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# Porin expression in clinical isolates of *Klebsiella pneumoniae*

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**Two porins, OmpK36 and OmpK35, have been described previously in *Klebsiella pneumoniae*, and they are homologous to the *Escherichia coli* porins OmpC and OmpF, respectively, at both the DNA and amino acid levels. Optimal resolution of the two *K. pneumoniae* porins by electrophoresis on polyacrylamide gels is not achieved using gel systems already described for *E. coli* and requires modifications of the bisacrylamide content of the resolving gels. Once resolved, identification of porins OmpK36 and OmpK35 cannot be based solely on their apparent molecular masses since in some strains the OmpK36 porin migrates faster than the OmpK35 porin, whilst in other strains OmpK35 is the faster-migrating porin. Expression of OmpK35 porin is increased in low-osmolarity medium and, combined with Western blot analysis, this allows for the identification of both porins. Application of this identification system showed that most isolates lacking expression of extended-spectrum $\beta$-lactamases express the two porins, whereas most isolates producing these $\beta$-lactamases express only porin OmpK36, and the OmpK35 porin is either very low or not expressed.**

Keywords: outer-membrane proteins, porins, *Klebsiella pneumoniae*, osmoregulation, antibiotic resistance

## INTRODUCTION

The outer membrane of enterobacterial species has significant medical importance because its constituents play major roles in the permeability of antimicrobial agents and substrates, and in interactions with the host defence mechanisms (Benz, 1994; Roth, 1988). In *Klebsiella pneumoniae*, most studies have been devoted to the roles of LPS and capsule in pathogenicity and other biological phenomena, whereas the outer-membrane proteins (OMPs) have attracted less attention (Williams & Tomás, 1990).

The OMPs of *K. pneumoniae* have been implicated in protection against lethal challenge with homologous strains (Serushago *et al.*, 1989), in activation of the complement system (Albertí *et al.*, 1993b), in iron acquisition (Williams *et al.*, 1987), and in permeability to antimicrobial agents (Martínez-Martínez *et al.*, 1996). Compared with close relatives such as *Escherichia coli*, less is known regarding the OMPs of *Klebsiella*. However, the existence of *K. pneumoniae* homologues to the phosphate-starvation-inducible PhoE porin (van der Ley *et al.*, 1987), the sucrose porin ScrY (Schmid *et al.*, 1991), and the LamB maltoporin (Werts *et al.*, 1992) have been shown. Concerning the non-specific pore proteins (porins), the molecular masses, pore sizes, and antimicrobial permeation of two porins, named 37kD and 39kD, have been characterized in *Enterobacter cloacae* strains later reidentified as *K. pneumoniae* (Kaneko *et al.*, 1984; Sawai *et al.*, 1982, 1987). These two porins are probably the equivalents of the two porins described by us in other *K. pneumoniae* strains: porin OmpK36, whose amino acid sequence is very homologous to that of *E. coli* OmpC porin (Albertí *et al.*, 1995), and porin OmpK35 (Hernández-Allés *et al.*, 1995), whose sequence (accession number AJ011501) is closer to that of *E. coli* OmpF porin.

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**Abbreviations:** 2D, two-dimensional; ESBL, extended-spectrum $\beta$-lactamase; OMP, outer-membrane protein; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

Porins are important in the permeability of antimicrobial agents (Nikaido, 1994). Loss of porins in *K. pneumoniae* strains producing expanded-spectrum *β*-lactamases (ESBLs) has been shown to cause resistance to cefoxitin, increased resistance to third-generation cephalosporins and monobactams, and decreased susceptibility to fluoroquinolones (Martínez-Martínez *et al.*, 1996). Porin loss was probably operating in other cases (Gutmann *et al.*, 1985; Pangon *et al.*, 1989; Sanders *et al.*, 1984; van de Klundert *et al.*, 1988), but the lack of a good definition of *K. pneumoniae* porins in the cited studies precludes the certainty of the porin nature of the OMP responsible for the resistance phenotypes. Enterobacterial porins have molecular masses compatible with the hypothesis that the OMPs cited in the above studies were in fact porins, but variations in molecular masses between these putative porins were observed depending on the study. It is clear that in the above situation one cannot conclude which porin (OmpC or OmpF type) was responsible for the observed phenotype. There is thus a need for an electrophoretic method to resolve and identify the two porins.

## METHODS

**Bacterial strains and culture media.** *K. pneumoniae* isolates preceded by M or SD were isolated respectively at Hospital Ramón y Cajal (Madrid) and Hospital Son Dureta (Palma de Mallorca) from blood (M) or other sites (SD); all of them were ESBL$^-$. *K. pneumoniae* strains expressing ESBLs were isolated at Hospital Universitario Virgen de la Macarena (Seville). *K. pneumoniae* isolates were identified following routine biochemical tests (Albertí *et al.*, 1993a). *K. pneumoniae* strains C3 (Albertí *et al.*, 1995) and 206 (Kaneko *et al.*, 1984) express the two porins OmpK36 and OmpK35 (strain C3) or their homologues, named 37kD and 39–40kD in the case of strain 206 (Kaneko *et al.*, 1984). *E. coli* strains JF568 (OmpA$^+$ OmpC$^+$ OmpF$^+$), JF699 (OmpA$^-$), JF701(OmpC$^-$) and JF703 (OmpF$^-$) were obtained from Dr Barbara J. Bachmann (*E. coli* Genetic Stock Center, Yale University, New Haven, CT, USA) and were used as controls to test the performance of electrophoretic porin separation systems.

