6, 10). Most clinical isolates of *K. pneumoniae* lacking extended-spectrum β-lactamase (ESBL) express both OmpK35 and OmpK36 porins, while most ESBL-expressing *K. pneumoniae* clinical isolates produce only OmpK36 (9). Until now, the few clinical isolates lacking both OmpK35 and OmpK36 have been ESBL-producing strains (14).

Loss of OmpK36 is related to cefoxitin resistance and increased resistance to oxyimino- and zwitterionic cephalosporins in strains producing ESBL and to carbapenem resistance in strains producing plasmid-mediated AmpC-type β-lactamase (3, 4, 13, 15). Loss of OmpK36 also results in a moderate increase in fluoroquinolone resistance in strains with altered topoisomerases and/or active efflux of quinolones.

Preliminary results (6) indicate that OmpK35 allows efficient penetration of cefoxitin, cefotaxime, and carbapenems, but there has been some controversy on the role of this porin in cephalosporin penetration in *K. pneumoniae* (18). Detailed studies on the importance of OmpK35 in antimicrobial resistance are lacking.

In order to investigate the role of OmpK35 in antimicrobial resistance, we cloned the *ompK35* gene. For this purpose, genomic DNA from *K. pneumoniae* strain KT755 (19) was digested with *Sau*3A. Fragments were ligated to cosmid pLA2917 (2) and used to transform *Escherichia coli* DH5α (17). Recombinants were screened for *ompK35* by PCR using primers U681 (5′-CGG TTA CGG CCA GTG GGA ATA-3′) and L1316 (5′-GAC GCA GAC CGA AAT CGA ACT-3′), specific for enterobacterial porins and located 215 and 850 bp downstream of the *ompK36* start codon, respectively (6). The sizes of PCR-amplified products from *ompF*-type genes are different from those of other porin genes (data not shown). One clone carrying a plasmid, designated pSHA15, produced an amplicon of the desired size. Outer membrane proteins (OMPs) were isolated as described previously (1, 13). Western blot analysis of OMPs was performed on Immobilon P filters (Millipore, Bedford, Mass.) using anti-OmpK35 antibody (diluted 1:1,000) and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (diluted 1:5,000) (13).

OMP profiles of *E. coli* DH5α carrying pSHA15 exhibited a band with the same mobility as that of OmpK35 expressed by *K. pneumoniae* KT755 (data not shown). The expression of OmpK35 was downregulated in a high-osmolarity culture medium, as occurs with the OmpF-like porins (9), and OmpK35 reacted with anti-OmpK35 in immunoblot experiments (data not shown). The OmpK35 protein expressed by the *E. coli* clone was extracted by porin extraction methods based on the trypsin resistance of porins and their strong noncovalent association with the peptidoglycan, and it also retained its heat modifiability.

The *ompK35* gene of *K. pneumoniae* KT755 was sequenced (EMBL database accession no. AJ011501). The amino acid sequence of OmpK35 was aligned with the sequences of other enterobacterial porins (5, 7) (Fig. 1) on the basis of the conservation of the β-strands and some key residues that are well conserved in porins: Lys16, Arg38, Glu58, Arg75, Asp106, Glu110, and Arg126. OmpK35 is an OmpF homologue and presents a typical 16 β-strand structure, with eight short periplasmic turns and eight extracellular loops of variable lengths. OmpK35 loop 3, which defines the size of the transmembrane pore in other porins, extends inside the barrel and is the most conserved loop and contains only one more residue than OmpF and OmpC from *E. coli* and OmpK36 from *K. pneumoniae*.

For susceptibility testing experiments, the *ompK35* gene was cloned in pWSK30 (20) and endowed with a kanamycin resis-

