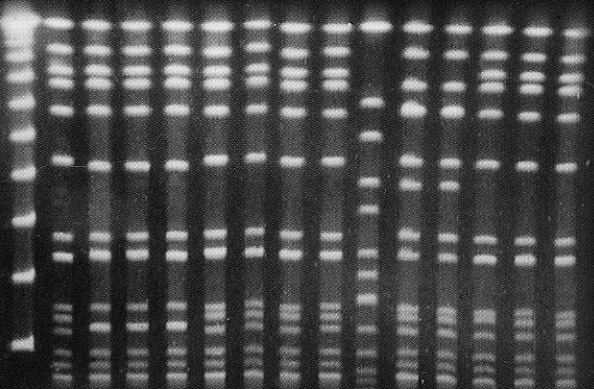


ANTIMICROBIAL RESISTANCE A LABORATORY APPROACH



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INTRODUCTION

The outer membrane of Gram-negative bacteria constitutes the physical and functional barrier between the cell and the environment. The natural environment, particularly the host of pathogenic organisms, is hostile for bacteria due to the possible presence of, for example, the toxic enzymes, components of the immune system and antibodies. For this reason, bacteria must develop surface structures or modify (mutate) the existing envelope components. Outer membrane proteins (OMPs) and in special cases, are important determinants of pathogenicity. Porins act as receptors of the complement system (2), and as channels for antibiotic agents (8). A well-known natural resistance mechanism to antimicrobial agents consists in the alteration of porin expression (14).

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate detergent (SDS-PAGE) is an important method for the characterization of OMPs and other membrane proteins. In this analysis, and the use of immunoblotting, protein expression

CHAPTER 3

Two groups of membrane proteins are found in the outer membrane (OM) of Gram-negative bacteria: integral membrane and peripheral proteins. They are found, respectively, on (1) and the outer membrane comprises phospholipids, inner and outer leaflets characterizing it can be separated in a phospholipid bilayer from the OM (2). The OM is made only by phospholipids and proteins.

ENTEROBACTERIAL OUTER MEMBRANE PROTEINS AND PORINS: ISOLATION AND POLYACRYLAMIDE GEL ELECTROPHORETIC ANALYSIS

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On the other hand, porin purification is also performed by immunoaffinity chromatography based on the strong but non-covalent interaction of porins to the polyclonal anti-porin antibodies (10). SDS-PAGE analysis of purified porins under the appropriate conditions indicates the presence of proteins which can

INTRODUCTION

The outer membrane of Gram-negative bacteria constitutes the physical and functional barrier between the cell and the environment. The natural environment, particularly the host of pathogenic organisms, is hostile for bacteria, due to the possible presence of, for example, bile salts, enzymes, components of the immune system, and antibiotics. For this reason, bacteria must develop surface structures or modify (mutate) the existing envelope components. Outer membrane proteins (OMPs), and in special porins, are important determinants of pathogenicity. Porins act as activators of the complement system (2), and as channels for antibiotic uptake (8). A well-characterized resistance mechanism to antimicrobial agents consists in the alteration of porin expression (14).

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate detergent (SDS-PAGE) is an important method for the initial characterization of OMPs and other enterobacterial surface antigens, and the method of choice in order to detect alterations of porin expression.

Two groups of methods have been developed for the isolation of outer membranes (OMs) of enterobacteria: gradient ultracentrifugation and differential solubilization. They are based, respectively, on: 1) since the outer membrane comprises phospholipids, proteins and lipopolysaccharide, it can be separated in an ultracentrifuge gradient from the inner (cytoplasmic) membrane made only by phospholipids and proteins. And 2) due to the different composition of the inner and outer membranes, they solubilize differently depending on the conditions and detergent used. Sodium lauryl sarcosinate, under the appropriate conditions, will solubilize preferentially the cytoplasmic membrane, leaving insoluble the outer membrane (OM), which can be recovered by centrifugation as a pellet and then solubilized in detergent sodium dodecyl sulphate (SDS).

To know more about the above two strategies, you are referred to the original references (6,22) and the excellent chapter of reference 26. It has to be pointed out that none method results in complete separation of one membrane from the other, as clearly demonstrated in the example (23), so small contamination with cytoplasmic membranes can be expected. This is particularly evident if the gels are finally stained with the more sensitive silver-staining method rather than with the usual Coomassie Blue. Because of its simplicity, we have chosen the differential solubilization method to isolate OMs from the species and strains under study. Usually, this method is used at a large scale (6). However, we describe here in detail a modification of the method that we have been using for years with success. This modified, or "miniprep" method, renders sufficiently clean OM preparations for their electrophoretic analysis and enough amounts to perform 2-4 minigel analyses. Since ultracentrifugation steps have been eliminated from the original method, and substituted by the less-expensive microfuge equipment, and given that small-scale cultures are processed (allowing the use of sonication instead or French-press for rupturing cells), the whole minipreps can be routinely used as a rapid preliminary analysis of OMs. Once required, confirmation with the complete large-scale method should be performed on selected strains. Recently we have submitted a manuscript describing this miniprep method (4).