Nutrient broth (Merck, Cat. No. 5443) or nutrient broth plus 20 % sorbitol, with osmolarities of 51 and 1513 mosmol kg$^{-1}$, respectively, were used as low- and high-osmolarity growth media. Strains were grown overnight at 37 °C with shaking.

**OMP and porin isolation.** Cells from overnight cultures were recovered by centrifugation, washed with 10 mM Tris/HCl (pH 7·2), 5 mM MgCl$_2$, and broken by sonication at 18–20 µm for 2 × 30 s cycles, each cycle comprised 6 × 5 s sonication steps separated by 1 s of no sonication, and 30 s of no sonication between the two cycles. Unbroken cells were eliminated by centrifugation at 3000 ***g*** for 10 min, and cell envelopes were recovered at 100 000 ***g*** for 1 h. After solubilization in 10 mM Tris/HCl (pH 7·2), 5 mM MgCl$_2$, 2 % sodium lauroyl sarcosinate for 30 min at 25 °C, the insoluble OMPs were recovered at 100 000 ***g***. A second solubilization step was performed, and the OMPs were again pelleted as above.

Porins were isolated from cell envelopes by a combination of methods based on the strong non-covalent association of porins with peptidoglycan (Nikaido, 1983) and on their resistance to proteases (Nurminen, 1978), as described by Albertí *et al.* (1993b). When required, a gel-permeation chromatography step which separates porins from LPS (Albertí *et al.*, 1993b) was performed.

OMP and porin samples were solubilized in electrophoresis sample buffer (see below) and boiled for 5 min before electrophoretic analysis.

**SDS-PAGE.** Electrophoretic analyses of *K. pneumoniae* OMPs were performed in polyacrylamide gels according to the following electrophoresis methods and recipes: the low bisacrylamide gel system of Lugtenberg (Lugtenberg *et al.*, 1975), A, B, C, and D separation gels (Pugsley & Schnaitman, 1978), and a urea-SDS-PAGE (Mizuno & Kageyama, 1978). After trying these methods, we routinely used the following for resolution of *K. pneumoniae* porins: resolving gels contained 11 % acrylamide and either 0·21 % (low), 0·35 % (medium) or 0·54 % (high) bisacrylamide, plus 0·2 % SDS, in 0·375 M Tris/HCl (pH 8·8). Gels were polymerized with 0·2 % TEMED and 0·025 % ammonium persulfate. Stacking gels contained 4 % acrylamide, 0·1 % bisacrylamide, 0·1 % SDS in 0·125 M Tris/HCl (pH 6·8), and were polymerized as above. Laemmli's sample buffer [62·5 mM Tris/HCl (pH 6·8), 2 % SDS, 10 % glycerol, 5 % *β*-mercaptoethanol] and electrode buffer [25 mM Tris (pH 8·3), 190 mM glycine, 0·1 % SDS] were used. We ran gels of 0·75 mm thickness in the mini gel format (Hoefer SE250, 8 cm long) at 20 mA, or in the long format (Hoefer SE600, 16 cm long) at 50 V, 20 mA (overnight) or at 30 V, 250 mA (for about 4 h). Staining was performed with 0·125 % Coomassie brilliant blue R250 in 45 % methanol, 10 % acetic acid for about 30 min. Destaining was performed in 45 % methanol, 10 % acetic acid.

**Two-dimensional gel electrophoresis (2D gels).** Analysis of OMPs from selected isolates by 2D gels was performed by using the O'Farrell method and buffers, with minor modifications (Albertí *et al.*, 1996).

**Antisera and Western blot analysis.** Antisera against OmpK36 and OmpK35 porins were raised by repeated immunization of rabbits with purified native (unboiled) porins (Albertí *et al.*, 1995). Western blot analysis of SDS-PAGE-separated OMPs was carried out with the buffers and conditions described by Towbin & Gordon (1984), Immobilon P membranes (Millipore), anti-porin serum, and alkaline-phosphatase-labelled goat anti-rabbit IgG (Sigma). Enzyme was detected as described by Blake *et al.* (1984).

***β*-Lactamase expression.** Production of ESBLs was studied by the double-disc synergy test (Legrand *et al.*, 1989) as described by Ardanuy *et al.* (1998).

