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Cloning of Porin OmpK35 and the Roles of the Three Porins of *Klebsiella pneumoniae* (Kp) in the Activity of Antimicrobial Agents

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Introduction

We have previously described porins OmpK36 and OmpK37 of *K. pneumoniae* (1, 4). They are the homologues of porins OmpC and OmpN of *Escherichia coli*, both by sequence similarity comparisons and by their functional characteristics. Both OmpK36 and OmpC porins are preferentially expressed in media of high osmolarity and they are under the control of a *micF* gene upstream of their porin-coding regions. Also, both OmpK37 and OmpN porins are expressed at very low levels under the usual laboratory conditions, so they cannot be detected in Coomassie Blue-stained polyacrylamide gels. *K. pneumoniae* clinical isolates express two or one porin: either porin OmpK36 alone or both OmpK36 and a second porin that is not OmpK37 (7). We have designated this second porin as OmpK35. Most *K. pneumoniae* clinical isolates expressing extended-spectrum §-lactamases (ESBL) express only porin OmpK36, while most ESBL ^{D} clinical isolates express both OmpK36 and OmpK35 porins (7).

To characterize in more detail the OmpK35 porin and evaluate its role in antimicrobial resistance, we have cloned and sequenced the *ompK35* gene. Expression of the cloned porin genes of *K. pneumoniae* in a porin-deficient isolate restored bacterial permeability to cephalosporins, carbapenems, and quinolones. Penetration of antimicrobials, except carbapenems, was much lower through the OmpK37 porin than through the other two porins. Among the three porins, OmpK35 allows more efficiently the penetration of antimicrobials than porins OmpK36 and OmpK37. Sequence comparison and functional properties demonstrated that OmpK35 is the *K. pneumoniae* equivalent to *E. coli* OmpF

Materials and Methods

Bacterial strains, plasmids, and culture media.

K. pneumoniae strains KT755 and C3 express both OmpK36 and OmpK35 porins, strain KT5002 derived from C3 expresses only OmpK35 due to Tn*5* inactivation of *ompK36* (1). CSUB10S and CSUB10R are porin-sufficient and -deficient clinical isolates of K.pneumoniae and express ESBL; they were isolated from a single patient during antimicrobial therapy (2). *E. coli* DH5 α was used as host for cloning experiments. Plasmids pWSK29 and pWSK30 were used as low-copy vector for cloning; plasmids pCSI2 and pSHA1 (10) were used as source of kanamycin and tellurite resistance cassettes, respectively; plasmids pLF4 (8) and pMY11 (11), and pSUV7 (1), were used as sources of cloned porins OmpF and OmpC (from *E. coli*), and OmpK36 (from *K. pneumoniae*), respectively. Luria Bertani and Nutrient Broth were used as media of high and low osmolarity: 447 and 104 mOsm/Kg, respectively.

Genetic methods.

A gene bank of strain KT755 was obtained by chromosomal digestion with *Sau 3A* and ligation of aprox. 20 kb fragments with *Bgl* II-digested cosmid pLA2917. Clones were packed using the Gigapack III gold kit (Stratagene) and were maintained in strain DH5 α. Clones were screened by PCR using primers directed against sequences conserved in porin genes (see Results). Southern blot analysis was performed by standard methods using the ECL kit (Amersham) for probe labeling. DNA sequencing was performed using an Applied Biosystem automatic sequencer.

Liposome assays.

Liposome swelling assays were performed following (9) as described (4)

Antimicrobial tests: MICs and §-lactamases.

MIC values were determined by microdilution following the NCCLS guidelines and by E-test (AB Biodisk). ESBL production was evaluated with amoxi/clavulanic, cefotaxime, and ceftazidime disks. §-lactamase production was measured spectrophotometrically after sonication.

Outer membrane proteins (OMPs) and porins.

OMPs were isolated as sodium-lauryl sarcosinate insoluble material (6) and porins were isolated by differential solubilization and trypsin treatment (1). OMPs and porins were analysed by SDS-PAGE as described (1).

Results

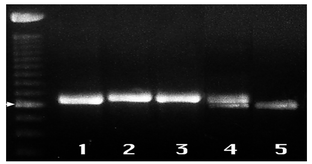

Fig. 1. Analysis PCR amplicons of cloned porin genes ompF (1) and ompC (2) from E. coli, and o mpK36 (3) from K. pneumoniae, and from chromosomal DNAs of OmpK36 $^{+}$ OmpK35 $^{+}$ (4) and OmpK36 ^{D} OmpK35 $^{+}$ K. pneumoniae strains (5). Arrowhead indicates the 600 bp marker.