On the other hand, porin purification is also performed by differential solubilization, based on the strong but non-covalent union of porins to the peptidoglycan layer. Sodium dodecyl sulphate (SDS), under the appropriate conditions, solubilize all the membrane proteins except porins, which can

be recovered by centrifugation as a pellet. Once purified, porins can be used for several purposes: for immunization to obtain anti-porin antibodies, liposome swelling assays (5), binding studies (2), or crystallization (5).

Regarding to the electrophoretic analysis of OMPs and porins, numerous variations of SDS-PAGE and many buffer systems have been described, but even minor variations may result in major changes in the electrophoretic profile of OMPs, as exemplified by porin separation of *Escherichia coli* (13). Despite the importance of a careful choice of electrophoretic conditions for separation of OMPs, there are not many reviews dealing with this topic. However, as a starting point to be followed by a detailed investigation for each species/strain case, one can learn a good deal of basic information from reference (27). Also, more specific references, such as (24), on the effect of SDS-PAGE variations on OM profiles, as well as basic information on electrophoresis (7) can be considered.

For the purposes of this book, we will use here two SDS-PAGE conditions that we routinely use with success for the analysis of *E. coli* and *Klebsiella pneumoniae* OMPs. These conditions, combined with the rapid miniprep isolation of OMs, produce reliable electrophoretic resolution of the so called "major outer membrane proteins" of these two species, even when gels are stained with the less sensitive Coomassie Blue protocol.

The term "major outer membrane proteins" is confusing and we will not use it later on. However, it deserves further explanation here, because "porins" and "major outer membrane proteins" have often been used as synonyms. Briefly, some "minor" OMPs can be seen as "major" (more abundant) depending on cultural conditions (ionic strength of culture medium, pH, temperature, carbon source, etc.). This is the case of, for example, OmpF-like porins and siderophores (whose expressions increase in low osmolarity media and under iron starvation conditions, respectively). Secondly, OmpA and murein lipoprotein, are by far the most abundant ("major") OMPs, but their roles as porins are debatable (OmpA, (28)) or null (lipoprotein). Porins are non-specific pore proteins of the outer membrane of gram-negative bacteria (20) that allow the passage of small hydrophilic solutes, including many antimicrobial agents (16-18). In *E. coli*, two inespecific porins have been described: OmpF and OmpC. In *K. pneumoniae*, our group has described porins OmpK36 (analogous to *E. coli* OmpC (3)) and OmpK35 (analogous to *E. coli* OmpF (9,10)).

The functional role of porins cannot be studied by SDS-PAGE, but there are other characteristics that, after SDS-PAGE analysis of an OM, may suggest a porin role for a given protein seen in the gel. Firstly, enterobacterial porin monomers are usually comprised in the 35-40 kDa molecular mass range. Secondly, they are strongly, but non-covalently, associated with the peptidoglycan layer. Thirdly, they are "heat-modifiable", which means that unless they are solubilized at high temperatures (above 60 °C) in the presence of SDS and a reducing agent, they are not fully solubilized, and therefore trimers, rather than monomers, are seen in the gels. Given the above properties, and after the suspicion (based on the absence of 35-40 kDa bands in the gel) that resistance to a certain antimicrobial agent can be partly due to the loss of porin expression, one may still proceed to simpler things before permeability-based studies are performed. For example, one can try to isolate the putative porin that is absent in the antibiotic resistant mutant from its corresponding wild-type (antibiotic sensitive) strain, in case this parent strain is available. If this protein is isolated

following a porin-isolation method, it is very likely that it will function as a porin. This strategy has been used with success to define *K. pneumoniae* porins OmpK36 and OmpK35 (1,10), whose porin role was further confirmed by DNA sequencing of their corresponding gene (3), by N-terminal sequencing of the isolated proteins (1,10), by crystallization of purified OmpK36 porin (5), and by the role of OmpK36 in permeabilization of β -lactams (14).

At this point, we have clarified many points that will facilitate the localization of bands by SDS-PAGE and the initial assumption that they can be porins. Now the protocols that follow will give you the details for their practical identification in *E. coli* and *K. pneumoniae*. However, remember that culture conditions alter porin expression, that the protocols may not be optimal for all enterobacterial species, and that for some *K. pneumoniae* clinical isolates, the below protocols have had to be slightly modified for optimal resolution of porins (9). Therefore, it is highly useful to include reference strain(s) of the species under study as controls on the acrylamide gels: strains whose OMPs and porin profiles have been previously defined (published).