## RESULTS

### Electrophoretic separation of *K. pneumoniae* porins

Polyacrylamide gel electrophoretic methods described for the resolution of *E. coli* porins were tested in *K. pneumoniae*. The use of previously described *E. coli* mutants with specific defects in some of their OMPs (OmpA$^-$, OmpC$^-$ or OmpF$^-$) facilitated the identification of porins and OmpA on the gels and helped in testing the resolution of the different electrophoretic systems. The best resolution of *E. coli* porins OmpC and OmpF was achieved by using the urea-SDS-PAGE system of Mizuno & Kageyama (1978), as shown in Fig. 1 (lanes 1–4). As is typical from gels containing urea, the OmpC porin migrated more slowly than OmpF porin, according to their molecular masses: 38·3 kDa and

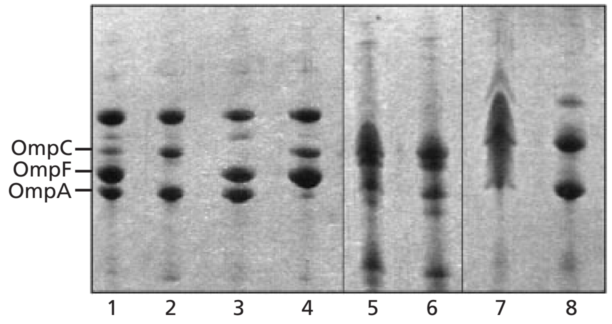

***Fig. 1.*** Electrophoretic separation of OMPs from *E. coli* (lanes 1–4) and *K. pneumoniae* (lanes 5–8) on the urea gel system of reference (Mizuno & Kageyama, 1978). Strains were grown in nutrient broth. Lanes 1–8, respectively, correspond to OMP preparations from *E. coli* strains JF568 (wild-type), JF703 (OmpF$^{-}$), JF701 (OmpC$^{-}$) and JF701 (OmpA$^{-}$), and *K. pneumoniae strains* C3, C3 after LPS removal, 206, and 206 after LPS removal. Only the relevant part of the gel is shown. The positions of *E. coli* OmpA, OmpC, and OmpF are labelled on the left.

37·0 kDa for the mature OmpC and OmpF monomers, respectively. The same gel system produced very diffuse bands when analysing OMPs of *K. pneumoniae* strains (Fig. 1, lanes 5 and 7). Depending on the *K. pneumoniae* strain, porins and other OMPs could be perceived on the gels, but separations were never as good as in the *E. coli* case. Removal of LPS from the OMP preparation seemed to improve protein separation on the gels (lanes 6 and 8). However, even after LPS elimination, resolution of *K. pneumoniae* OMPs in the porin molecular mass range was not as good as for *E. coli* OMPs.

When tested with *K. pneumoniae* strains, other gel systems described for *E. coli* produced variable results (not shown) depending on the isolate under study. For some strains, Lugtenberg's system (Lugtenberg *et al.*, 1975) and its close relative system A (Pugsley & Schnaitman, 1978) resolved the OMPs of interest well (data not shown), except that the latter system produced broader bands, perhaps because of the presence of urea in the resolving gel.

Variations in the bisacrylamide content of the resolving gel resulted in variable resolution of porins in *K. pneumoniae* (Fig. 2). The porins of each strain were optimally resolved in one of three SDS-PAGE systems: 11% polyacrylamide, 0·2% SDS resolving gels with (i) low, 0·21% bisacrylamide content, as in the Lugtenberg system (Fig. 2a); (ii) medium, 0·35% bisacrylamide content (Fig. 2b); or (iii) high, 0·54% bisacrylamide content (Fig. 2c). Although the porins of many strains could be well resolved independently of the bisacrylamide concentration used, for example strain C3 (Fig. 2, lane 7), optimal resolution was achieved at a given bisacrylamide concentration (0·54%, Fig. 2c, in the case of strain C3). In other strains, the two porins were seen as a single, unresolved band or as two bands, depending on the gel system used: e.g. strains SD17 and 206 (lanes 3 and 5, respectively) show only one porin band in high bisacrylamide gels (Fig. 2c) but two well resolved porin bands in low bisacrylamide gels (Fig. 2a). Finally, some isolates produced only one porin band in all three gel systems.

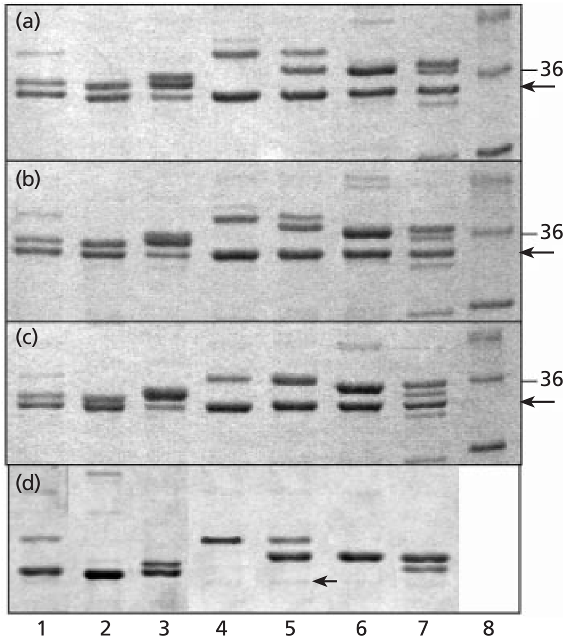

***Fig. 2.*** Effect of bisacrylamide concentration on the electrophoretic separation of *K. pneumoniae* porins. OMPs of strains M35, M32, SD17, M28, 206, 52145 and C3 (lanes 1–7, respectively) grown on nutrient broth, were isolated and analysed on gels with 0·21% (a), 0·35% (b), or 0·54% (c) bisacrylamide in the resolving gel. Alternatively (d), porins were first purified from the same strains and growth conditions, and then separated in the gel system (one of the three cited above) that best resolved the porins of each particular strain. The 36 kDa marker of the molecular-mass markers run in lane 8 is indicated on the right. The putative OmpA is indicated by arrows. Molecular-mass markers are not shown for (d) because this panel is a composite of porins separated in different gel systems (as in a, b and c), which also affect migration of the markers. The arrow in (d) indicates the position of the putative OmpA, which is barely visible, as a contaminant of porin preparation, in lane 5.

