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A Porin from *Klebsiella pneumoniae*: Sequence Homology, Three-Dimensional Model, and Complement Binding

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A recombinant plasmid containing *ompK36*, the gene coding for the *Klebsiella pneumoniae* outer membrane protein OmpK36, was constructed by transposon mutagenesis and subcloning. Clones were identified in a cosmid library in *Escherichia coli* on the basis of their reaction with antiserum against the OmpK36 protein and by the presence in gel electrophoretic analysis of a band in *E. coli* outer membranes migrating with a mobility corresponding to 36 kDa. The *ompK36*-encoded protein exhibited characteristic properties of porins, such as heat modifiability and resistance to trypsin. The sequence of the gene revealed that OmpK36 is a close relative of the enterobacterial porin family, with a high degree of homology with *E. coli* OmpC, PhoE, and OmpF. On the basis of the structures of OmpF and PhoE porins, determined previously by X-ray analysis, it appears likely that the three-dimensional structure of OmpK36 also contains the motif of a 16-stranded β-barrel, with long loops on one end and short turns on the other. Like the OmpC porin from *E. coli*, OmpK36 contains a long insertion in loop 4. The results of a binding study of complement component C1q to OmpK36 and the analysis of the OmpK36 model suggest that C1q binding sites are covered by the lipopolysaccharide core in the native porin.

The outer membrane of gram-negative bacteria plays a significant role in a variety of functions; it serves as a diffusion barrier to extracellular solutes and interacts with the bacterial environment. This membrane is composed of a bilayer containing phospholipids, lipopolysaccharide (LPS), and proteins. One family of proteins, the porins, is represented in large amounts in the outer membrane and forms nonspecific diffusion channels, allowing small, polar molecules (<600 Da) to diffuse across the membrane barrier. Porins also serve as receptors for bacteriophages and bacteriocins and, in conjunction with peptidoglycan and LPS, play a significant role in maintaining the integrity of the cells. Porins from *Escherichia coli* (OmpF, OmpC, and PhoE) are the best characterized concerning their biochemical, functional, genetic, immunochemical, and structural properties (9, 32). The correlation between biological properties and structure was recently resolved by X-ray analysis of OmpF porin at a resolution of 0.24 nm (10).

Klebsiella pneumoniae is a nosocomial pathogen of particular relevance to immune-deficient individuals (4, 21). Capsular polysaccharide, LPS, and some outer membrane proteins (OMP) determine its virulence (48), but the role of *K. pneumoniae* porins in pathogenesis has not yet been studied in detail. Two of them, homologous to phosphate-repressible porin (PhoE) and the maltose-induced maltoporin (LamB) in *E. coli*, have been cloned and sequenced (46, 47). The derived *Klebsiella* protein sequences, and the regulation of their expression, closely resemble those of the corresponding *E. coli* proteins. Two other porins have been described for *Enterobacter cloacae* strains (26) that were later identified as *K. pneumoniae* (40). These proteins have been presumed initially to be the analogs of *E. coli* OmpC and OmpF, but the assumption was based on their pore sizes only (26). Presumably, they are identical to the *K. pneumoniae* OMP OmpK36 and OmpK35 that, on the basis of their modes of isolation, molecular masses, and N-terminal sequences, have been suggested to be the porins of this bacterial species (2). It has been shown that they can form a complex with C1q, the first component of the classical pathway of the complement system, and that they can activate the complement cascade, a property observed neither with other OMP nor with rough LPS (2). Potentially relevant observations are twofold. First, the interaction between porins and C1q is due to electrostatic forces: porins have a pI of around 5.0 (30, 41) and C1q has one of >9.0. Second, all of the enterobacterial porins tested to date have been shown to bind C1q (2, 18, 28, 41). The formation of porin-C1q complexes activates the complement classical pathway. Together with an activated complement alternative pathway, the result is the effective elimination of serum-sensitive *K. pneumoniae* strains. Since this interaction is antibody independent (2), and *K. pneumoniae* is an important pathogen for immunocompromised hosts, this may represent a relevant mechanism for the elimination of serum-sensitive strains and for the protection of susceptible hosts. There also seems to be a common way that enterobacteria have developed to protect themselves against lysis by complement, i.e., the LPS (24). Many enterobacterial species are susceptible to complement if long-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
K. pneumoniae		
C3	O1:K66 OmpK36$^+$ OmpK35$^+$	42
KT707	Rough LPS mutant derived from C3 (chemotype Rd); OmpK36$^+$ OmpK35$^-$	42
KT141	Rough LPS mutant derived from C3 (chemotype Ra); OmpK36$^+$ OmpK35$^+$	43
KT793	Noncapsulated and rough LPS mutant (chemotype Rb or Rc) derived from C3; OmpK36$^+$ OmpK35$^+$	2
KT793Rif	Spontaneous rifampin-resistant mutant derived from KT793; OmpK36$^+$ OmpK35$^+$	This work
KT5002	KT793Rif *ompK36*::Tn*5*	This work
E. coli		
HB101	*supE44 hsd20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	5
DH5α	F$^-$ *endA1 hsdR17 SupE44 thi* λ$^-$ *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)-U169 Φ80d*lacZ*ΔM15	19
UH302	OmpA$^-$ OmpC$^-$ OmpF$^-$	8
Plasmids		
pACYC184	Medium-copy-number cloning vector	6
pBluescript SK(−)	High-copy-number cloning vector	Stratagene
pPH1JI	IncP1; gentamicin resistant	22
pRK2073	Helper plasmid for conjugation	16
pVK102	Mobilizable cosmid vector	27
pSUV1	pVK102 clone carrying *ompK36*	This work
pSUV7	pACYC184 carrying a *Sal*I-*Xho*I *ompK36*-containing fragment	This work
pSUV9, −10, −11, −12	pBluescript SK(−) derivatives used for *ompK36* sequencing	This work
pSUV100	pSUV1, *ompK36*::Tn*5*	This work

chain LPS is removed, presumably allowing the porins to bind complement components. Given the high homology between enterobacterial porins and the binding of C1q to the porins of all enterobacteria tested to date, the existence in the porins of common motifs for C1q binding and antibody-independent activation of the classical complement pathway is suggested.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids are indicated in Table 1 and Fig. 1. Luria-Bertani medium supplemented with the appropriate antibiotic concentrations (39) was used throughout.

