Porin expression in clinical isolates of Klebsiella pneumoniae

Santiago Hernández-Allés, Sebastián Albertí, Dolores Álvarez, Antonio Doménech-Sánchez, Luis Martínez-Martínez, José Gil, and Vicente J. Benedí

Author for correspondence: Vicente J. Benedí. Tel: +34 971 173335. Fax: +34 971 173184. e-mail: dbsjbb0@ps.uib.es

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 (Alberti 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 (Alberti et al., 1993), and porin OmpK35 (Hernández-Allés et al., 1995), whose sequence (accession number AJ011501) is closer to that of E. coli OmpF porin.
Porins are important in the permeability of anti-
to cefoxitin, increased resistance to third-generation
cephalosporins and monobactams, and decreased sus-
cceptibility 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 \textit{K. pneumoniae} porins in the cited
studies precludes the certainty of the porin nature of the
OMP responsible for the resistance phenotypes. Entero-
bacterial 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.

\section*{METHODS}

\subsection*{Bacterial strains and culture media.} \textit{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. \textit{K. pneumoniae} strains expressing ESBLs were isolated at
Hospital Universitario Virgen de la Macarena (Seville). \textit{K.
\textit{pneumoniae} isolates were identified following routine bio-
chemical tests (Alberti et al., 1993a). \textit{K. pneumoniae} strains C3
(Alberti 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). \textit{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 \degree C with shaking.

\subsection*{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 \mu m
for 2 x 30 s cycles, each cycle composed of 6 x 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 100000 g for 1 h. After solubiliza-
tion in 10 mM Tris/HCl (pH 7.2), 5 mM MgCl$_2$, 2\% sodium
lauryl sarcosinate for 30 min at 25 \degree C, the insoluble OMPs
were recovered at 100000 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
Alberti et al. (1993b). When required, a gel-permeation
chromatography step which separates porins from LPS
(Alberti 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.

\subsection*{SDS-PAGE.} Electrophoretic analyses of \textit{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 \textit{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\% \textit{f}-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.

\subsection*{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 modifica-
tions (Alberti et al., 1996).

\subsection*{Antisera and Western blot analysis.} Antisera against OmpK36
and OmpK35 porins were raised by repeated immunization
of rabbits with purified native (unboiled) porins (Alberti 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 (Milli-
pore), anti-porin serum, and alkaline-phosphatase-labelled
goat anti-rabbit IgG (Sigma). Enzyme was detected as de-
scribed by Blake et al. (1984).

\subsection*{\textit{\beta}-Lactamase expression.} Production of ESBLs was studied by
the double-disc synergy test (Legrand et al., 1989) as described by
Ardanuy et al. (1998).

\section*{RESULTS}

\subsection*{Electrophoretic separation of \textit{K. pneumoniae} porins.}
Polyacrylamide gel electrophoretic methods described for
the resolution of \textit{E. coli} porins were tested in \textit{K.
\textit{pneumoniae}. The use of previously described \textit{E. coli}
mutants with specific defects in some of their OMPs (OmpA$^-$, OmpC$^-$ or OmpF$^-$) facilitated the identifi-
cation of porins and OmpA on the gels and helped in
testing the resolution of the different electrophoretic
systems. The best resolution of \textit{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
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*Fig. 1.* Electrophoretic separation of OMPs from \textit{E. coli} (lanes 1–4) and \textit{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 \textit{E. coli} strains JF568 (wild-type), JF701 (OmpC), and \textit{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 \textit{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 \textit{K. pneumoniae} strains (Fig. 1, lanes 5 and 7). Depending on the \textit{K. pneumoniae} strain, porins and other OMPs could be perceived on the gels, but separations were never as good as in the \textit{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 \textit{K. pneumoniae} OMPs in the porin molecular mass range was not as good as for \textit{E. coli} OMPs.

When tested with \textit{K. pneumoniae} strains, other gel systems described for \textit{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 \textit{K. pneumoniae} (Fig. 2). The porins of each strain were optimally resolved in one of three SDS-PAGE systems: (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.

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 \( \approx 25 \) 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-\textit{K. pneumoniae} clinical isolates. This resulted in up to
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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

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, and one 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 pl and molecular mass and the putative OmpA was well separated from the porins (Fig. 3b).

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.
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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 (Alberti et al., 1995) and OmpK35 (Hernández-Álles 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 mceF-, 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+ 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; Martinez-Martinez et al., 1998). Porin loss in ESBL+ strains as a result of antimicrobial therapy has been described (Ardanuy et al., 1998; Martinez-Martinez 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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