The identity of the putative porin bands was proven after porin isolation (Fig. 2d). The faster-migrating OMPs shown in Fig. 2(a–c) (the band labelled with an arrow that runs just below the porins) had the same apparent molecular mass in all strains examined. Given its molecular mass and its absence in porin preparations (Fig. 2d), this OMP must correspond to OmpA. This was further confirmed by the heat modification of the putative OmpA protein, which runs as a protein of ≈ 25 kDa in membranes solubilized at 37 °C (data not shown).

The above variations in electrophoretic mobility of porins were characterized for a total of 30 ESBL$^{-}$ *K. pneumoniae* clinical isolates. This resulted in up to

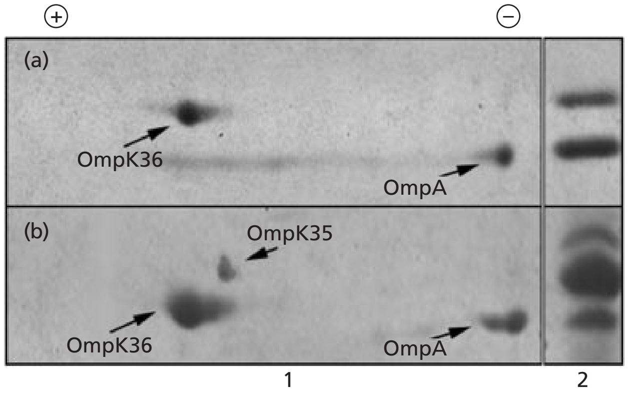

***Fig. 3.*** Electrophoretic 2D gel analysis of porins. Analysis of OMPs from isolates which expressed (a) only one porin (strain 52145) or (b) two porins (strain 206) as revealed by SDS-PAGE. In lane 1 in both (a) and (b), only the relevant portion of the 2D gels is shown. Lane 2 in both (a) and (b) corresponds to the SDS-PAGE separation (one-dimension gel) of the OMPs shown in lane 1.

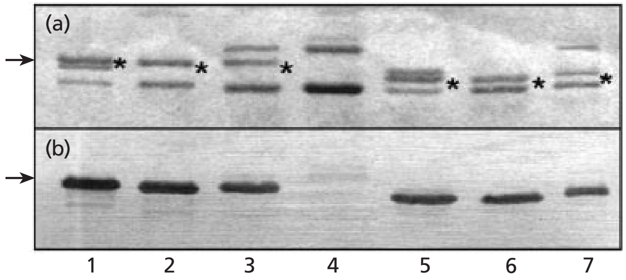

***Fig. 4.*** SDS-PAGE (a) and Western blot analysis with anti-OmpK36 porin serum (b) of OMPs from *K. pneumoniae* strains C3, 52145, 206, M28, SD17, M32, and M35 (lanes 1–7, respectively) grown on nutrient broth. Asterisks in (a) indicate the porin detected by the antiserum in (b). Arrowheads on the left indicate the position of the 36 kDa molecular-mass marker. Only the relevant parts of the gel and Western blot membrane are shown.

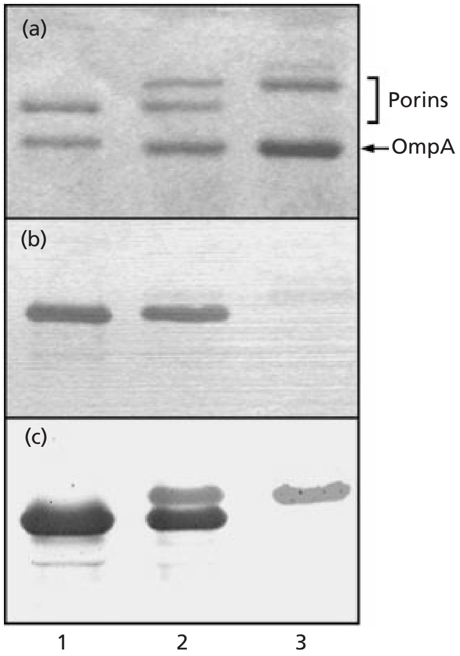

***Fig. 5.*** SDS-PAGE (a) and Western blot analyses with anti-OmpK36 (b) or anti-OmpK35 (c) sera of OMPs from strains 52145 (lane 1), 206 (lane 2) and M28 (lane 3). Strains were grown in low-osmolarity medium.

seven different patterns, depending on (i) the presence of one or two porins, and (ii) their apparent molecular mass on the gels. These porin patterns can be observed in Fig. 2(d) (lanes 1–7). Most strains (14) belong to the pattern shown on lane 5, followed by patterns shown on lanes 6 and 7 (four strains each). Patterns shown on lanes 1–4 were represented by three, two, one and two strains, respectively. Overall, we could resolve two porins in 22 isolates (73 %), and only one porin in eight isolates.