OMP isolation and analysis and crystallization of OmpK36. Bacterial cell envelopes containing cytoplasmic and outer membranes were obtained by French press cell lysis and centrifugation. OMP were isolated as sodium lauryl sarcosinate-insoluble material (17). Porin proteins, including OmpK36, were isolated and purified by a combination of methods (31, 34) as described before (2). Electrophoretic analysis of OMP was performed in 11.5% acrylamide–0.5% bisacrylamide–0.1% sodium dodecyl sulfate (SDS) gels. Samples (about 5 μg of OMP or 1 μg of purified porins) were boiled for 5 min in Laemmli's sample buffer before electrophoresis. Western blot (immunoblot) analysis of proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by transfer to polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.) at 1.3 A for 1 h in Towbin's buffer (44). Membranes were then incubated sequentially with 5% skim milk–phosphate-buffered saline, anti-OmpK36 serum, alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (IgG), and 5-bromo-4-chloro-3-indolylphosphate disodium–nitroblue tetrazolium (BCIP-NBT) (3). Incubations were carried out for 1 h, and washing steps with 0.05% Tween 20–PBS were included after each incubation step.

Purification of the OmpK36 protein from strain KT707 was performed by published procedures (2).

Antiserum. New Zealand White rabbits were injected subcutaneously three times every 2 weeks with 40 μg of purified OmpK36 and bled 2 weeks after the last injection. This antiserum was absorbed (twice for 2 h at 37°C and once overnight at 4°C) with *E. coli* HB101 cells (approximately 10^{11} CFU per absorption step), and the complement was inactivated (30 min at 56°C) before its use in Western and dot blot experiments.

DNA procedures. Restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were from Pharmacia (Uppsala, Sweden) and used as specified by the manufacturer. Large-scale and miniscale plasmid isolations were carried out by alkaline lysis (39). Genomic DNA was isolated as described previously (13). Standard DNA procedures for agarose gel electrophoresis, Southern blotting, plasmid transformation, and recovery of DNA fragments from gels were used throughout (39). Conjugation with pVK102 derivatives was performed by triparental mating, using pRK2073 to provide in *trans* Tra functions. Southern blot analysis of genomic DNA from *K. pneumoniae* KT793Rif and KT5002 digested with *Eco*RV or *Xho*I was carried out as described previously (39). A 500-bp *Hin*dII-*Hin*dII fragment from pSUV7 (Fig. 1) was labeled with [α-^{32}P]dCTP by use of the Megaprime labeling system (Amersham Ibérica SA, Madrid, Spain) and used as a probe.

Construction and screening of a *K. pneumoniae* DNA library. Genomic DNA

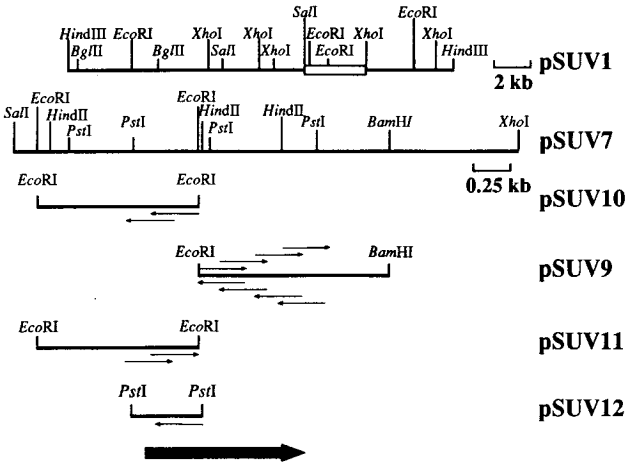

FIG. 1. Cloning and sequencing strategy of the *ompK36* gene. The *ompK36* gene was located within the *Sal*I-*Xho*I fragment of pSUV1 (open box) by immunoreactivity with anti-OmpK36, expression of OmpK36 in *E. coli*, and mutagenesis with Tn*5*. The boxed *Sal*I-*Xho*I fragment was cloned in pACYC184, resulting in plasmid pSUV7. The *Bam*HI-*Eco*RI from pSUV7 was cloned in pBluescript (SK$^-$) (pSUV9). The *Eco*RI-*Eco*RI from pSUV7 was also cloned in pBluescript (SK$^-$), generating pSUV10 (oriI) and pSUV11 (oriII) plasmids. Plasmid pSUV12 resulted from the cloning of fragment *Pst*I-*Pst*I from pSUV7 in pBluescript (SK$^-$). Deletions from these plasmids were produced with exonuclease III (Erase-a-base; Promega, Madison, Wis.) and sequenced by the dideoxy method (Sequenase 2.0; U.S. Biochemical Corp., Cleveland, Ohio). Arrows indicates the direction of sequencing. The region coding for OmpK36 is indicated by a thick arrow.

