ELSEVIER

Biophysical Chemistry 109 (2004) 215–227

Biophysical Chemistry

www.elsevier.com/locate/bpc

Molecular and functional characterisation of the *Serratia marcescens* outer membrane protein Omp1

Neus Ruiz[a], Elke Maier[b], Christian Andersen[b], Roland Benz[b], Miguel Viñas[a,*]

[a]*Laboratory of Microbiology. Biomedical Research Centre of Bellvitge, University of Barcelona, E-08907 L'Hospitalet, Barcelona, Spain*

[b]*Lehrstuhl für Biotechnologie, Theodor-Boveri-Institut (Biozentrum) der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany*

Received 27 May 2003; received in revised form 17 November 2003; accepted 20 November 2003

Abstract

Serratia marcescens outer membrane contains three different general diffusion porins: Omp1, Omp2 and Omp3. Omp1 was cloned and sequenced and it shows a great homology to the family of outer membrane porins that comprises the general porins of enteric bacteria. The gene for Omp1 was transferred into an expression plasmid and was expressed in *Escherichia coli* UH302 (*E. coli* UH302 pOM100), a porin deficient strain. Its expression confers a higher susceptibility towards different antibiotics to this strain. Omp1 was purified to homogeneity from outer membrane of *E. coli* UH302 pOM100. Reconstitution of the purified protein into black lipid bilayers demonstrated that it is a channel-forming component with a single-channel conductance of approximately 2 nS in 1 M KCl similar to that of other porins from enteric bacteria. Omp1 is slightly cation-selective. Its homology to already crystallised members of the family of enteric porins whose three-dimensional-structures are known and allowed the design of a topology model for Omp1. The charge distribution within a porin monomer is similar as in other general diffusion pores. The positively charged amino acids localised at the β-strands opposite the external loop L3, which restrict the pore diameter in the porin monomer.

Keywords: Porin; General diffusion pore; Channel formation; Topology model; *Serratia marcescens*

1. Introduction

The outer membrane of Gram-negative bacteria constitutes the interface between bacteria and the surrounding medium. It forms an efficient barrier that protects the cell against external harmful agents, including antibiotics, detergents, heavy atoms and other damaging substances [1]. The lipopolysaccharide outer envelope of these bacteria is crucial in determining susceptibility patterns, since many antibiotics cannot penetrate this barrier to reach their intracellular target [2]. Thus, the outer membrane opposes resistance to certain hydrophilic molecules that cross the membrane through a few major proteins called 'porins' [3].

Porins form transmembrane channels in the outer membrane, which can be divided into two

*Corresponding author. Tel: +34-93-402 42 65; fax: +34-93-402-18-86.

E-mail address: mvinyas@ub.edu (M. Viñas).

0301-4622/04/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2003.11.005

classes according to functional properties [4]. General diffusion pores form water filled channels that allow the energy independent passage of the substrates across the outer membrane according to their molecular masses (exclusion limit approx. 600 Da in *Escherichia coli*). The diameter and ion selectivity of the porins determine which solutes could permeate through. General diffusion pores are constitutively expressed and can reach 10^4–10^6 copies per cell. The second class of porins comprises substrate specific channels ('specific porins') such as LamB for carbohydrates [5], or Tsx for nucleosides [6]. Specific porins are very often induced in the outer membrane of Gram-negative bacteria under special growth conditions. These 'specific porins' contain binding sites inside the channels thus facilitating the diffusion of specific classes of nutrients across the cell wall [7,8].

Some of the outer membrane porins from different organisms have been crystallised [9–11]. The structurally characterised members of the porin superfamily are homotrimeric proteins made up of three identical polypeptide subunits of 250–450 amino acid residues. The monomer consists of 16 or 18 β-strands arranged in a cylindrical barrel configuration [4]. According to their three-dimensional-structure each of the three monomers contains a channel with a diameter between 0.8 and 1.4 nm. The pore is stabilised and its diameter is limited by an internal eyelet region formed by the third extracellular loop bent into the channel that restrict the channel width, thereby defining the diffusion properties of the pore [3].