PROTOCOLS

A. Large scale isolation of OMPs. Adapted from (6)

1. Grow strains in 0.5-1 l of Luria Broth or Nutrient Broth overnight at 37°C in a shaker (200 rpm).
2. Centrifuge at 10,000 $\times$ g for 20 min. Discard supernatant fluids.
3. Resuspend cell pellets in 500 ml of cold Tris-Mg buffer, and centrifuge as in step 2. Repeat resuspension and centrifugation, and finally resuspend cells in 20 ml of chilled Tris-Mg buffer.
4. Break cells using a French press. Alternatively, sonicate cell suspension, keeping samples at 4°C. We routinely use these conditions: 15 cycles of 30 sec each. Each cycle consists of 6 $\times$ 5 sec sonication (amplitude 18-20 microns) separated by 1 sec of no sonication. After completion of each cycle, leave 30 sec without sonication. Remember that efficiency of cell lysis using sonication is lower than when using a French press.
5. Microfuge sonicates at 3,000 $\times$ g for 10 min to eliminate unbroken cells. If pellet is still big, repeat step 4.
6. Carefully transfer supernatant fluids (representing cytoplasmic contents and cell envelopes) to a clean centrifuge tube. Ultracentrifuge at 100,000 $\times$ g for 1 h at 4°C to pellet cell envelopes. Discard supernatant fluids.
7. Resuspend pellets in 10 ml of 2% sodium lauryl sarcosinate in Tris-Mg buffer, and incubate for 20-30 min at room temperature.
8. Pellet outer membranes for 1 h at 70,000 $\times$ g. Eliminate supernatant fluids (cytoplasmic membranes). Repeat steps 7 and 8 twice.
9. Remove completely the supernatant fluids and resuspend pellets in (depending on the pellet size) 1-2 ml of H₂O.
10. Dilute samples with 1 volume of Laemmli's electrophoresis sample buffer, boil samples for 5 min, microfuge for 1 min to pellet insoluble material, and load 2-10 μ l of supernatant fluid.

B. Rapid isolation of small amounts of OMPs

1. Grow strains in 4 ml of Luria Broth or Nutrient Broth overnight at 37°C in a shaker (200 rpm).

2. Transfer 1.5 ml of each culture to a microfuge tube and spin down cells at full microfuge speed (13,000 rpm) for 4 min. Discard supernatant fluids. If pellet is too small, add again 1.5 ml of culture to the same tube and repeat centrifugation and elimination of supernatant fluids.
3. Resuspend cell pellets in 1 ml of cold Tris-Mg buffer, and centrifuge as in step 2. Repeat resuspension and centrifugation, and finally resuspend cells in 1 ml of chilled Tris-Mg buffer.
4. Sonicate cell suspensions in a Soniprep 150 MSE-sonifier or similar, keeping samples at 4°C. (For sonication conditions, see previous protocol).
5. Microfuge sonicates at 5,000 rpm for 2 min to eliminate unbroken cells.
6. Carefully transfer supernatant fluids (representing cytoplasmic contents and cell envelopes) to a new microfuge tube. Microfuge at full speed for 30 min at 4°C to pellet cell envelopes. Discard supernatant fluids.
7. Resuspend pellets in 0.8 ml of 2% sodium lauryl sarcosinate in Tris-Mg buffer, and incubate for 20-30 min at room temperature.
8. Pellet outer membranes for 30 min at full microfuge speed. Eliminate supernatant fluids (cytoplasmic membranes). Steps 8 and 9 may be repeated to obtain cleaner outer membrane preparations, but each time this is done you could lose some outer membrane proteins.
9. Remove completely the supernatant fluids and resuspend pellets in 20-40 µl (depending on the pellet size) of Laemmli's electrophoresis sample buffer.
10. Boil samples for 5 min, microfuge for 1 min to pellet insoluble material and load 2-10 µl of supernatant fluid, depending on the pellet size.

C. Isolation of porins. Adapted from (19) and (21)

1. Grow strains in 1-2 liters of Luria Broth or Nutrient Broth overnight at 37°C in a shaker (200 rpm).
2. Centrifuge at 10,000 × g for 20 min. Discard supernatant fluids.
3. Resuspend cell pellets in 500 ml of chilled Tris-HCl 50 mM buffer (pH 7.7), and centrifuge as in step 2. Repeat resuspension and centrifugation, and finally resuspend cells in 20 ml of chilled Tris HCl 50 mM buffer (pH 7.7).
4. Break cells using a French press. Alternatively, sonicate cell suspension, keeping samples at 4°C (see conditions on previous protocol).
5. Centrifuge sonicates at 3,000 × g for 10 min to eliminate unbroken cells. If pellet is too large, repeat step 4.
6. Carefully transfer supernatant fluids (representing cytoplasmic contents and cell envelopes) to a clean centrifuge tube. Ultracentrifuge at 100,000 × g for 1 h at 4°C to pellet cell envelopes. Discard supernatant fluids.
7. Resuspend pellets in 10 ml of Tris 10 mM (pH 8.0), containing 0.1 mg trypsin per 1 mg protein (w/w). Add sodium azide (0.002%, final concentration) to avoid sample contamination. Incubate o/n at 37°C.
8. Add 10 ml of 4% sodium dodecyl sulphate (SDS) in Tris 10 mM (pH 7.7), and incubate for 1 h at 32°C.
9. Pellet outer membranes for 30 min at 100,000 × g. Eliminate supernatant fluids (cytoplasmic membranes), and resuspend pellet with 10 ml of 2% SDS in Tris 10 mM (pH 7.7). Incubate for 1 h at 32°C.
10. Repeat step 9.