We then studied the possibility that the significant number of strains (8 out of 30) expressing only one porin could be due to a failure to resolve two porins co-migrating as a single band in all gel systems used, rather than to expression of a single porin. To address this question we performed 2D electrophoresis with selected isolates (Fig. 3). The results showed that isolates for which only one porin band was seen by SDS-PAGE produced only one single spot in 2D gels (Fig. 3a). In these gels, the two porins could be well resolved due to their differences in both pI and molecular mass and the putative OmpA was well separated from the porins (Fig. 3b).

## Identification of OmpK36 and OmpK35

The porin nature of the OMPs analysed in Fig. 2 was confirmed by Western blot analysis with specific anti-porin serum. After electrophoretic resolution of OMPs (Fig. 4a), in Western blot experiments (Fig. 4b) anti-OmpK36 porin serum detected a single protein in most of the isolates. These experiments also demonstrated: (i) the absence of OmpK36 porin expression in some isolates, such as strain M28 (Fig. 4a, lane 4), and (ii) that the OmpK36 porin in different isolates cannot be identified just by its apparent mobility on the polyacrylamide gels. Examples of the latter can be seen by comparing strains C3 (lane 1) and 206 (lane 3): of the two porins, OmpK36 is the slower-migrating porin of strain C3, whereas OmpK36 porin is the faster-migrating porin of strain 206. Identification of the OmpK35 porin was performed by Western blot experiments with anti-OmpK35 serum. As shown in Fig. 5, antiserum against OmpK35 identified a porin that was not detected by the anti-OmpK36 serum (lanes 2 and 3). Although anti-OmpK35 did not detect non-porin OMPs, like the

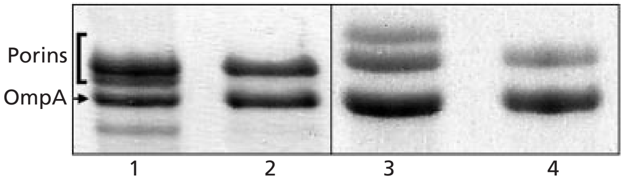

***Fig. 6.*** Porin expression in low-osmolarity (odd lanes) and high-osmolarity (even lanes) culture media. Strains analysed: C3 (lanes 1 and 2) and 206 (lanes 3 and 4).

putative OmpA, it strongly cross-reacted with the OmpK36 porin.

### Osmoregulation of OmpK35 porin

Further identification of porins OmpK36 and OmpK35 was obtained by studying their expression in culture media with different osmolarities (Fig. 6). It is well known in *E. coli* that osmolarity of the culture medium affects porin expression, and that expression of OmpF porin is enhanced in low-osmolarity medium and repressed in a medium with high osmolarity (van Alphen & Lugtenberg, 1977). Besides OmpA, whose expression was not affected by osmolarity, strains C3 and 206 expressed two porins in low-osmolarity medium (lanes 1 and 3). The expression of one of these porins was greatly reduced when the strains were grown in high-osmolarity medium (lanes 2 and 4). This shows that osmoregulation also affects the expression of *K. pneumoniae* porins. Furthermore, Fig. 6 demonstrates that the porin whose expression is downregulated under high-osmolarity conditions cannot be identified by its apparent molecular mass because, depending on the strain, the faster-migrating porin on the gels may be either OmpK36 or OmpK35. Western blot analyses with antisera against the OmpK36 and OmpK35 porins (data not shown) demonstrated that the porin whose expression increased under low-osmolarity conditions is the OmpK35 porin. This suggests that OmpK35 porin is the homologue of the *E. coli* porin OmpF.

### Porin expression in ESBL-producing *K. pneumoniae* isolates

Porin expression was also studied in 29 *K. pneumoniae* clinical isolates expressing ESBL. Porins from cultures grown in low- and high-osmolarity media were electrophoretically resolved by all gel systems described in this work, including 2D gels on some selected isolates (data not shown). Results from these studies showed that 83 % of these ESBL$^+$ isolates express only one porin, whilst the rest (five isolates, 17 %) express two porins. Further analyses by Western blot experiments with anti-OmpK36 and anti-OmpK35 sera demonstrated that the expressed porin was in all cases the OmpK36 porin. Additionally, in overloaded gels, a very low expression of the OmpK35 porin could be detected with the anti-OmpK35 serum in nine isolates, for which only one porin band was seen in Coomassie-blue-stained gels (data not shown). In summary, in 83 % ESBL$^+$ isolates we could detect expression of only one porin (OmpK36) by SDS-PAGE, and by the more sensitive Western-blotting analysis 52 % of the ESBL$^+$ isolates showed no expression of the OmpK35 porin and expressed the OmpK36 porin.