Serratia marcescens is a major opportunistic pathogen, isolated from urinary tract infections, nosocomial pneumoniae, surgical wounds and bloodstream infections, mostly in intensive care unit patients [12]. Most of the strains involved are highly resistant to an increasing number of non-related antibiotics, such as β-lactam antibiotics, aminoglycosides, quinolones, trimethoprim and chloramphenicol [13]. Reduced permeability can lead to cross-resistance to several families of antibiotics but this mechanism is particularly effective when combined with other methods, as the synergistic action of various mechanisms provides bacteria with higher levels of resistance [14].

We have studied the outer membrane proteins from *Serratia* and three porins have been described and named Omp1, Omp2 and Omp3 with molecular weights of 42, 40 and 39 kDa, respectively [15].

Little is known about Omp1 and its contribution to the bacterial resistance, although we have observed that mutants lacking this porin show significantly decreased susceptibility. In this paper, we describe the cloning and sequencing of a porin locus, named omp1 and we further characterise the protein, including channel properties and a topology model derived from the sequence.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. marcescens 2170 is an environmental isolate [16]. *E. coli* XL1Blue was used for cloning and *E. coli* UH302 [17], a porin-deficient strain, was used for expression experiments.

All strains were grown in Luria Broth (LB) for DNA manipulations and in Trypticase Soy Broth (TSB) for porin purification. Trypticase Soy Agar (TSA), supplemented with ampicillin 100 μg/ml, IPTG and X-gal, was used to pick up recombinant clones. Mueller–Hinton Broth was used to test susceptibility to antibiotics. All media were purchased from Scharlau (Barcelona, Spain). Ciprofloxacin kindly supplied by CENAVISA Laboratories (Reus, Spain) and the rest of antibiotics were purchased from SIGMA (Sigma Aldrich Corporation, St. Louis, Missouri, USA).

2.2. Cloning and sequencing

S. marcescens 2170 DNA was isolated following the method described by Leranoz [18]. The vector used for cloning was pUC19 (MBI Fermentas; Germany). Two oligonucleotides, SMF1 (5′-CGCAAGCTTCTAATTTATAGGATCCCACGT-3′) and SMF2 (5′–CGATCTTATGAACGTGAAGGATCCGACAGA-3′), deduced from the sequence of the ompF gene of *S. marcescens* [19], were used as primers to obtain a 1.7 kb region containing a porin gene by PCR amplification with the genomic DNA of *S. marcescens*

2170 as a template. Insertion of this fragment, after BamHI cleavage, into the BamHI site of the pUC19 plasmid, yielded the recombinant plasmid pOM100, which was propagated in *E. coli* XL1Blue. The sequence of the gene was obtained using the ABI PRISM BigDye Terminator Sequencing Ready Reaction Kit, version 2.0.

2.3. Expression and purification of the cloned porin in E. coli UH302

E. coli UH302 pOM100 was cultured in TSB supplemented with ampicillin 100 μg/ml overnight, bacteria were harvested by centrifugation (1000×*g*, 10 min), washed once with a 10 mM Tris–HCl pH 7.4 and resuspended in the same buffer. Cells were broken by ultrasonic treatment and unbroken cells removed by centrifugation (1000×*g*, 10 min). The supernatant was centrifuged (100 000×*g* for 1 h). The sedimented bacterial envelopes were resuspended in a buffer containing 2% SDS, 10 mM Tris–HCl pH 7.4. The peptidoglycan layer and the associated proteins were pelleted by centrifugation at 100 000×*g* for 30 min. The pellet was subjected to a second SDS wash. The final pellet was suspended in a buffer containing 2% Genapol, 10 mM Tris–HCl pH 7.4 and 2 mM ethylenediamine tetraacetic acid (EDTA). The supernatant of the subsequent centrifugation (100 000×*g*, 30 min) contained pure Omp1 porin. SDS-PAGE was performed by using a Bio-Rad apparatus (miniprotean II) by a modification of the method of Laemmli [20], gels were stained with 0.25% Coomassie brilliant blue, destained and finally dried using a gel dryer (BioRad 543).