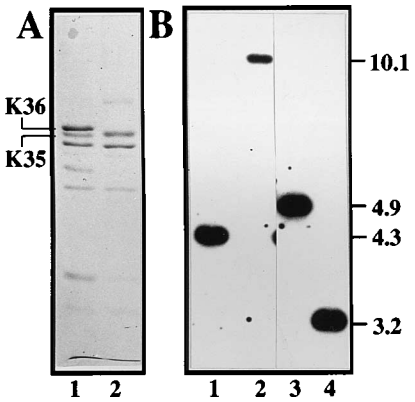

FIG. 2. Analysis of the cloned gene. A mutant strain (KT5002) which lacks OmpK36 was obtained by recombination between chromosomal DNA from *K. pneumoniae* KT793Rif (OmpK36$^+$) and plasmid pSUV100 (*ompK36*::Tn*5*). (A) SDS-PAGE profiles of OMP from *K. pneumoniae* KT793Rif (lane 1) and KT5002 (lane 2). (B) Southern blot analyses of chromosomal DNA from KT793Rif (lanes 1 and 3) and KT5002 (lanes 2 and 4) digested with *Eco*RV (lanes 1 and 2) or *Xho*I (lanes 3 and 4). A [α-^{32}P]dCTP-labeled intra-*ompK36* fragment (*Hin*dII-*Hin*dII) isolated from plasmid pSUV9 was used as a probe. Sizes (in kilobases) are indicated to the right of panel B.

from *K. pneumoniae* C3 was isolated and partially digested with *Hin*dIII. DNA fragments of 23 to 25 kb were separated in a sucrose gradient and ligated to dephosphorylated *Hin*dIII-linearized pVK102 DNA. The ligation mixture, packed in phage heads with the lambda DNA in vitro packaging kit from Amersham Ibérica SA, was used to infect *E. coli* HB101. A collection of 1,900 tetracycline-resistant, kanamycin-sensitive colonies was obtained and tested for OmpK36 expression by dot blot analysis with anti-OmpK36 serum. The dot blot screening with anti-OmpK36 was carried out as follows. *K. pneumoniae* KT707 and *E. coli* HB101 (as positive and negative controls, respectively) and clones were grown overnight at 37°C with shaking in wells of microtiter plates containing Luria-Bertani medium plus tetracycline. For each culture, 100 μl was transferred to nitrocellulose filters by Milliblot filtration (Millipore). Filters were blocked in 1% bovine serum albumin (BSA) in PBS. After washing, the filters were incubated sequentially with anti-OmpK36 (1:4,000) and alkaline phosphatase-labeled goat anti-rabbit IgG (1:3,000). The filters were developed with BCIP-NBT. All of the incubations were carried out at room temperatures for 1 h in 1% BSA–0.05% Tween 20–PBS, and after incubations with the antisera, washing steps with 0.05% Tween 20–PBS were performed.

Identification, cloning, and sequencing of the OmpK36-encoding gene. Sixteen clones were selected and studied further because of their strong reaction with anti-OmpK36 in the dot blot screening. Analysis of the OMP of these clones by SDS-PAGE and comparison with the OMP of strain HB101 showed that eight clones produced an extra OMP that migrated in the gels slightly above the band containing both OmpC and OmpF porins from *E. coli*. The protein expressed by the clones had a molecular mass of about 36 kDa (the size of OmpK36). Restriction analysis of the plasmid-carrying clones demonstrated that all of them contained the same chromosomal fragment in the two possible orientations. These two forms were designated pSUV1 (Fig. 1) and pSUV2. The former was selected for further analysis.

Localization of the gene coding for OmpK36 to pSUV1 was done by random mutagenesis of *E. coli* HB101(pSUV1) with λ::Tn*5* (11). For the selection of the Tn*5*-mutated pSUV1 plasmid, the kanamycin- and tetracycline-resistant colonies were pooled and their plasmid DNA was isolated and used to transform DH5α. Transposon insertions in plasmid pSUV1 were identified by selection of tetracycline- and kanamycin-resistant DH5α(pSUV1) strains. The OMP of 20 selected mutants were analyzed by SDS-PAGE. One of them, *E. coli* DH5α (pSUV100), did not produce OmpK36. Physical mapping of pSUV100 showed that transposon insertion occurred within a 1.1-kb *Eco*RI fragment of the *K. pneumoniae* DNA insert originally contained in pSUV1. Conjugal transfer of pSUV100 to *K. pneumoniae* KT793Rif (OmpK36$^+$), followed by mobilization of the incompatible plasmid pPH1JI into KT793Rif(pSUV100), resulted in the replacement of the *ompK36* gene of KT793Rif by its corresponding Tn*5*-mutated allele of pSUV100. The mutant obtained above (strain KT5002) had lost OmpK36, as confirmed by SDS-PAGE and Southern blot analyses (Fig. 2). These results indicated that the 1.1-kb *Eco*RI fragment of plasmid pSUV1 contains the gene (or a part of it) coding for OmpK36. Subcloning of the 3.5-kb *Sal*I-*Xho*I fragment from pSUV1 for further sequencing of the OmpK36 gene was performed in the high-copy-number vector pBluescript (SK$^-$) and in medium-copy-number vector pACYC184. Stable expression of OmpK36 was obtained only with pACYC184 subclones (pSUV7). Further subcloning and sequencing of truncated OmpK36 gene were carried out in pBluescript (SK$^-$).