Attempts to screen the gene bank with antibodies raised against porin OmpK35 isolated from strain KT5002 (OmpK36D, OmpK36+) were unsuccessful due to the strong cross-reactivity of the antiserum with the porins of the *E. coli* host strain. We then decided to use a PCR approach. As shown in Fig. 1, the PCR products from cloned *ompF*-type genes are clearly distinct from those of the *ompC*-type, the latter showing a slightly higher molecular mass: compare *ompF* amplicons (lane 1) with *ompC* amplicons (lanes 2 and 3). These differences were also observed when starting from chromosomal DNAs (lanes 4 and 5): strain C3 (OmpK36 $^{+}$, OmpK35 $^{+}$) produced two amplicons, whereas strain KT5002 (OmpK35 $^{+}$, but OmpK36 ^{D}) produced one single amplicon with a molecular mass compatible with that of the *ompF*-type amplicons. Since the putative *ompK35* gene produced by PCR an amplicon clearly distinguishable from those of other porin genes, we used this approach to screen the gene bank from *K. pneumoniae* KT755. Eleven groups of 90 clones each were separately analysed as follows: the 90 clones of each group were pooled, and plasmids were isolated by a miniprep method, and amplified by PCR with the selected primers. One group produced an amplicon with the desired size, and successive analyses within this group of 10-clones groups, and the individual clones of the positive group, resulted in the identification of a single clone carrying a plasmid designated pSHA15 which gave the desired PCR amplicon

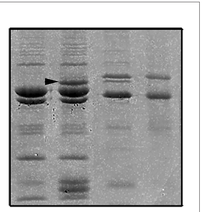

Fig. 2. Analysis of the expression of the cloned ompK35 porin gene. OMPs from the host strain E. coli DH5 α (1) and with cloned ompK35 (2). Arrow indicates the expression of the cloned OmpK35 porin. Lanes 3 and 4 show the osmoregulation of OmpK35 in strain KT755 grown in low (3) and high (4) osmolarity medium.

As shown on Fig. 2, analysis of the OMPs of the pSHA15-containing clone showed that this plasmid coded for an OMP with a molecular mass compatible with that of the OmpK35 porin. The OmpK35 porin of strain KT755, was identified on the gel because its expression is downregulated on high osmolarity culture medium.

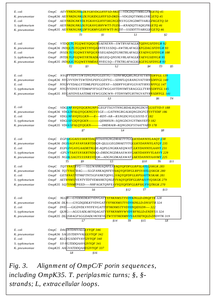

Fig. 3. Alignment of OmpC/F porin sequences, including OmpK35. T. periplasmic turns; §, §-strands; L, extracellular loops.

Further evidence that pSHA15 carried the OmpF-type porin of *K. pneumoniae*, or OmpK35, came from sequencing of the corresponding gene and comparison of its deduced amino acid sequence with those of other related porins. Since the crystal structures of porins OmpK36 and OmpF are known (3, 5), the secondary structures of OmpK35 and other porins could be easily predicted. As shown in Fig. 3, for OmpK35 a typical 16 §-strand structure, with 8 short periplasmic turns and 8 extracellular loops of variable length was predicted. Alignment with other porin sequences was straightforward due to the conservation of the §-strands and of some key residues that are well conserved in enterobacterial and non-enterobacterial porins: Lys16, Arg38, Glu58, Arg75, Asp106, E110, and Arg126. In the know porins structures (including that of *Rhodopseudomonas capsulatus*), these residues are distributed across the pore, with basic residues (Lys and Arg) on one side, and Asp, Glu, and main carbonyl residues of L3 on the other side. OmpK35 and other OmpF-type porins show a shorter extracellular loop L4 than the OmpC-type porins.

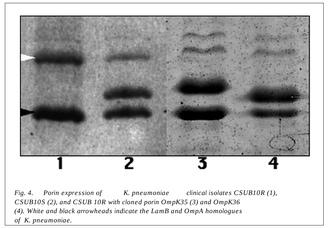

Fig. 4. Porin expression of K. pneumoniae clinical isolates CSUB10R (1), CSUB10S (2), and CSUB 10R with cloned porin OmpK35 (3) and OmpK36 (4). White and black arrowheads indicate the LamB and OmpA homologues of K. pneumoniae.