## DISCUSSION

Polyacrylamide gel electrophoresis is a routine technique for the analysis of bacterial OMPs. Numerous variations of SDS-PAGE and buffer systems have been described for the above purpose, but even minor variations may result in major changes in the electrophoretic profile of OMPs, as exemplified by porin separation of *E. coli* (Lugtenberg *et al.*, 1975). Despite the importance of a careful choice of electrophoretic conditions for the separation of porins, there are not many reviews dealing with this topic; thus the tendency when studying porins from other species is to use the methods described for *E. coli*. Electrophoretic methods that have successfully been used for the separation of *E. coli* porins (Lugtenberg *et al.*, 1975; Mizuno & Kageyama, 1978; Pugsley & Schnaitman, 1978) were shown not to be optimal for the same purpose in *K. pneumoniae*.

Two non-specific pore proteins (porins) have been identified in *K. pneumoniae* strain C3, namely porins OmpK36 (Albertí *et al.*, 1995) and OmpK35 (Hernández-Allés *et al.*, 1995), the homologues of *E. coli* porins OmpC and OmpF, respectively. *E. coli* porins OmpC and OmpF have a high degree of identity in their primary sequences, but they also differ in other properties, such as pore size and expression in different environmental conditions (temperature, osmolarity, pH, etc.). Whilst in *E. coli* it is possible to determine in a gel which porins correspond to OmpC and OmpF, the situation in *K. pneumoniae* was more complex because of the lack of porin definition in different clinical isolates. We have shown here that the majority of ESBL$^-$ *K. pneumoniae* clinical isolates express two porins which could be resolved by SDS-PAGE systems with different bisacrylamide concentrations. For a few isolates, the expression of only a single porin was detected by SDS-PAGE and confirmed by 2D gels.

The use of anti-OmpK36 and anti-OmpK35 sera allowed identification of both porins in different isolates. As a result, we observed that the apparent molecular masses of both porins varied among different isolates. Furthermore, in some isolates the OmpK36 porin ran faster in the gels than the OmpK35 porin, whereas in other strains it was the OmpK35 porin that ran faster than OmpK36. These results were confirmed by the differential expression of porins OmpK36 and OmpK35 in media with high and low osmolarity.

Clearly, the expression of *K. pneumoniae* porins OmpK36 and OmpK35 in response to osmolarity changes resembles that of *E. coli* and *Salmonella typhi*

OmpC and OmpF porins (Puente *et al.*, 1991). This should be due, at least partially, to the existence in the regulatory region upstream of *ompK36* (accession number Z33506) of sequences homologous to those that in *E. coli* are involved in the adaptive response of porins to osmolarity and other environmental conditions, such as *micF*-, OmpR-, SoxS- and Lrp-binding sites (Esterling & Delihas, 1994).

In conclusion, porins from *K. pneumoniae* ESBL$^-$ isolates were separated by an optimized SDS-PAGE technique and identified by their differential expression in different media and by their reaction with specific antibodies. When these methods were applied to the study of porins from *K. pneumoniae* ESBL$^+$ isolates, we observed that a high proportion of them expressed only a single porin. Western blot analyses demonstrated that all ESBL$^+$ isolates expressed the OmpK36 porin, and that expression of the OmpK35 porin was either not detectable or greatly reduced. It has been reported that deficiency or loss of porin expression, either OmpF or OmpC, or both, is accompanied by an increased resistance to antimicrobials (Harder *et al.*, 1981; Medeiros *et al.*, 1987; Misra & Benson, 1988). Most reports have shown (Benson *et al.*, 1988) that, of the two porins, expression of the OmpF porin is preferentially lost during the development of antimicrobial resistance, and this is probably due to its wider pore, compared to OmpC (Nikaido, 1983). In this study, we have observed that those *K. pneumoniae* isolates that express only one porin have lost the OmpK35 porin (the OmpF homologue) and this confirms the situation described in *E. coli*. Furthermore, our study revealed a strong correlation between the expression of both porins and ESBL$^-$, or expression of only one porin (OmpK36) and ESBL$^+$. We have as yet no suggestions to explain the possible mechanism(s) linking ESBL$^+$ and porin deficiency/loss. However, it has been clearly demonstrated that both porin deficiency and ESBL production interact to increase resistance to antimicrobials (Nikaido & Normark, 1987). Thus, it is possible that strains expressing ESBLs may respond to antibiotic pressure either by losing porins or by increasing expression of ESBL, or both, with the subsequent decrease in antibiotic uptake or increase in antibiotic degradation. In ESBL$^-$ strains, the above responses are unlikely, but still possible, in accordance with the low percentage of ESBL$^-$ isolates expressing only one porin that we have observed.

Strains producing ESBLs have a great clinical importance because they are difficult to treat. Furthermore, these strains often acquire additional mechanisms of resistance, such as mutations in the *gyrA* gene or/and expression of efflux pumps (Ardanuy *et al.*, 1998; Martínez-Martínez *et al.*, 1998). Porin loss in ESBL$^+$ strains as a result of antimicrobial therapy has been described (Ardanuy *et al.*, 1998; Martínez-Martínez *et al.*, 1996): these strains are difficult to treat because they are multiresistant and the choice of drug is very restricted. The fact that most ESBL$^+$ isolates in this study are also deficient in one porin makes them good candidates for developing a major increase in antimicrobial resistance by losing the expression of the remaining porin. We are currently studying the ability of this type of isolate to develop multiresistance.