Sequence and homology studies. Translation to amino acids of the DNA sequence coding for OmpK36 was done with the DNA Strider program (C. Mark and Commissariat à l'Énergie Atomique, Gif-sur-Yvette, France). The Genetics Computer Group programs (12) PILEUP and LINEUP were used for the alignment of porin amino acid sequences and to construct a phylogenetic tree of enterobacterial porins. Pairwise similarity between selected porins was further studied with DISTANCES (Genetics Computer Group), with a threshold of comparison equal to 0.60. The alignment between OmpF and PhoE was corrected subsequently in a few places to conform with the alignment obtained from the superposition of their respective X-ray structures (10).

Homology modeling of OmpK36 was performed on the basis of the X-ray structures of *E. coli* porins OmpF and PhoE (10) with the interactive graphics program FRODO (25). The X-ray structure of OmpF was taken for the barrel scaffold and the internal loop L3. The residue numbering is based on the OmpF sequence.

For homologous turn and external loop regions, that structure of OmpF or PhoE whose sequence showed closer resemblance to OmpK36 was used. Side chains were replaced according to OmpK36 sequence and manually adjusted on the graphics system to avoid steric interferences. For nonhomologous loops, no efforts were undertaken to optimize their structure.

C1q binding assays. Purification, labeling with biotin or ^{125}I, and C1q binding assays were performed as described previously (2). Two types of binding experiments were performed. (i) OMP from the strains under study were isolated, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes as described above. C1q binding proteins were detected by incubations with biotin-labeled purified C1q and with alkaline phosphatase-labeled avidin. (ii) Binding of C1q to whole cells was quantitated with ^{125}I-labeled C1q. Briefly, bacterial cells were incubated with radiolabeled C1q for 45 min at 0°C, microcentrifuged for 5 min. Bound and unbound ^{125}I-labeled C1q was measured from the counts in the pellet and supernatant fluids, respectively.

Nucleotide sequence accession number. The nucleotide sequence of OmpK36 gene has been submitted to EMBL (accession number Z33506).

RESULTS

Expression of OmpK36 in *E. coli*. Both plasmids pSUV1 and pSUV7 contained the gene coding for OmpK36. This was demonstrated by introduction of these plasmids into *E. coli* DH5α and into the porinless mutant *E. coli* UH302. As revealed by SDS-PAGE analysis, only *E. coli* carrying pSUV1 or pSUV7 exhibited a band with the same mobility as that of OmpK36 expressed by *K. pneumoniae* C3 and KT707 (Fig. 3A). Figure 3A also shows that expression of the *ompK36*-encoded protein leads to a reduced or abolished expression of endogenous *E. coli* porins. The novel OMP expressed by *E. coli* carrying pSUV1 or pSUV7 retained its antigenic properties, as it reacted with anti-OmpK36 in immunoblot experiments (Fig. 3B). Some minor nonspecific reactions were seen in the Western blot with endogenous *E. coli* porins, presumably because of the high degree of homology between enterobacterial porins. The OmpK36 expressed by the *E. coli* clones was extracted by porin extraction methods based on the trypsin resistance to porins and their strong noncovalent association with the peptidoglycan (Fig. 3C); it thus also retained its original porin properties. Figure 3D shows that the *ompK36*-encoded protein has also retained its heat modifiability.

Sequence homology and three-dimensional model. The comparison between the sequences of known enterobacterial porins and the deduced protein sequence of OmpK36 clearly supports our previous observation (based on the N-terminal sequence analysis) that OmpK36 belongs to the porin family. The phylogenetic tree (not shown), derived from the known sequences of enterobacterial porins, including OmpK36, shows divergence into four groups. One is formed by the osmoporins (OmpC), a second consists of the phage-related porins (Lc and NmpC), a third consists of the phosphate-repressible PhoE porins, and a fourth consists of OmpF porin. Clearly, OmpK36 falls within the OmpC group and is closer to the OmpC porin of *E. coli* than to that from *Salmonella typhi*. Given the high homology within the OmpC group, and considering only enterobacterial porins, the new member OmpK36 does not alter the phylogenetic tree established (23). The detailed alignment

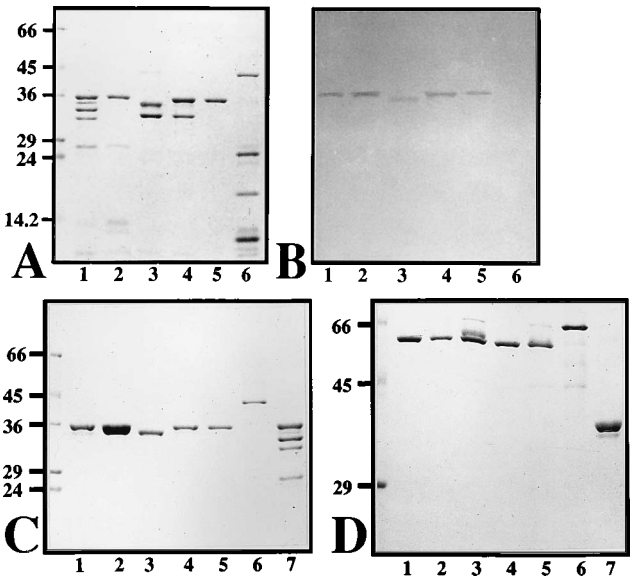

FIG. 3. Expression of OmpK36 in *E. coli*. (A and B) Electrophoretic (A) and Western blot (B) analysis with anti-OmpK36 of OMP isolated from *K. pneumoniae* and *E. coli* strains expressing the *ompK36*-encoded protein. Lanes in both panels: 1, *K. pneumoniae* C3; 2, *K. pneumoniae* KT707; 3, *E. coli* DH5α; 4, *E. coli* DH5α(pSUV1); 5, *E. coli* UH302(pSUV7); 6, *E. coli* UH302. Molecular mass markers (in kilodaltons) are indicated on the left. (C and D) SDS-PAGE analysis of porins isolated from *K. pneumoniae* and *E. coli* strains expressing the *ompK36*-encoded protein. Samples were either boiled for 5 min in electrophoresis sample buffer (C) or incubated at 37°C for 30 min in the same buffer (D) to study their heat modifiability. Lanes: 1, *K. pneumoniae* C3; 2, *K. pneumoniae* KT707; 3, *E. coli* DH5α; 4, *E. coli* DH5α(pSUV1); 5, *E. coli* UH302(pSUV7); 6, *E. coli* UH302; 7, OMP (panel C) and isolated porins (panel D) from *K. pneumoniae* C3. Samples in lane 7 (both panels) were boiled for 5 min in sample buffer. Molecular mass markers (in kilodaltons) are indicated on the left.