- 11. Remove the supernatant fluids and resuspend pellets in 0.5-1 ml of H₂O. Alternatively, you can eliminate lipopolysaccharidic material from samples following this protocol.
- 12. Resuspend pellets in 10 ml of Solubilization buffer. Incubate 2 h at 37°C.
- 13. Centrifuge 30 min at 100,000 x g.
- 14. Chromatography: recover supernatant fluid and load a Sephacryl-S200 column (100 cm x 2.5 cm) previously washed with Solubilization buffer. Recover fractions of 2 ml, and determine absorbance at 280 nm. Dialyze protein-containing fractions with 3 mM sodium azide for several days.
- 15. Quantify protein content with the BCA protein assay. Check purity of samples in a polyacrylamide gel. Store porin solution at 4°C.

D. Polyacrylamide gel electrophoretic analysis

I. *K. pneumoniae* porins

It is impossible to describe a single gel system for the optimal resolution of *K. pneumoniae* porins. Porin separation is not an easy task. The molecular masses of porins, for example, of *E. coli* porins OmpC and OmpF (38,284 Da and 37,061 Da, respectively, as derived from their corresponding gene sequences) are close enough to difficult their resolution. Also, as we mentioned before, it is wise to analyze OMPs from strains grown in low and high osmolarity medium, because porins expression is greatly affected by cultural conditions. For this reason, it is important to grow strains in a medium/conditions where both porins are expressed in similar amounts. Also, load the gel with different amounts of OMPs, because if gels are overloaded the bands corresponding to the two porins can be seen as a single band.

In our experience, *K. pneumoniae* porins can be resolved in 11% acrylamide resolving gels by controlling the final concentration of bisacrylamide. We use 3 final bisacrylamide concentrations in our gels: 0.2%, such as in (13), 0.35% as in (11), or 0.55% (our lab). Porins from different clinical isolates are optimally resolved in one of the 3 above bisacrylamide concentrations without having to add urea to the resolving gel. We have observed that for most of *K. pneumoniae* strains, 0.35% bisacrylamide is the concentration that gives best results, but optimal resolution of porins from some strains may require lower or higher bisacrylamide concentration (0.2 or 0.55%, respectively). Assemble the glass plates and spacers (usually of 0.75 mm) according to the manufacturer instructions, and fill them with the "resolving gel". To make this gel, prepare the solutions described in table I.

TABLE I. Resolving gel solutions, volumes added, and final concentrations.

stock solution	final volume	concentration
30% Acrylamide	7.33 ml	11%
1.5% Bisacrylamide	4.66 ml	0.35%
10% SDS0.	40 ml	0.2%
Resolving gel buffer	5.00 ml	0.375 M Tris
H2O	2.61 ml	
TEMED (1)	40 µl	0.2%
10% APS(1)	50 µl	0.025%

(1) Before adding TEMED and APS, mix the components in a vacuum flask and degas by connecting it to a vacuum pump for a couple of minutes.

Mix carefully, and fill the gels up to the 3/4 parts of them. Then add 200-300 µl of resolving gel overlay (the upper phase of it, i. e., the Tris-equilibrated butanol) and leave gels to polymerize for 1 hour. After the gel polymerizes, rinse its top part with water and add the stacking gel solution (table 2).

TABLE 2. Stacking gel solution, volumes added, and final concentrations.

stock solution	final volume	concentration
30 %-0.8 % Acryl-Bis	1.34 ml	4 % Acryl- 0.1 % Bis
10 % SDS	0.10 ml	0.1 %
stacking gel buffer	2.50 ml	0.125 M Tris
H2O	6 ml	
TEMED(1)	10 µl	0.1 %
10 % APS(1)	50 µl	0.05 %

⁽¹⁾ Before adding TEMED and APS, mix the components in a vacuum flask and degas by connecting it to a vacuum pump for a couple of minutes.

Mix well, fill completely the gel, and insert the combs. Leave it to polymerize for 1 hour. After polymerization, disassemble the gel caster and run the gels. Alternatively, gels can be stored in a closed container at 4°C for 2-3 days, provided they are wrapped in filter paper wetted with tank buffer.

Before running the gels, remove the combs and wash the wells with tank buffer. Mount the gels in the electrophoresis chamber, and fill the lower and upper reservoirs with tank buffer. Boil samples for 5 min in sample buffer, and load samples in the wells.

Connect the electrodes and run the gels at 10 mA (milliamperes) per gel until the samples enter the resolving gel, and then at 20 mA per gel until the blue front reaches the end of the plates. These electrophoretic conditions are suitable for mini-gels (8-10 cm long). For longer gels (20 cm), 50 V-20 mA overnight or 30 mA-250 V for ≈ 4 h can be used. Remember that under the described conditions, proteins will migrate from the negative electrode (cathode) towards the positive one (anode). Most power supplies plugs and electrophoresis chambers are color-coded (red, anode; black, cathode).