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## REFERENCES

**Albertí, S., Hernández-Allés, S., Gil, J., Reina, J., Martínez-Beltrán, J., Camprubí, S., Tomás, J. M. & Benedí, V. J. (1993a).** Development of an Enzyme-Linked Immunosorbent Assay method for typing and quantitation of *Klebsiella pneumoniae* lipopolysaccharide: application to serotype O1. *J Clin Microbiol* **31**, 1379–1381.

**Albertí, S., Marqués, G., Camprubí, S., Merino, S., Tomás, J. M., Vivanco, F. & Benedí, V. J. (1993b).** C1q binding and activation of the complement classical pathway by *Klebsiella pneumoniae* outer membrane proteins. *Infect Immun* **61**, 852–860.

**Albertí, S., Rodríguez-Quiñones, F., Schirmer, T., Rummel, G., Tomás, J. M., Rosenbusch, J. P. & Benedí, V. J. (1995).** A porin from *Klebsiella pneumoniae*: sequence homology, three-dimensional structure, and complement binding. *Infect Immun* **63**, 903–910.

**Albertí, S., Marqués, G., Hernández-Allés, S., Rubires, X., Tomás, J. M., Vivanco, F. & Benedí, V. J. (1996).** Interaction between complement subcomponent C1q and the *Klebsiella pneumoniae* porin OmpK36. *Infect Immun* **64**, 4719–4725.

**van Alphen, W. & Lugtenberg, B. (1977).** Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. *J Bacteriol* **131**, 623–630.

**Ardanuy, C., Liñares, J., Domínguez, M. A., Hernández-Allés, S., Benedí, V. J. & Martínez-Martínez, L. (1998).** Outer membrane profiles of clonally related *Klebsiella pneumoniae* isolates from clinical samples and activities of cephalosporins and carbapenems. *Antimicrob Agents Chemother* **42**, 1636–1640.

**Benson, S. A., Occi, J. L. L. & Sampson, B. A. (1988).** Mutations that alter the pore function of the OmpF porin of *Escherichia coli* K12. *J Mol Biol* **203**, 961–970.

**Benz, R. (1994).** Uptake of solutes through bacterial outer membranes. In *Bacterial Cell Walls*, pp. 397–424. Edited by J.-M. Ghuysen & R. Hakenbeck. Amsterdam: Elsevier.

**Blake, M. S., Johnston, K. H., Russell-Jones, G. J. & Gotschlich, E. C. (1984).** A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal Biochem* **136**, 175–179.

**Esterling, L. & Delihas, N. (1994).** The regulatory RNA gene *micF* is present in several species of Gram-negative bacteria and is phylogenetically conserved. *Mol Microbiol* **12**, 639–646.

**Gutmann, L., Williamson, R., Moreau, N., Kitzis, M.-D., Collatz, E., Acar, J. F. & Goldstein, F. W. (1985).** Cross-resistance to nalidixic acid, trimethoprim, and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella*, *Enterobacter*, and *Serratia*. *J Infect Dis* **151**, 502–507.

**Harder, K. J., Nikaido, H. & Matsuhashi, M. (1981).** Mutants of *Escherichia coli* that are resistant to certain beta-lactam compounds lack the OmpF porin. *Antimicrob Agents Chemother* **20**, 549–552.

**Hernández-Allés, S., Albertí, S., Rubires, X., Merino, S., Tomás, J. M. & Benedí, V. J. (1995).** Isolation of FC3–11, a bacteriophage specific for the *Klebsiella pneumoniae* porin OmpK36, and its use for the isolation of porin-deficient mutants. *Can J Microbiol* **41**, 399–406.

**Kaneko, M., Yamaguchi, A. & Sawai, T. (1984).** Purification and characterization of two kind of porins from the *Enterobacter cloacae* outer membrane. *J Bacteriol* **158**, 1179–1181.

**van de Klundert, J. A. M., van Gestel, M. H., Meeerdink, G. & de Marie, S. (1988).** Emergence of bacterial resistance to cefamandole in vivo due to outer membrane protein deficiency. *Eur J Clin Microbiol Infect Dis* **7**, 776–777.

**Legrand, P., Fournier, G., Buré, A., Jarlier, V., Nicolas, M. H., Decré, D., Durval, J. & Philippon, A. (1989).** Detection of extended broad-spectrum beta-lactamases in *Enterobacteriaceae* in four French hospitals. *Eur J Clin Microbiol Infect Dis* **8**, 527–529.

**van der Ley, P., Bekkers, A., van Meersbergen, J. & Tommassen, J. (1987).** A comparative study on the *phoE* genes of three enterobacterial species. *Eur J Biochem* **164**, 469–475.

**Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P. & van Alphen, L. (1975).** Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. *FEBS Lett* **58**, 254–258.