2.4. Black lipid bilayer membrane experiments

Membranes were prepared from a 1% (wt./vol.) solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in *n*-decane in a Teflon cell consisting of two aqueous compartments connected by a circular hole with an area of approximately 0.4 mm^2 [21,22]. The aqueous salt solutions (analytical grade; Merck, Darmstadt, Germany) were used unbuffered at pH 6. The temperature was kept at 20 °C throughout the experiments. The single-channel measurements were performed with a pair of Ag/AgCl electrodes (with salt bridges) switched in series with a voltage source and a current amplifier (Keithley 427). The amplified signal was monitored with a storage oscilloscope and recorded with a strip chart recorder. Stock solutions containing the pure Serratia porin were added after the lipid membrane turned optically black to reflected light.

For the zero-current membrane potentials, the membranes were prepared in a 100 mM KCl solution and insertion of pores was detected until a sufficient number of porin channels was reached in the membrane. The instrumentation was then switched to the measurement of the zero-current potentials and a KCl gradient was established by adding 3 M KCl solution to one side of the membrane while stirring. The zero-current membrane voltage reached its stationary value approximately 2–5 min after addition of the concentrated KCl solution and was analysed using the Goldman–Hodgkin–Katz equation [23].

2.5. Determination of the MIC and quinolone accumulation

Minimal inhibitory concentrations (MIC) were determined by the broth dilution method. Overnight cultures of the bacterial strain in Mueller–Hinton broth were diluted 1000-fold in fresh broth, and 5 μl of the bacterial suspension (0.5×10^4 cfu/ml approximately) was inoculated into Mueller–Hinton broth containing serial dilutions of the antimicrobial agents. MICs were determined after 18 h of incubation at 37 °C as the minimum concentration of antibiotic that inhibits growth.

Quinolone accumulation was measured following Mortimer and Piddock [24] with minor modifications [25]. Isolates were incubated at 37 °C until $A_{600\ nm}=0.5–0.7$. Bacteria were harvested by centrifugation (9000×*g*) at room temperature, washed and concentrated 10-fold in phosphate buffer saline (PBS) pH 7.5. Quinolone was added to 1-ml-aliquots to a final concentration of 10 μg/ml. At time intervals of 0.25, 0.5, 1.5, 3, 6, 8, 10, 15 and 20 min, samples were centrifuged in a microfuge at 10 000 rev./min at 4 °C for 1 min. Pellets were resuspended in 1 ml of 0.1 M gly-

cine–HCl buffer at pH 3.0, and finally incubated at room temperature overnight to allow bacterial lysis. Thereafter, the suspensions were centrifuged at 20 °C for 25 min to remove bacterial debris. The concentration of antibiotic in the supernatants was determined fluorometrically using an SLM Aminco 8100 spectrofluorometer.

2.6. Structure modelling

The structure modelling is based on the sequence alignment of Omp1 and PhoE of *E. coli* performed with the Homology module of Insight II-software package (Accelrys). The structures of highly conserved regions, which comprise the hole β-barrel, were directly translated. The loops search command was used to create new loops structures in areas of deletion or insertions. The transition between the created loops and the β-barrel was refined by the splice repair command. A check for sterical overlaps was performed by the check bump command and sterical overlaps were removed by relaxing the structure (relax-command) followed by an energy minimization of the concerned region. Finally, a relax and energy minimisation was performed at the same time for the five major loops (loop4–loop8), which computed the final structure.