After disassembling gels, proceed to section III.

II. *E. coli* porins

The same general comments made before for *K. pneumoniae* are valid for *E. coli* (and are probably true for other species). Resolution (separation) of porins depends on each strain (24), and therefore it is necessary to try different gel systems. We have been using the gel system described below with success for the separation of *E. coli* porins. If it does not work for your strains, try other methods (13,24). It is important to assay different gel systems before concluding that a given strain expresses just one porin.

The method we use for optimal separation of *E. coli* porins (15) requires the use of urea in the acrylamide gel (tables 3 and 4). It is well known that porins OmpC and OmpF from *E. coli* in urea-containing gels migrate differently than in urea-free gels: in urea gels, OmpF migrates above the OmpC porin, whereas in plain (no urea) gels, OmpC migrates above OmpF. Therefore, the "appa-

rent molecular mass" of a porin can change drastically depending on the electrophoretic conditions used.

TABLE 3. Resolving gel solution containing urea, volumes added, and final concentrations.

stock solution	volume	final concentration
30 %-0.8 % Acryl-Bis	5.3 ml	10% Acryl-0.27% Bis
Urea	5.76 g	6 M
10% SDS	0.16 ml	0.1%
Resolving gel buffer	4.00 ml	0.375 M Tris
H2O	up to 16 ml (add 2.5 ml)	
TEMED ⁽¹⁾	10 µl	0.06%
10% APS ⁽¹⁾	60 µl	0.0375%

⁽¹⁾ Before adding TEMED and APS, mix the components in a vacuum flask and degas by connecting it to a vacuum pump for a couple of minutes.

Mix well, fill the gels up to the 3/4 parts of them, and polymerize gels for 1 h. Then add 200-300 µl of resolving gel overlay, and leave it to polymerize for 1 hour. After the gel polymerizes, rinse its top part with water and add the stacking solution (table 4).

TABLE 4. Stacking gel solution containing urea, volumes added, and final concentrations.

stock solution	volume	final concentration
30 %-0.8 % Acryl-Bis	0.75 ml	4.5% Acryl-0.12% Bis
Urea	1.8 g	6 M
10% SDS	0.05 ml	0.1%
Stacking gel buffer	1.25 ml	0.125 M Tris
H2O	up to 5 ml (add » 1.67 ml)	
TEMED(I)	10 µl	0.06%
10% APS(I)	15 µl	0.0375%

⁽¹⁾ Before adding TEMED and APS, mix the components in a vacuum flask and degas by connecting it to a vacuum pump for a couple of minutes.

Mix carefully, fill gels, and polymerize gels with the combs in place. Disassemble the gel caster and proceed as before for the *Klebsiella* case. However, since the gels here contain urea, they will form crystals if stored at 4°C, so keep them at room temperature in a humid chamber/box for no more than 2 days.

We use the following control strains, whose OMPs and porins are well known and resolved in this system: JF568 (wild type), JF699 (OmpA), JF703 (OmpF), and JF701 (OmpC). These strains were obtained from Dr. Barbara J. Bachmann (*E. coli* Genetic Stock Center, Department of Biology 355 OML, Yale University, P.O. Box 6666, New Haven, CT 06511-7444, USA). These strains have been described in:

- strain JF568: Foulds and Chai, J. Bacteriol. 133:1478 (1978).
- strains JF699 and JF701: Foulds and Chain, Can. J. Microbiol. 25:423 (1979).
- strain JF703: Chai and Foulds, J. Bacteriol. 130:781 (1977).

After disassembling gels, proceed to section E.

E. Staining, fixing, and reporting gels.

After electrophoresis run, gels are disassembled and immersed in Coomassie Blue staining solution for 20-30 min. Since the described staining solution contains acetic acid, proteins are fixed and stained in a single step. To destain gels, discard staining solution, rinse briefly with destaining solution and shake gels in several changes of destaining solution until background is judged to be low enough for drying. Alternatively, proteins can be stained using the more sensitive silver stain. For this purpose, we use the Silver Staining kit from Pharmacia.

After staining, gels can be dried using vacuum pump-operated dryers such as Hoefer's SEI 160 or similar. Gels can also be dried without a vacuum pump by framing them between two porous cellophane sheets (we use the "DryEasy" kit from Novex).

Once dried, gels can be stored for several years. Dried gels can be photographed, scanned, or captured with a video camera. The resulting images can be acquired with a computer for subsequent editing (elimination of background and lanes, lanes numbering, composition of gels, etc.), and printing with a printer (Mitsubishi digital colour printer CP-DIE or similar) or instant photographing of the computer screen.

RESULTS.