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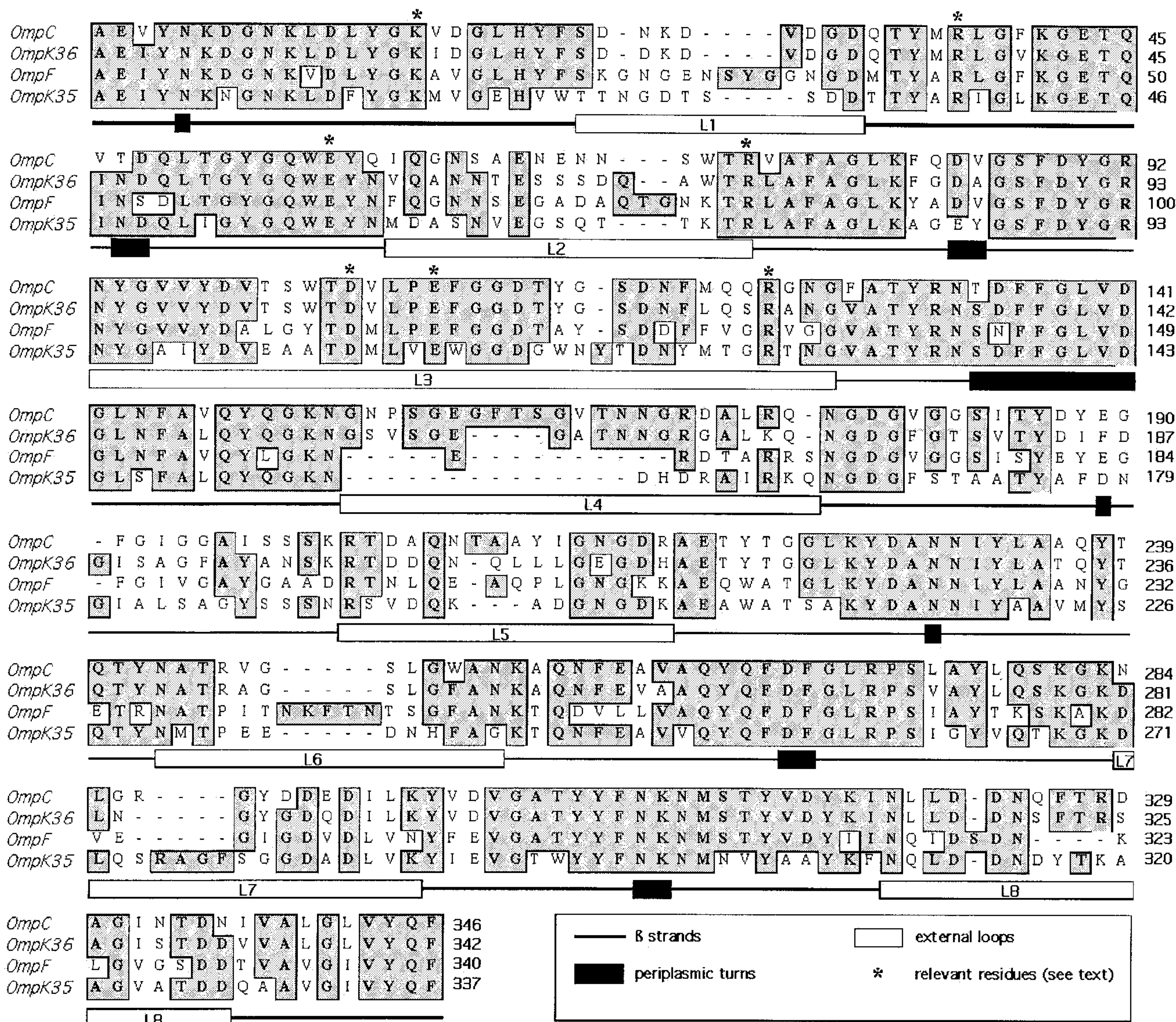

FIG. 1. Comparison by alignment of the deduced OmpK35 sequence from *K. pneumoniae* with the sequences of OmpF and OmpC from *E. coli* and OmpK36 from *K. pneumoniae* available in GenBank, EMBL, and DDBJ. Secondary structure motifs are described on the basis of the crystal structure of OmpK36. The numbering is based on the mature OmpK36. Conserved amino acids (shaded and boxed) and gaps introduced to maximize alignment (hyphens) are indicated.

tance cassette from pCS12 (8) to give pSHA16K. The pSHA16K plasmid was cloned into the porin-deficient *K. pneumoniae* CSUB10R clinical isolate and into the clonally related (3) isolate *K. pneumoniae* CSUB10S (expressing OmpK36). The expression of OmpK35 does not interfere with β-lactamase activity in CSUB10R, as determined spectrophotometrically with crude supernatants from sonicated cells as the enzyme source (15) (data not shown).

MICs of cefoxitin (Sigma, Madrid, Spain), cefotetan (Zeneca, Madrid, Spain), ceftazidime (Glaxo, Barcelona, Spain), cefotaxime (Sigma), cefepime (Bristol-Myers Squibb, Madrid, Spain), cefpirome (Hoechst Marion-Roussel, Romainville, France), imipenem (Merck Sharp & Dohme, Madrid, Spain), meropenem (Zeneca), ciprofloxacin (Sigma), clinafloxacin (Parke-Davis, Ann Arbor, Mich.), amikacin (Sigma), gentamicin (Sigma), tetracycline (Sigma), and chloramphenicol (Sigma) for strains CSUB10S and CSUB10R and OmpK35-expressing transconjugants derived from these two strains (Table 1) were determined by microdilution, according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (16). Antimicrobial agent MICs for *K. pneumoniae* CSUB10R containing plasmids pKSK (vector) and pSHA25K (OmpK36) were also determined for comparison.