3. Results and discussion

3.1. S. marcescens omp1 cloning and sequencing

E. coli XL1Blue pOM100 (Fig. 1) expressing Omp1 was used to determine the sequence. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 2. The omp1 gene had an ORF of 1125 bp with characteristic sequences of outer membrane protein genes. The promoter region of the *S. marcescens* omp1 gene was located by comparison with the *E. coli* ompF and ompC genes. The −10 and −35 regions did not follow the consensus sequence. Transcription may be controlled by cis-acting activation sequences upstream of the −35 region, similar to the ompC and ompF regions of *E. coli*. We identified a non-translated leader region of mRNA (Osmobox A) that may be involved in osmoregulation, and two

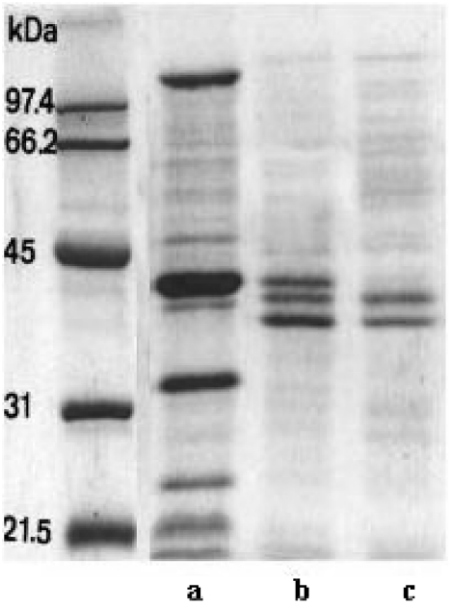

Fig. 1. SDS-PAGE of outer membrane of *S. marcescens* 2170 (a), *E. coli* XL1 Blue pOM100 (b) and *E. coli* XL1 Blue (c). Omp1 porin is expressed in recombinant strain.

regions similar to the ompF gene of *E. coli*, Osmobox B and Osmobox C, upstream of the transcriptional start, which are probably the site of interaction with the activator OmpR. A typical Shine–Dalgarno (SD) sequence centred nine nucleotides upstream of the start codon ensured the translation of initiation complexes. Finally, we found a potential terminator hairpin downstream. Referring to the amino acid sequence, two points could be mentioned: (i) a N-terminal phenylalanine, which is required for the assemble of proteins of the outer membrane [26,27] and (ii) the conserved PEFGGD motif, which forms a turn in the third external loop of the porin, and is responsible for the size constriction of porin channels [9].