Our group has been using routinely the miniprep method described in this chapter for the isolation of OMPs, due its simplicity and because this rapid method allows the analysis of a high number of samples (up to 24), rendering a quality comparable to the purification of OMPs. Figure 1 shows the SDS-PAGE analysis of OMPs isolated with the miniprep method from *K. pneumoniae* clinical isolates. After Coomassie blue staining, just a few (approximately a dozen) OMPs are detected in the acrylamide gel (lanes 1 to 4). This OMPs profile is qualitatively comparable with the large scale OMPs isolation method (data not shown). The so called "major" proteins are situated in the 30-40 kDa range. These major proteins include the OmpA protein (marked with an asterisk) whose molecular mass is conserved in all strains, and porins (running close above the OmpA). It is noticeable that the molecular mass of porins varies among the analyzed strains; this variability was previously described in *K. pneumoniae* (9). As mentioned previously, the expression of some OMPs depends on the culture conditions. In this acrylamide gel, lane 4 shows OMPs extracted after bacterial growth in LB plus 2% of maltose, a medium that induces the expression of the LamB maltoporin (marked with an arrow). OMPs shown in lanes 1 to 3 were obtained after bacterial growth in NB, a medium that increases expression of OmpK35 porin (an OmpF-like porin). However, both porins OmpK36 and OmpK35 are seen as a single band (except in lane 3), because samples were overloaded in order to increase the amount of minor proteins. In our experience, resolution of porins is achieved by loading low amounts of sample (approximately 2-3 times less than in figure 1), because the molecular masses of both porins are usually very similar.

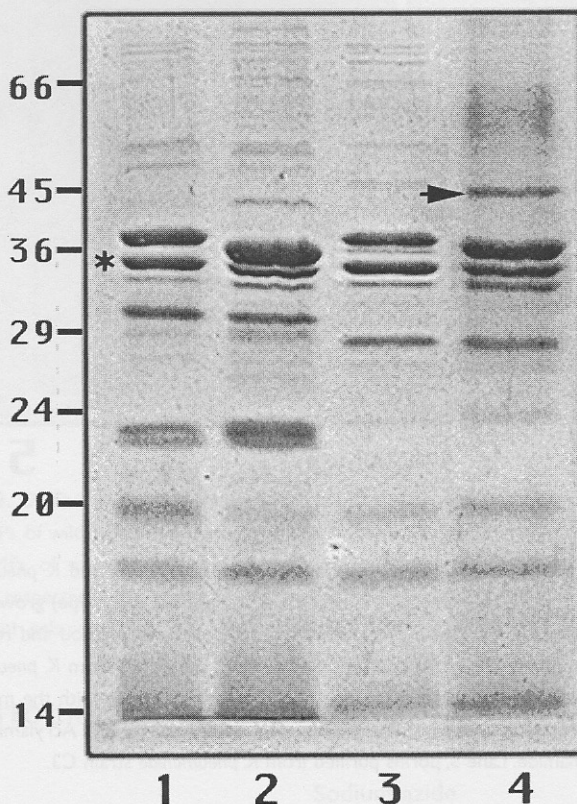


FIGURE 1. SDS-PAGE analysis of OMPs from *K. pneumoniae* strains. Lanes 1 to 3 show OMPs isolated after bacterial growth in NB. Lane 4 shows OMPs extracted after growth in LB containing 2% of maltose, medium that induces expression of LamB protein (labeled with a black arrow). The OmpA protein is labeled with an asterisk in lane 1. The molecular mass marker (in kDa) is shown on the left.

Figure 2 shows the SDS-PAGE analysis of OMPs isolated with the miniprep method from *E. coli* and *K. pneumoniae* strains. In this example, when *E. coli* wild type strain JF568 grows in LB (a high osmolarity medium), OmpF porin expression decreases, and increases that of OmpC (lane 1). On the other hand, in a low osmolarity medium (NB), OmpF expression increases in detriment of OmpC expression (lane 2). The *E. coli* OMPs were resolved with the urea-containing polyacrylamide gel system described in this chapter.

In *K. pneumoniae*, regulation of porin expression is similar to that of *E. coli*. OmpK35 porin, the analogue to OmpF from *E. coli*, increases its expression in NB (figure 2, lane 4), compared with the OmpK35 expression in LB (lane 3). Lane 5 shows the purified porins from *K. pneumoniae* strain C3 after growth in NB. We have used an SDS-PAGE system composed by 11% Acrylamide-0.55% Bisacrylamide for the resolution of OMPs from *K. pneumoniae* C3 (figure 1, lanes 3, 4, and 5). Other systems used (11% Acrylamide with 0.35% or 0.2% Bisacrylamide) do not resolve well OmpK36 and OmpK35 porins from this strain (data not shown). Its important to note that OMPs other than OmpA and porins are extracted less efficiently with the miniprep method, compared with the large scale isolation of OMPs (data not shown).