of the OmpK36 amino acid sequence with the members of the OmpC family and with OmpF and PhoE from *E. coli*, for which the three-dimensional structures are known to high resolution (9), is given in Fig. 4. OmpK36 exhibits between 88.3 and 72.6% sequence identity with these porins, and therefore, the alignment is well defined. As can be seen from the figure, the homology is most pronounced within the region that, in OmpF, forms the transmembrane β-strands (β1 and β16). All insertions and deletions are found in the loops facing the cell exterior (external loops L1, L2, and L4 to L8), with the exception of the insertions or deletions at the end of β8 at the periplasmic side. For loops L1 and L2, OmpK36 resembles more closely PhoE in that the numbers of residues are identical. Loop 3, which in the known porin structures extends inside the barrel and defines the size of the transmembrane pore, is the most conserved loop and of identical length to that in OmpC from *E. coli*, OmpC from *S. typhi*, and OmpF. Loop L4 carries the longest insertion (10 residues) in OmpK36, L6 and L8 exhibit small insertions or deletions, and L5 and L7 are unchanged in length.

A three-dimensional model of OmpK36 was obtained by modeling, using the X-ray structures of the homologous porins OmpF and PhoE (Fig. 5). Most of the model building required only exchange and adjustment of amino acid side chains at the interactive graphics. Almost no substitutions were found in the lining of the pore at the height of the constriction. The cluster of R-42, R-82, and R-132 and residues D-113 and E-117 that are opposed across the pore are conserved. Clearly, it is this part of the model that could be devised most reliably. The model shows that the cross-section of the pore is smaller than it is in OmpF. This is due to the presence in OmpK36 of the more bulky side chains of W-80 and Y-123, which are K and A, respectively, in OmpF (the residue numbering is based on the OmpF sequence). The net charge within the pore constriction of OmpK36 is 1 charge unit more negative than in OmpF, while the difference of negative charges relative to PhoE porin is 4 charge units. Residues contributing to the interface between the internal loop L3 and the barrel wall show considerable variation between *E. coli* OmpF and PhoE and *K. pneumoniae* OmpK36, with replacements of hydrophobic by hydrophilic residues occurring in several places. The hydrophobic interface between the subunits of the trimeric structure is well conserved. The only substitutions found are conservative replacements at positions 17, 41, 65, and 336. The smooth side of the barrel that is exposed to the periplasm also shows but a few differences among the three porins.

C1q binding. The ability of the *ompK36*-encoded protein to bind complement component was investigated in immunoblot experiments with biotinylated C1q (Fig. 6A). OmpK36 (lane 1), purified from the outer membrane of *K. pneumoniae* KT707, with its known C1q binding ability, served as a positive control. OMP preparations from *K. pneumoniae* KT707 and *E. coli* DH5α(pSUV1) and UH302(pSUV7) contain a protein that binds C1q with a mobility corresponding to that of the C1q-binding OmpK36 protein. Weaker reactions were also observed in OMP preparations of *E. coli* DH5α and UH302. They were consistently above the background of the negative control (BSA) and were located in the position of the unresolved porins of strain DH5α and of a high-molecular-mass OMP of strain UH302. In vivo, binding of C1q is affected by the LPS composition of the outer membrane. As shown in Fig. 6B, the binding of C1q to cells of isogenic LPS mutant strains increases with shorter LPS: chemotype Ra and wild-type (O^+) strains bind C1q poorly, strains with Rb chemotypes are intermediate, while cells with an Rd chemotype bind at least four times more C1q than wild-type LPS cells.

DISCUSSION

Activation of the complement system is relevant for immune-deficient individuals whose defense mechanisms are severely impaired against bacterial infections as well (15). We have recently shown that two OMP from *K. pneumoniae*, involved in the sensitivity of this pathogen to the bactericidal complement system, are presumably porins (2). Therefore, we have now investigated this property in more detail by performing the following steps. First, we have cloned, expressed, and sequenced the OmpK36 porin. Second, we have studied the sequence homology that it may share with those *E. coli* porins for which the structure has been solved to high resolution. Third, we have modeled the structure of the OmpK36 protein with the *E. coli* porin structure, keeping in mind the localization of binding sites resembling those known to mediate interactions between the C1q component and the Fc portion of the IgG antibodies. The expression of the clones studied inhibited the expression of endogenous *E. coli* porins, a fact that has been documented before for other cloned OMP-coding genes (1, 36, 49). It is due, in part, to a gene dosage effect (7). To ensure that the selected clones carried the gene for OmpK36, we mobilized them to an $OmpC^-$ $OmpF^-$ *E. coli* strain and confirmed the expression of OmpK36. Furthermore, recombination of a Tn*5*-mutated pSUV1 plasmid with the *K. pneumoniae* chromosome was followed by loss of OmpK36 expression in the resulting strain. The deduced protein sequence of the OmpK36 gene confirmed the previously published N-terminal sequence of OmpK36 (2) and the assignment of