3.2. Expression and purification of the S. marcescens porin from a porin-deficient E. coli strain

SDS-PAGE gels showed highly purified Omp1 preparation (Fig. 3) which was used for the rest of the experiments. UH302 pOM100 was also used to determine the functionality of the protein.

```
1     CGTATCTTTG  GGGACACGTG  CAATCGATTT  GGTGCCCGAC  CATCCCACGT
51    TTTTTAAACG  ATTTCGTTAC  AAAACAAAGG  CCAGCCTCGC  TGGCCTTTCG
101   CATTTTTTAA  TATTGACGCA  CGTCACAAAG  TTCCCGAAAA  ATCACATTTA
                                Osmobox C
151   GTTACACATA  CTTTCCTTTT  GCAACCTCAT  TGCGACATTG  GAATCATTTT
                  Osmobox B
201   CGTCCTAGAT  TAACCCGCCT  GTGAATGGAA  CACTTTTTTC  AGACACAGGA
                                          -35
251   CGACACCAAT  CTATCTACAA  TAGTTCCCAA  GGAATTATTG  GCGGCAGTGG
                    -10                   Osmobox A
301   CAAAGGTGTC  CGAATAACAC  CAATGAGGGT  AATAGTGATG  AAGCGCAACA
                              SD          M  K  R  N
351   TTCTTGCAGT  GGTTATCCCG  GCTCTGTTGG  CTGCTGGTGC  AGCAAACGCA
      I  L  A  V  V  I  P  A  L  L  A  A  G  A  A  N  A
401   GCTGAAATCT  ACAACAAAGA  CGGCAACAAG  CTGGATCTGT  ACGGCAAAGT
      A  E  I  Y  N  K  D  G  N  K  L  D  L  Y  G  K  V
451   TGACGGTCTG  CACTACTTCT  CCGACGACAA  AGGTAATGAC  GGCGATCAGA
      D  G  L  H  Y  F  S  D  D  K  G  N  D  G  D  Q
501   CCTATGTTCG  TTTCGGCTTC  AAAGGTGAAA  CTCAGATTAC  TGACCAACTG
      T  Y  V  R  F  G  F  K  G  E  T  Q  I  T  D  Q  L
551   ACCGGTTACG  GCCAGTGGGA  ATACAACGTC  CAGGCTAACC  ACTCCGAATC
      T  G  Y  G  Q  W  E  Y  N  V  Q  A  N  H  S  E  S
601   TCAGGGCACC  GAAGGCACCA  AAACCCGTCT  GGGCTTCGCG  GGTCTGAAAT
      Q  G  T  E  G  T  K  T  R  L  G  F  A  G  L  K
651   TCGCTGACTA  CGGCTCCTTC  GACTACGGCC  GTAACTACGG  CGTACTGTAC
      F  A  D  Y  G  S  F  D  Y  G  R  N  Y  G  V  L  Y
701   GACGTGGAAG  GCTGGACCGA  TATGCTGCCA  GAGTTCGGTG  GCGATACTTA
      D  V  E  G  W  T  D  M  L  P  E  F  G  G  D  T  Y
751   CACCTACACC  GACAACTTCA  TGACCGGGCG  TACCTACGGG  GGTACGACCT
      T  Y  T  D  N  F  M  T  G  R  T  Y  G  G  T  T
801   ATCGTAACAA  CAACTTCTTC  GGTCTGGTTG  ACGGTCTGAA  CTTCGCCGTT
      Y  R  N  N  N  F  F  G  L  V  D  G  L  N  F  A  V
851   CAGTACCAGG  GCAAAAACCA  GAACGACGGC  CGTAACGTCA  AGAAACAAAA
      Q  Y  Q  G  K  N  Q  N  D  G  R  N  V  K  K  Q  N
901   CGGCGACGGC  TGGGGCATCT  CCTCTACTTA  TGACATCGGC  GAAGGCGTAA
      G  D  G  W  G  I  S  S  T  Y  D  I  G  E  G  V
951   GCTTCGGCGC  TGCATACGCG  TCTTCAAACC  GTACCGACGA  CCAGAAACTG
      S  F  G  A  A  Y  A  S  S  N  R  T  D  D  Q  Q  L
1001  CGTTCCAACG  AGCGTGGCGA  CAAGGCTGAC  GCATGGACCG  TAGGTGCGAA
      R  S  N  E  R  G  D  K  A  D  A  W  T  V  G  A  K
1051  ATACGACGCC  AACAACGTTT  ACCTGGCGGC  GATGTACGCA  GAAACCCGTA
      Y  D  A  N  N  V  Y  L  A  A  M  Y  A  E  T  R
1101  ACATGACCCC  GTTCGGCGGC  GGTAACTTCG  GTGCAGGCTG  TGCAGCTACC
      N  M  T  P  F  G  G  G  N  F  G  A  G  C  A  A  T
1151  GATGACAAGT  GCGGCGGCTT  CGCGAGCAAA  ACTCAGAACT  TCGAAGTGAC
      D  D  K  C  G  G  F  A  S  K  T  Q  N  F  E  V  T
1201  TGCTCAGTGC  CAGTTCGACT  TCGGTCTGCG  TCCAGAAGTG  TCTTACCTGC
      A  Q  C  Q  F  D  F  G  L  R  P  E  V  S  Y  L
1251  AATCTAAAGG  TAAAAACCTG  AACGTTCCTG  GCGTGGGTTC  TGACCAGGAT
      Q  S  K  G  K  N  L  N  V  P  G  V  G  S  D  Q  D
1301  CTGGTTAAAT  ATGTTTCTGT  TGGTACCACT  TACTACTTCA  ACAAGAACAT
      L  V  K  Y  V  S  V  G  T  T  Y  Y  F  N  K  N  M
1351  GTCCACCTAC  GTTGATTACA  AAATCAACCT  GCTGGATGAC  AACGAGTTCA
      S  T  Y  V  D  Y  K  I  N  L  L  D  D  N  E  F
1401  CCAAAGCAAC  CGGCACCGCT  ACCGACGATA  TCGTAGCTGT  TGGTCTGGTA
      T  K  A  T  G  T  A  T  D  D  I  V  A  V  G  L  V
1451  TACCAGTTCT  AAGTTGTCTC  GCTTAACGGC  GGTTCACCGT  CGGCTAAGTT
      Y  Q  F
1501  AAAAAAACAG  GGCTTCGGCC  CTGTTTTTAT  TTGTGTCTTC  CGGTAACGTT
1551  TTATCCTGCA  GGTTTCCCCT  TCCCCCTCAT  TTTTTCGGTC  GGATGGTTCT
1601  ACCCTCAAAT  GCAGGCATGA  CTTTCGAAAG  TTC
```