**Martínez-Martínez, L., Hernández-Allés, S., Albertí, S., Tomás, J. M., Benedí, V. J. & Jacoby, G. A. (1996).** In vivo selection of porin deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and third generation cephalosporins. *Antimicrob Agents Chemother* **40**, 342–348.

**Martínez-Martínez, L., García, I., Ballesta, S., Benedí, V. J., Hernández-Allés, S. & Pascual, A. (1998).** Energy-dependent accumulation of fluoroquinolones in quinolone-resistant *Klebsiella pneumoniae* strains. *Antimicrob Agents Chemother* **42**, 1850–1852.

**Medeiros, A. A., O'Brien, T. F., Rosenberg, E. Y. & Nikaido, H. (1987).** Loss of OmpC porin in a strain of *Salmonella typhimurium* causes increased resistance to cephalosporins during therapy. *J Infect Dis* **156**, 751–757.

**Misra, R. & Benson, A. (1988).** Isolation and characterization of OmpC porin mutants with altered pore properties. *J Bacteriol* **170**, 528–533.

**Mizuno, T. & Kageyama, M. (1978).** Separation and characterization of the outer membrane of *Pseudomonas aeruginosa*. *J Biochem* **84**, 179–191.

**Nikaido, H. (1983).** Proteins forming large channels from bacterial and mitochondrial outer membranes: porins and phage lambda receptor protein. *Methods Enzymol* **97**, 85–113.

**Nikaido, H. (1994).** Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264**, 382–387.

**Nikaido, H. & Normark, S. (1987).** Sensitivity of *Escherichia coli* to various beta-lactams is determined by the interplay of outer membrane permeability and degradation by periplasmic beta-lactamases: a quantitative predictive treatment. *Mol Microbiol* **1**, 29–36.

**Nurminen, M. (1978).** A mild procedure to isolate the 34K, 35K and 36K porins of the outer membrane of *Salmonella typhimurium*. *FEMS Microbiol Lett* **3**, 331–334.

**Pangon, B., Bizet, C., Buré, A., Pichon, F., Philippon, A., Regnier, B. & Gutman, L. (1989).** *In vivo* selection of a cephamycin-resistant, porin deficient mutant of *Klebsiella pneumoniae* producing a TEM-3 beta-lactamase. *J Infect Dis* **159**, 1005–1006.

**Puente, J. L., Verdugo-Rodríguez, A. & Calva, E. (1991).** Expression of *Salmonella typhi* and *Escherichia coli* OmpC is influenced differently by medium osmolarity; dependence on *E. coli* OmpR. *Mol Microbiol* **5**, 1205–1210.

**Pugsley, A. P. & Schnaitman, C. A. (1978).** Identification of three genes controlling production of new outer membrane pore proteins in *Escherichia coli* K-12. *J Bacteriol* **135**, 1118–1129.

**Roth, J. A. (1988).** *Virulence Mechanisms of Bacterial Pathogens*. Washington, DC: American Society for Microbiology.

**Sanders, C. C., Sanders, W. E., Goering, R. V. & Werner, V. (1984).** Selection of multiple antibiotic resistance by quinolones, beta-lactams and aminoglycosides with special reference to cross-resistance between unrelated drug classes. *Antimicrob Agents Chemother* **26**, 797–801.

**Sawai, T., Hiruma, R., Kawana, N., Kaneko, M., Taniyasu, F. & Inami, A. (1982).** Outer membrane permeation of beta-lactam antibiotics in *Escherichia coli*, *Proteus mirabilis*, and *Enterobacter cloacae*. *Antimicrob Agents Chemother* **22**, 585–592.

**Sawai, T., Hirano, S. & Yamaguchi, A. (1987).** Repression of porin synthesis by salicylate in *Escherichia coli*, *Klebsiella pneumoniae* and *Serratia marcescens*. *FEMS Microbiol Lett* **40**, 233–237.

**Schmid, K., Ebner, R., Jahreis, K., Lengeler, J. W. & Titgemeyer, F. (1991).** A sugar-specific porin, ScrY, is involved in sucrose uptake in enteric bacteria. *Mol Microbiol* **5**, 941–950.

**Serushago, B. A., Mitsuyama, M., Handa, T., Koga, T. & Nomoto, K. (1989).** Role of antibodies against outer-membrane proteins in murine resistance to infection with encapsulated *Klebsiella pneumoniae*. *J Gen Microbiol* **135**, 2259–2268.

**Towbin, H. & Gordon, J. (1984).** Immunoblotting and dot immunobinding. Current status and outlook. *J Immunol Methods* **72**, 313–340.

**Werts, C., Charbit, A., Bachellier, S. & Hofnung, M. (1992).** DNA sequence of the *lamB* gene from *K. pneumoniae*. *Mol Gen Genet* **233**, 372–378.

**Williams, P. & Tomás, J. M. (1990).** The pathogenicity of *Klebsiella pneumoniae*. *Rev Med Microbiol* **1**, 196–204.

**Williams, P., Chart, H., Griffiths, E. & Stevenson, P. (1987).** Expression of high affinity iron uptake systems by clinical isolates of *Klebsiella*. *FEMS Microbiol Lett* **44**, 407–412.

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