To study the role of *K. pneumoniae* porins in antimicrobial resistance, we separately expressed the three cloned porins in the porin-deficient clinical isolate CSUB10R. For selection purposes, plasmids containing the cloned genes were doted with a tellurite-resistance cassette. As shown in Fig. 4, CUSB10R does not express porins (lane 1) by comparison with its parent strain CSUB10S (lane 2). Lanes 3 and 4 show the expression of the cloned porins OmpK36 and OmpK35, respectively. A high-molecular mass proteins which migrates just above the cloned porins in all strains and constructs was indeed isolated from CSUB10S as a porin, and its N-terminal sequence demonstrated that it is the LamB porin. Expression of porin OmpK37 was achieved also by cloning but its demonstration required Western blot experiments that are not shown here.

Table 1. MIC values against antimicrobials of K. pneumoniae clinical isolates and constructs expressing different porins.

Strains	Porin expression	MIC values (µg/ml) against antimicrobials															
		FOX	CF	CAZ	CT	CTT +CLV	CAU +CLV	CEX +CLV	FEP	PIR	IP	MP	CIP	CLI	AK	G	CL	TE	CH	SXT
			T		S													Y	L	
CSUB10R	None	128	32	>2048	512	4	4	+0.06	512	512	1	4	2	0.12	1	8	16	2	64	4
CSUB10S	OmpK36	1	0.06	512	16	0.06	1	+0.06	2	4	0.12	0.06	0.25	0.06	1	4	16	2	64	0.5
CSUB10R(pK35K)	None (control)	128	8	2048	256	8	>256	8	128	256	1	4	2	0.12	2	8	8	2	64	4
CSUB10R(pQE1K)	OmpK37 [1]	64	4	2048	128	2	4	0.06	128	125	1	1	2	0.12	1	8	32	2	32	4
CSUB10R(pQE7K)	OmpK37 [1]	32	1	1024	64	0.06	0.5	+0.06	32	32	0.06	0.06	1	0.12	0.5	2	16	0.5	16	4
CSUB10R(pSH15K)	OmpK36	2	0.25	512	16	<0.01	0.25	+0.06	4	4	0.25	0.01	0.5	0.06	0.5	4	32	1	16	4
CSUB10R(pSH21)	OmpK35	1	0.03	2	1	<0.01	<0.06	+0.06	0.125	0.25	0.25	0.03	0.12	0.01	1	4	16	0.75	1	1

[1] FOX, cefoxitin; CTT, cefotetan; CAZ, ceftazidime; CTL, cefotaxime; CLV, clavulanic acid; FEP, cefepime; PIR, cefpirome; IP, imipenem; MP, meropenem; CIP, ciprofloxacin; CLI, clindfoxacin; AK, amikacin; G,gentamicin; TET, tetracycline; CHL, chloramphenicol; SXT, sulfamidas, trimethoprim.

[2] Expression not detected by Coomassie Blue staining but detected by Western blotting with anti OmpK37; [3] Expression detected by Coomassie Blue staining

Discussion

We have described the existence of three porins in *K. pneumoniae*: porins OmpK37, OmpK36, and OmpK35. Most clinical isolates of this species express porins OmpK36 and OmpK35, but when isolates produce ESBL, most of them does not express or express very reduced levels of porin OmpK35. Here we have demonstrated by cloning and sequencing that porin OmpK35 is the homologue of *E. coli* OmpF porin. Both OmpF and OmpK35 porins are preferentially expressed in media with low osmolarity, and both porins are preferentially lost, over OmpK36 and OmpC, in response to antibiotic pressure. This is due to the fact that OmpK35 and OmpF porins allow more efficiently the penetration of antimicrobials than the OmpK36/OmpC porins. The following porin, in terms of efficiency of antibiotic diffusion, is OmpK36 (or OmpC in *E. coli*). Thus, a *K. pneumoniae* ESBL $^{+}$ isolate, which does not express or express low levels of porin OmpK35, could increase its MIC values by loosing expression of the remaining OmpK36 porin. This type of porin-deficient isolate would be visualized in Coomassie-stained polyacrylamide gels as OmpK36 ^{D} OmpK35 ^{D}. Most of these isolates would still be sensitive to carbapenems due to the existence of a third porin OmpK37, which is essentially not permeable to §-lactams but does allow passage of imipenem and meropenem.

Conclusions

We have characterized porin OmpK35, the *K. pneumoniae* homologue of *E. coli* porin OmpF. Among the three porins described in *K. pneumoniae*, OmpK35 allows more efficiently than porins OmpK36 and OmpK37 the penetration of antimicrobials.

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