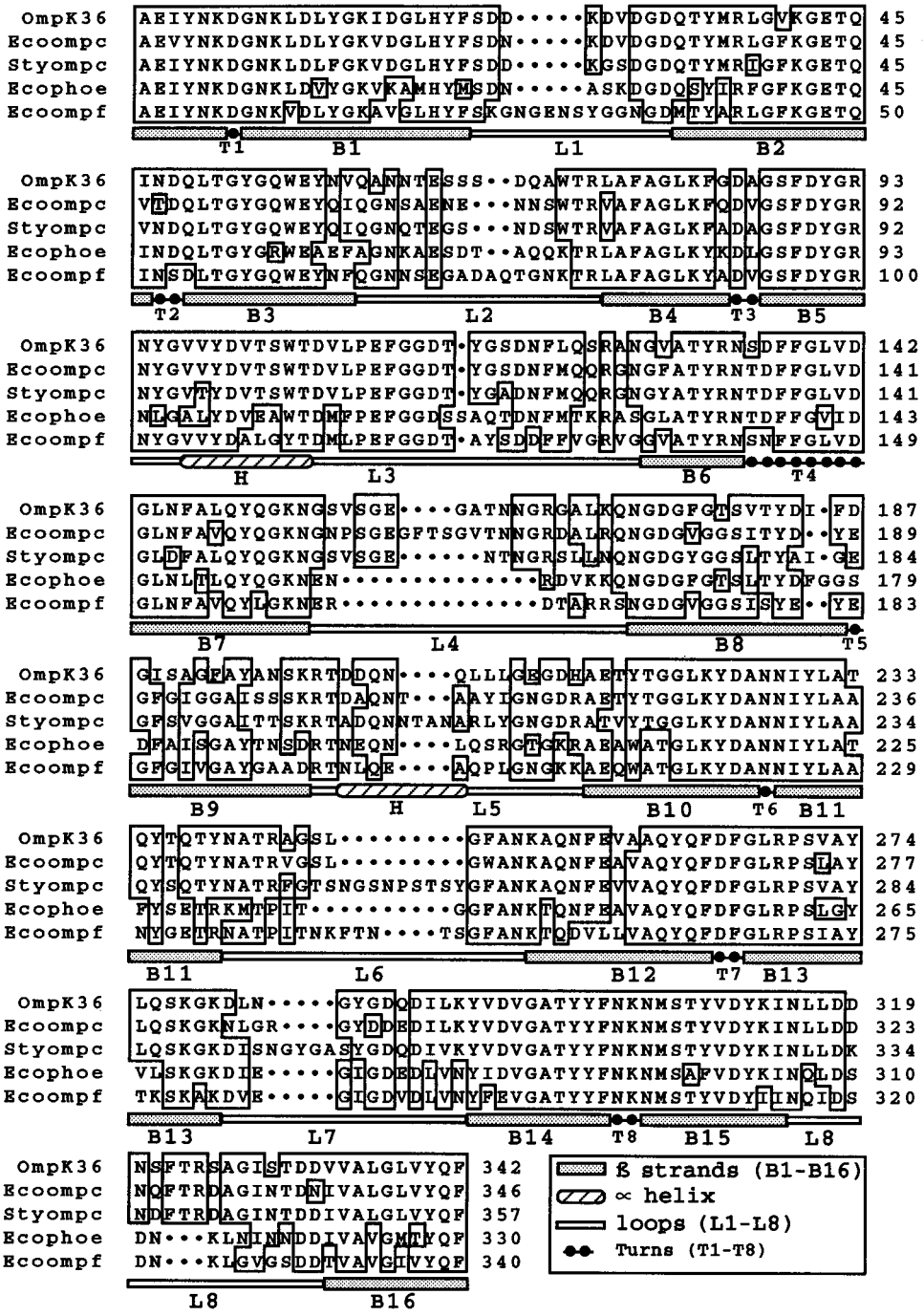

FIG. 4. Alignments of the OmpK36 sequence with selected sequences from other enterobacterial porins. Protein sequences were derived from nucleotide sequences. Porins were selected on the basis of their high homology with OmpK36. Secondary structural motifs are those of OmpF structure (10).

OmpK36 to the OmpC subgroup of enterobacterial porin proteins. Nonspecific porins (33) from *K. pneumoniae*, which are the putative equivalents of *E. coli* OmpC and OmpF, have been suggested to play a role in the permeation of small solutes (26), in antibiotic resistance (20, 35, 37, 45), and in activation of the complement system (2). The porin nature of the *K. pneumoniae* OMP involved in those phenomena is thus demonstrated directly. Furthermore, we have recently reported that OmpK36 allows diffusion of cefoxitin and expanded-spectrum cephalosporins (29).