Fig. 2. Nucleotide and amino acid sequences of the *S. marcescens* Omp1 porin gene are shown. The hypothetic osmobox A, B and C and the characteristic Shine–Dalgarno sequence are underlined. A potential terminator hairpin downstream is also underline.

Whereas the lack of porins in UH302 resulted in an extremely low growth rate, the clone grew much more rapidly (data not shown). It has been shown that in *E. coli*, most β-lactam and other small hydrophilic agents such as chloramphenicol, tetracyclines and aminoglycosides use predominantly the porin pathway to penetrate the cells [28]. The MIC values (Table 1) in wild-type (*E. coli* UH302) were higher than in the recombinant (*E. coli* UH302 pOM100), due to the higher permeability of the OM of *E. coli* UH302 pOM100 to different hydrophilic antimicrobial agents such as β-lactams and chloramphenicol. On the contrary, no changes in MICs values of tetracycline and ciprofloxacin were seen. The recombinant plasmid in *E. coli* UH302 partially restored antibiotic susceptibility. Ciprofloxacin accumulation was significantly lower in *E. coli* UH302 than in recombinant clone or in *S. marcescens* (Fig. 4).

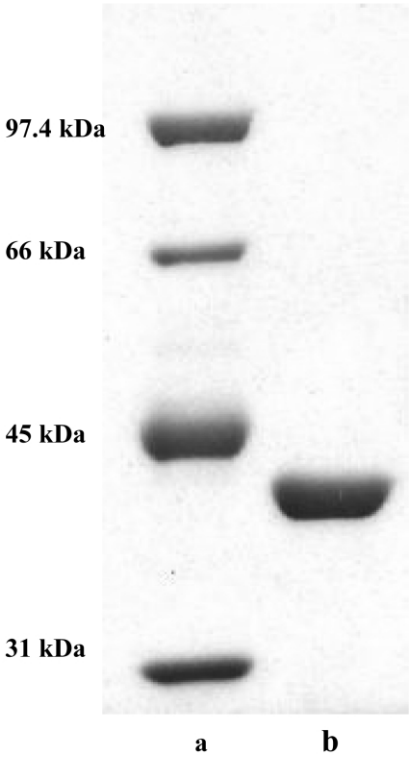

Fig. 3. SDS-PAGE of the Omp1 purification protein from E. coli UH302 pOM100. (a) standard molecular weigh, (b) Omp1 purified and heated previously.