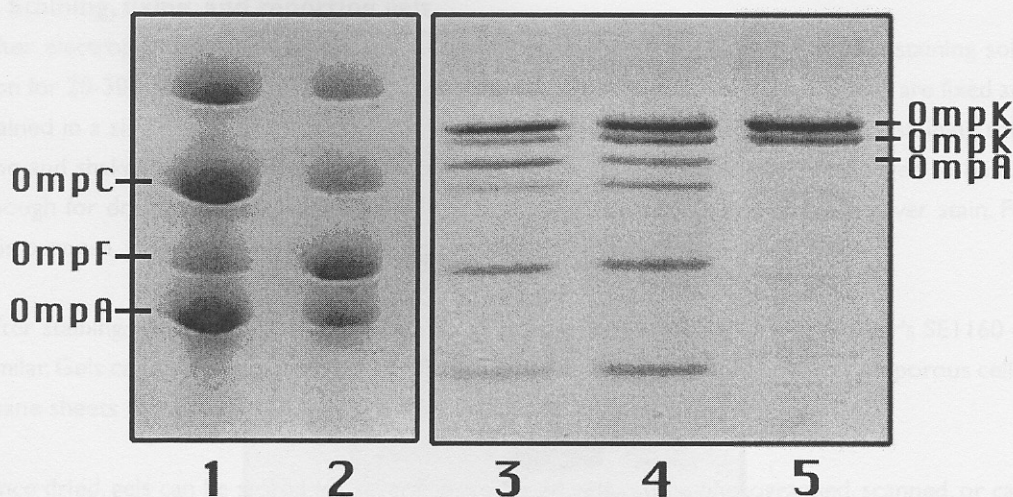


FIGURE 2. SDS-PAGE analysis of OMPs and porins from *E. coli* and *K. pneumoniae* strains. Lanes 1 and 2 show the OMPs from *E. coli* strain JF568 (wild type) grown in LB and NB, respectively. OMPs were extracted with the miniprep method and resolved with the urea-SDS-PAGE system. Lanes 3 and 4 show the OMPs from *K. pneumoniae* strain C3 grown in LB and NB, respectively. OMPs were extracted with the miniprep method, and resolved with the SDS-PAGE system composed by 11% Acrylamide and 0.55% Bisacrylamide. Lane 5, porins purified from *K. pneumoniae* strain C3.

At the present, our group is studying the expression of porins in clinical isolates of *K. pneumoniae*. For this purpose, we have applied the miniprep method to 30 clinical isolates producing expanded spectrum β -lactamase (ESBL+), and 30 strains non-producing extended spectrum β -lactamase (ESBL-). We have demonstrated that most ESBL- strains express both OmpK36 and OmpK35 porins, while most ESBL+ strains produce only OmpK36 porin (9). Figure 3 shows the OMPs from an ESBL- clinical isolate (strain M30, lane 1), and OMPs from an ESBL+ clinical isolate (strain HUS62/94, lane 3). Beginning from these data, we selected *in vitro* resistant mutants to several cephalosporins. We observed that most mutants derived from ESBL- strains decreased expression of OmpK35 porin or even loosed completely its expression (lane 2), demonstrating that alteration of porin expression is the main resistance mechanism of this type of strains. On the other hand, porin expression was unaltered in most mutants derived from ESBL+ strains, and few of them were porin deficient mutants (lane 4). We are currently studying the resistance mechanism of these mutants. The OMPs shown in figure 3 were resolved with the SDS-PAGE system described in this chapter (11% Acrylamide-0.35% Bisacrylamide).

In conclusion, the miniprep method described in this chapter for the isolation of OMPs, together with the SDS-PAGE systems, are useful tools for the study of OMPs and porins of *E. coli* and *K. pneumoniae*.

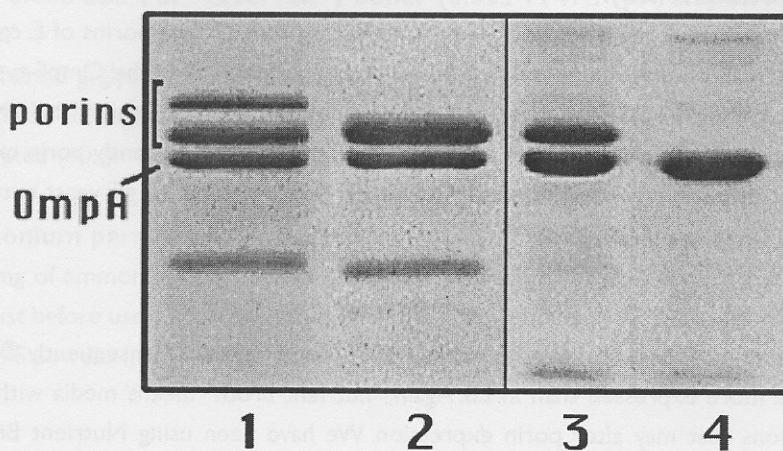


FIGURE 3. OMPs analysis of *K. pneumoniae* mutants resistant to cephalosporins. Lanes 1 and 3, OMPs of wild type *K. pneumoniae* strains M30 and HUS62/94, respectively. Lanes 2 and 4, OMPs of mutants resistant to cephalosporins derived from strains M30 and HUS62/94, respectively. In all cases, OMPs were extracted with the miniprep method, and resolved with gels containing 11% Acrylamide-0.35% Bisacrylamide.