The OmpK36 porin sequence shares a typical pattern with enterobacterial porins (23): the N and C termini of OmpK36 show better homology with other enterobacterial and nonenterobacterial porins than the central part, where the conserved PEFXG motif (23) is located. Given the high sequence homology of the OmpK36 sequence, the alignment with the sequences of OmpF and PhoE was unambiguous for most parts. In the regions of the β-scaffold, most of the short turns at the smooth side, and the internal loop L3, model building of OmpK36 was straightforward and a very similar backbone was

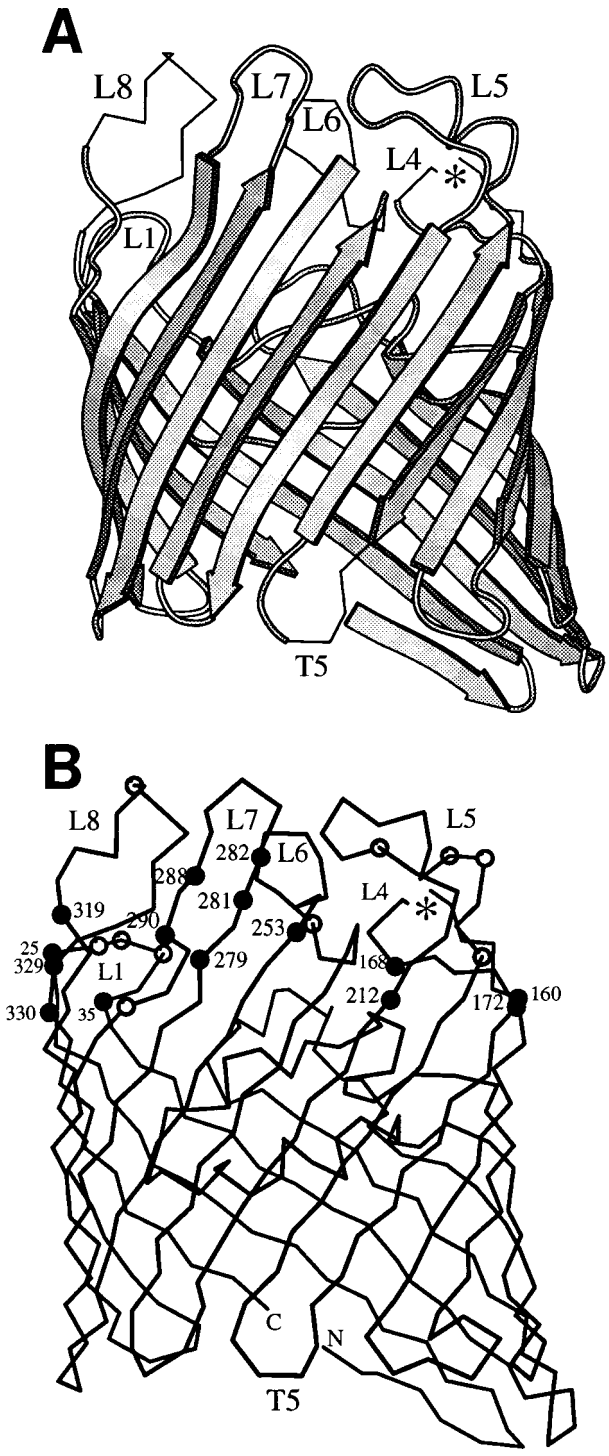

FIG. 5. Structural model of the OmpK36 monomer from *K. pneumoniae* based on the X-ray structures of *E. coli* OmpF and PhoE (10). (A) Overall structure. β-strands are symbolized by arrows, and the connecting loops and turns are symbolized by double lines. In the native membrane, the side of the barrel with the long loops (top) is facing the extracellular space. The parts of the structure that could not be modeled reliably are represented as thin lines (loops L4, L6, and L8 and turn T5). Loop L4 is not shown in full since it carries a 10-residue insertion of unknown conformation (see break). Loop L3 (not labeled) extends inside the barrel and constricts the pore. In this view, the threefold symmetry axis of the trimer (not shown) is behind the monomer. Loop L2 (not labeled) is protruding towards the rear and is involved in subunit interactions. (B) Cα tracing of the OmpK36 model in the same view as that in panel A. The positions of the acidic and basic residues (D, E, K, and R) that are part of the external loops or the adjacent hydrophilic segments of the β-strands are highlighted by open circles. Filled circles indicate ionizable residues that are identical in OmpK36 and at least in two of the three *E. coli* porins, OmpC, OmpF, and PhoE. All of these residues are exposed except E-71 and R-196 (not shown). Open circles represent acidic and basic residues that are not conserved. The residue numbering scheme is based on the OmpF sequence.

to be anticipated. The location and orientation of amino acid side chains could be approximated easily, but the determination of exact conformations would be premature. The model of the pore constriction in OmpK36 revealed mostly conserved residues. The net charge of the constriction is close to that of OmpF but, due to the presence of a few bulkier side chains, the cross-section of the pore is slightly smaller. The loops facing the cell exterior show the most sequence variability.

C1q binding sites were examined with scrutiny for conserved charged patches on the side of the porin potentially exposed to the extracellular space. This domain consists of the external loops and those parts of the β-strands that extend beyond the hydrophobic core of the membrane. Obviously, the X-ray structures can serve as a reliable model only for those long external loops of OmpK36 carrying neither insertions nor deletions compared with OmpF or PhoE (i.e., L1, L2, L5, and L7). Conserved motifs on the porin surface are the exposed ionizable residues found mainly in the regions in or near L6 or L8 (Fig. 5B). In the native membrane, these may constitute the binding sites for LPS molecules (10). However, it is conceivable that these sites interact competitively with C1q. This is corroborated by our previous finding that LPS O side chain (but not capsular polysaccharide) is the major barrier for binding of C1q to *K. pneumoniae* porins (2) and by the present results demonstrating C1q binding to whole cells (Fig. 6B).

The natural and best-characterized target of C1q is the Fc portion of IgG and IgM. By site-directed mutagenesis, Duncan and Winter (14) have localized the binding site for C1q on an IgG molecule to three side chains, i.e., E-318, K-320, and K-322. These residues are adjacent to each other on the external face of a β-strand. In OmpK36, the motif of a string-like arrangement of one negative and two positive side chains is also present. The two basic side chains of K-279 and K-281 are adjacent on the external face at the end of β-strand β13 and are followed by the acidic side chain of D-282 (loop L7) that also protrudes in an outward direction. Further studies with synthetic peptides covering residues 279 to 294 in the β13-L7 region of OmpK36 and by site-directed mutagenesis of this region are being conducted.