Expression of OmpK35 in *K. pneumoniae* CSUB10R reduced (Fig. 2) the MICs of all agents tested two or more times. The highest reductions (≥128-fold) were observed for cephamycins, oxyimino-cephalosporins, zwitterionic cephalosporins, and meropenem. Significant reductions (≥8-fold) were also noted for imipenem, ciprofloxacin, and chloramphenicol. The lowest reductions were obtained for those agents to which *K.*

TABLE 1. MICs of antimicrobial agents against *K. pneumoniae* strains with different patterns of porin expression

Strain	Porin(s)	MIC (μg/ml)[a]													
		FOX	CTT	CAZ	CTX	FEP	PIR	IPM	MEM	CIP	CLX	AMK	GEN	TET	CHL
CSUB10S	OmpK36	2	0.06	256	8	2	4	0.125	0.03	0.5	0.06	1	4	2	16
CSUB10R	None	128	32	>512	512	512	512	1	4	4	0.125	1	8	4	64
CSUB10R/pSHA16K	OmpK35	1	0.03	2	0.5	0.125	0.5	0.125	0.03	0.25	0.06	0.5	4	1	8
CSUB10R/pSHA25K	OmpK36	2	0.125	256	4	0.5	4	0.25	0.03	0.5	0.125	0.5	4	1	16
CSUB10S/pSHA16K	OmpK35 and OmpK36	1	0.03	4	1	0.125	0.5	0.125	0.03	0.5	0.06	1	4	1	8
CSUB10R/pKSK	None	128	16	>512	256	128	256	1	4	2	0.125	1	8	2	64

[a] Abbreviations: FOX, cefoxitin; CTT, cefotetan; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; PIR, cefpirome; IPM, imipenem; MEM, meropenem; CIP; ciprofloxacin; CLX, clinafloxacin; AMK, amikacin; GEN, gentamicin; TET, tetracycline; CHL, chloramphenicol.

pneumoniae CSUB10R was already susceptible: clinafloxacin, tetracycline, amikacin, and gentamicin. Expression of OmpK36 in CSUB10R also decreased the MICs of all antimicrobial agents tested, except clinafloxacin, to values similar to the MICs for the related clinical isolate CSUB10S. MIC reductions caused by OmpK35 expression were 4 times (cefepime), 8 times (cefotetan, cefotaxime, and cefpirome), or 128 times (ceftazidime) higher than those caused by OmpK36 expression. These results, however, do not necessarily mean that OmpK35 should be considered specific for these agents, as expression of OmpK36 also significantly reduced their MICs. MIC reductions caused by OmpK35 were the same (meropenem, amikacin, gentamicin, and tetracycline) or 1 dilution step lower (cefoxitin, imipenem, ciprofloxacin, clinafloxacin, and chloramphenicol) than those caused by OmpK36.

Expression of OmpK35 in *K. pneumoniae* CSUB10S, leading to the simultaneous expression of the two major porins of *K. pneumoniae* (Fig. 2), resulted in the MICs of the evaluated agents being the same or one dilution step higher than those against the transformant expressing only OmpK35.

Expression of both OmpK35 and to a lesser extent OmpK36 decreases the MICs of ciprofloxacin for *K. pneumoniae* CSUB10R (which contains a Ser83Phe change in the A subunit of DNA gyrase and expresses active efflux of fluoroquinolones [12]), indicating that both porins allow penetration of this drug.

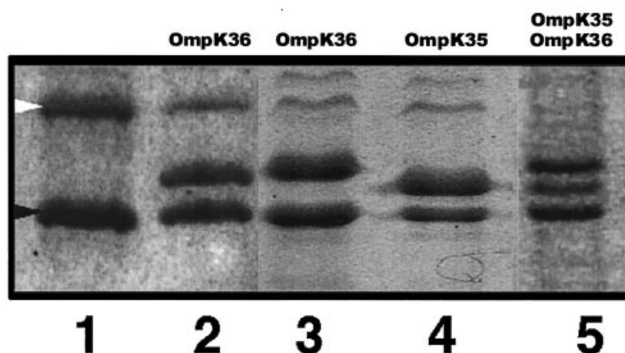

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of OMPs from *K. pneumoniae* strain CSUB10R (lane 1) and clones derived from CSUB10R carrying plasmids pSHA25K (lane 3) and pSHA16K (lane 4). *K. pneumoniae* isolate CSUB10S and its clone carrying pSHA16K are also shown in lanes 2 and 5, respectively. Porins expressed by each strain are indicated above each lane. The positions of LamB (white arrowhead) and OmpA (black arrowhead) homologues of *K. pneumoniae* are indicated to the left of lane 1.

Porin expression was minimally relevant for the activity of clinafloxacin, a fluoroquinolone much more active against CSUB10R than ciprofloxacin. OmpK35 and OmpK36 expression also decreased the MICs of tetracycline and chloramphenicol. These data support the general role of both porins as hydrophilic pores. MICs of aminoglycosides did not significantly change after porin expression, presumably because of the penetration of these agents by porin-independent pathways.

Most ESBL-producing *K. pneumoniae* strains lack OmpK35 (11). Loss of this porin may be one of the factors contributing to antimicrobial resistance in ESBL-producing *K. pneumoniae* and may favor the selection of additional mechanisms of resistance, including loss of OmpK36 and/or active efflux (14).

OmpK35 is not normally expressed in high-osmolarity media, which may result in repression of its expression in *K. pneumoniae* in vivo. This may be of therapeutic importance because of the limited entrance of certain antimicrobial agents in *K. pneumoniae*. New studies on porin expression in *K. pneumoniae* grown in vivo are needed.

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