REAGENTS and STOCKS

Chemicals:

Tryptone	Sodium azide
Yeast extract	SDS (sodium dodecyl sulfate)
NaCl	Acrylamide
Meat extract	Bis-acrylamide
Peptic digest of meat	TEMED
Glycerol	Ammonium persulfate
Dithiothreitol	Butanol
β -mercaptoethanol	Urea
Bromophenol blue	Comassie Brilliant Blue R250
This-base	Methanol
MgCl ₂	Acetic acid
Sodium lauryl sarcosinate (sarkosyl)	Silver staining kit (Pharmacia)
Distilled sterile water	Dry easy Kit (novex)
Trypsin	Glycine
	Sephacryl S200 column

Equipment

Shaker	Spectrophotometer
Centrifuge	Vacuum pump
French press	Electrophoresis chamber
Sonicator (optional)	Power supply
Microfuge	Hoefer SE 1160 (or similar)
Ultracentrifuge	Magnetic stirring
Soniprep 150 MSE sonifier (or similar)	

Luria-Bertani medium (LB):

This is a high-osmolarity medium (447 mosmol/kg), so OmpC-type porins of *E. coli*, *K. pneumoniae* and other enterobacteria are preferentially expressed over the OmpF-type porins. Many culture media are abbreviated as LB, but their compositions (mainly their NaCl content) differ. Since this clearly affects the final osmolarity, and consequently porin expression, we point out here LB composition as in reference (25): tryptone, 10 g/l; yeast extract, 5 g/l; and NaCl, 10 g/l (pH 7.0).

Nutrient Broth:

This can be used as a low osmolarity medium (51 mosmol/kg), and consequently OmpF-type porins are more expressed than in LB. Again, "nutrient broth" means media with different compositions that may alter porin expression. We have been using Nutrient Broth from Merck (cat. No. 5443) or the same homemade culture: meat extract, 3g/l (Adsa cat. No. 7-075) and peptic digest of meat, 5 g/l (Adsa cat. No. 7-152), adjust to pH 7.0.

Tris-Mg buffer:

10 mM Tris-HCl, and 5 mM MgCl₂ (pH 7.3). Store at 4°C.

Laemmli's sample buffer:

0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and, as reducing agent, 10% β-mercaptoethanol or 150 mM dithiothreitol. Mix 2.5 ml stacking gel buffer solution, 4 ml of 10% SDS, 2 ml of glycerol, 116 mg of dithiothreitol (or 20% β-mercaptoethanol), and 1.5 ml H₂O. Add 0.004% of bromophenol blue, dissolve, aliquot and store at -20°C. These amounts are calculated to give double-concentrated (2x) sample buffer.

Acrylamide-Bisacrylamide:

Acrylamide, 29.2 g Bisacrylamide, 0.8 g; H₂O up to 100 ml. Filter through a 0.45 μm filter and store at 4°C protected from light.

Resolving (or "separating") gel buffer (1.5 M Tris-HCl pH 8.8):

Tris-Base, 18.15 g; H₂O up to 100 ml. Adjust pH to 8.8. Store at 4°C.

Stacking gel buffer (0.5 M Tris-HCl pH 6.8):

Tris-Base, 3.0 g; H₂O UP to 50 ml. Adjust pH to 6.8. Store at 4°C.

10% SDS:

Sodium dodecyl sulfate, 10 g (Sigma); H₂O up to 100 ml. Store at room temperature.

Resolving gel overlay:

Mix equal volumes of butanol and stacking gel buffer, shake well for a few minutes, and let stand to separate the two phases. Store at room temperature.

Tank (or "electrode", or "reservoir") buffer (0.025 M Tris (pH 8.3)-0.192 M glycine-0.1% SDS):

Tris-Base, 3 g; glycine, 14.4 g; 10 ml of 10 % SDS solution; H₂O up to 1 l. Store at room temperature. Although the above amounts are 1x, we usually prepare this buffer 10 times concentrated (10x).

10% Ammonium persulphate (APS):

100 mg of ammonium persulphate (BioRad) dissolved in 1 ml of H₂O. It should be prepared just before use. Or preferentially, it can be prepared, aliquoted and stored at -20°C. Keep the APS powder in a sealed container under P₂O₅ or other desiccant since it is very hygroscopic.

TEMED:

(N, N, N', N'-tetra methylethylenediamine) (BioRad).

Coomassie Blue Staining solution:

0.125% Coomassie Brilliant Blue R250 in 45% methanol-10% acetic acid. Dissolve overnight with a magnetic stirring bar and filter through a filter paper. Keep at room temperature.

Destaining solution:

45% methanol-10% acetic acid.

Solubilization buffer (chromatography buffer):

SDS 1%-Tris 50 mM-NaCl 0.4 M- β-mercaptoethanol 0.05%-EDTA 5 mM-sodium azide 3 mM (pH 7.7).

BCA protein assay (Pierce).

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POLYMERASE CHAIN REACTION-SINGLE-STRAND CONFORMATIONAL POLYMORPHISM (PCR-SSCP)

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