As one removes specific parts of the LPS, the bacterial cells, through porins, bind more C1q. Binding of LPS by porins thus appears to be a mechanism that renders the bacterial cells resistant to the classical complement pathway. This resistance does not need the presence of a complete LPS molecule: as shown here, affinities of binding of C1q by cells with complete LPS (O^+) and chemotype Ra LPS (O^-) are of the same magnitude and very reduced compared with those of chemotypes Rb or Rc and Rd. The O side chain and lipid A parts of the LPS have been studied the most and have been implicated in biological phenomena (in antigenic variation and resistance to complement and in endotoxicity, respectively) (38). Our results suggest a new role for the relatively conserved core part of the LPS: binding to and protection of the C1q binding motifs in porins may have developed a common mechanism of resistance in response to a general mechanism of activation of the complement classical pathway. It will now be interesting to determine whether transient, and partial, access to the core part of the LPS allows C1q to bind, and thus to activate, the

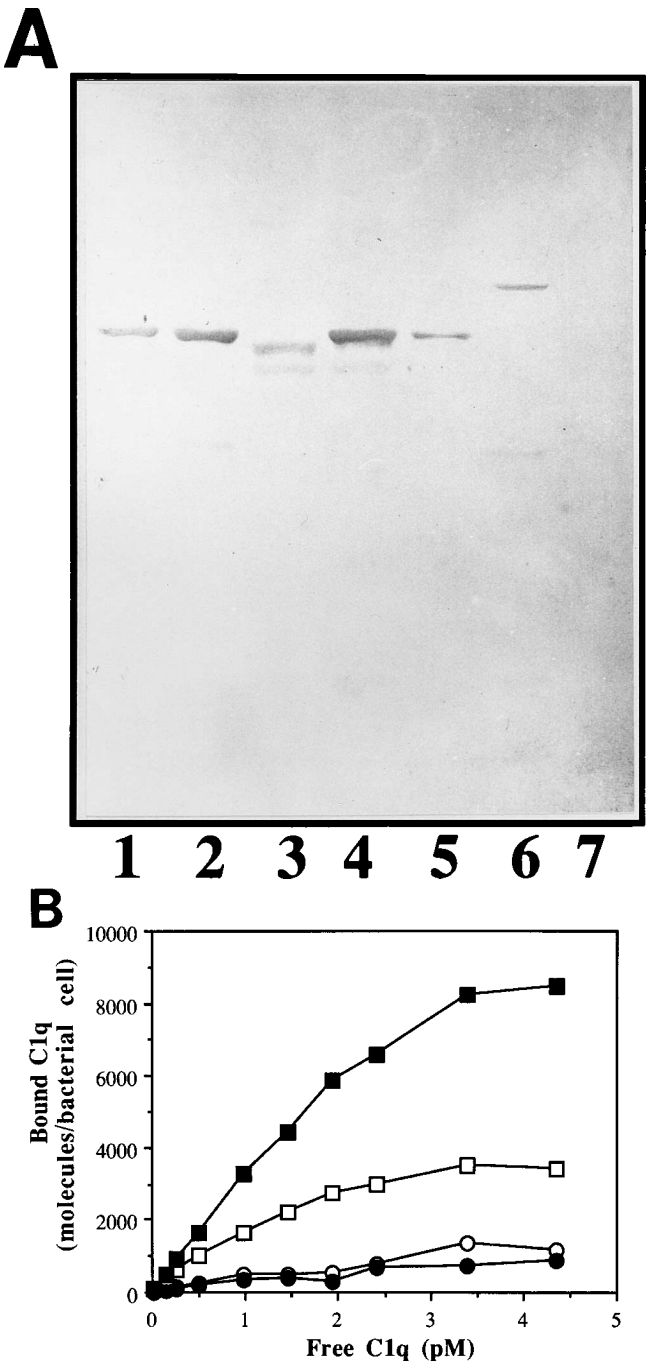

FIG. 6. Binding of the complement component C1q to the *ompK36*-encoded protein. (A) The lanes represent complexes with purified OmpK36 (lane 1) and OMP from *K. pneumoniae* KT707 (lane 2) and from *E. coli* DH5α, DH5α(pSUV1), UH302(pSUV7), and UH302 (lanes 3 to 6, respectively). Lane 7 contains BSA as a negative control. Binding was assayed by immunoblot analysis with biotinylated C1q and alkaline phosphatase-labeled avidin. (B) Binding of C1q to whole cells of *K. pneumoniae* wild-type strain C3 (O1:K66) (open circles). The binding of isogenic mutant strains with rough LPSs of chemotypes Ra (strain KT141) (closed circles), Rb or Rc (strain KT793) (open squares), and Rd (strain KT707) (closed squares) is also shown. Binding was studied with ^{125}I-labeled C1q. The results are the averages of four independent experiments.

complement cascade. We have recently obtained three-dimensional crystals (not shown), and their further study is in progress. This will help to confirm the proposed structural model and define the C1q-binding site(s) in OmpK36 porin.

In summary, we have provided several independent lines of evidence showing that OmpK36 is an OmpC-like porin in *K. pneumoniae*. Guided by the OmpK36 structural model, the porin-C1q interaction can now be studied in detail by site-directed mutagenesis. As a complementary approach, the three-dimensional structure of the OmpK36 porin of *K. pneumoniae* can be studied by X-ray crystallography.

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