Table 1
MICs (microgram per millilitre) of different antimicrobial agents to *E. coli* UH302 and *E. coli* UH302 pOM100

	E. coli UH302 pOM100	*E. coli* UH302
Cefoxitin	15.6	31.2
Ceftriaxone	0.5	4
Cefotaxime	0.5	2
Moxalactam	125	500
Ciprofloxacin	0.1	0.1
Tetracycline	2	2
Cloramphenicol	2	8

Ciprofloxacin is a fluoroquinolone that uses three different ways to penetrate the bacteria [29]: (i) an hydrophobic way throughout the lipid bilayer; (ii) a *self-promoted* still not well known; and (iii) a hydrophilic way due to the channel forming activity of porins. Although the estimation of the MIC values did not show clear differences between recombinant and wild-type bacteria, accumulation of ciprofloxacin measured in spectrofluorometric experiments demonstrate that ciprofloxacin uses Omp1 protein to penetrate *Serratia*. Furthermore, it should be noted that MIC values are determined after an incubation period as long as 18 h, thus, ciprofloxacin can penetrate into the bacteria using one or more of the three ways cited above. If one of these penetration ways is not functional (*E. coli* UH302) the ciprofloxacin permeation should be slower, although long incubations will allow similar inner final concentrations. Thus, one should not expect great differences in MIC values due to changes in permeability. On the contrary, short incubation periods (e.g. 20 min) allow quantitatively in determining the kinetics antibiotic accumulation (e.g. the lack of porins in *E. coli* UH302 leads to reduced ciprofloxacin accumulation when compared with *E. coli* UH302 pOM100).

3.3. Pore conductance analysis

For proofing the pore forming ability Omp1 we performed conductance measurements in black lipid bilayer. Small amounts of the purified 42 kDa protein (approx. 100 ng/ml) were added to the aqueous phase on one or both sides of the membrane. After a delay of 1–2 min, probably due to the slow aqueous diffusion of the protein, the current increased in a step-wise fashion similar to that observed for other Gram-negative bacterial porins [6,21]. The current increase was gradual in these conditions and the conductance of these steps was approximately 4 nS when the porin was freshly added to the pre-existing membrane (Fig. 5). However, the conductance of the current steps changed when the membrane was reformed in the same aqueous solution. The channels opened more rapidly, i.e. the time resolution had to be increased. The channels often showed under these conditions a conductance of 2 nS, about half that of the first onset. Fig. 5 shows a recording of the *S. marcescens* porin from a reformed diphytanoyl phosphatidylcholine/*n*-decane membrane. We would like to emphasize that 2 nS is close to the conductance of other Gram-negative bacterial porins, e.g. OmpC or OmpF of *E. coli*, which show conductances of 1.5 to 2.0 nS under the same conditions [6,9,22,30].

The channels had a long lifetime because only on-steps lasted at least several minutes. The distribution of channel conductances had two maxims (Fig. 6), one corresponding to an average of 2 nS (left) and one corresponding to approximately 4 nS (right). These two maxims may represent the reconstitution of the monomeric and dimeric trimers of the *S. marcescens* porin. It is obvious that the 2 nS channel represents the sum of the conductance of three individual channels in a trimer. However, this could not be shown here, because it was not possible to close the channels even at

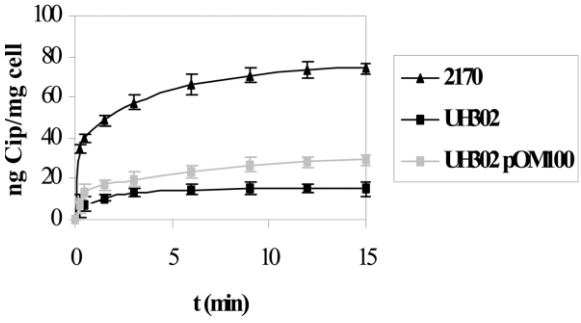

Fig. 4. Ciprofloxacin accumulation of *S. marcescens* 2170, *E. coli* UH302 and *E. coli* UH302 pOM100.

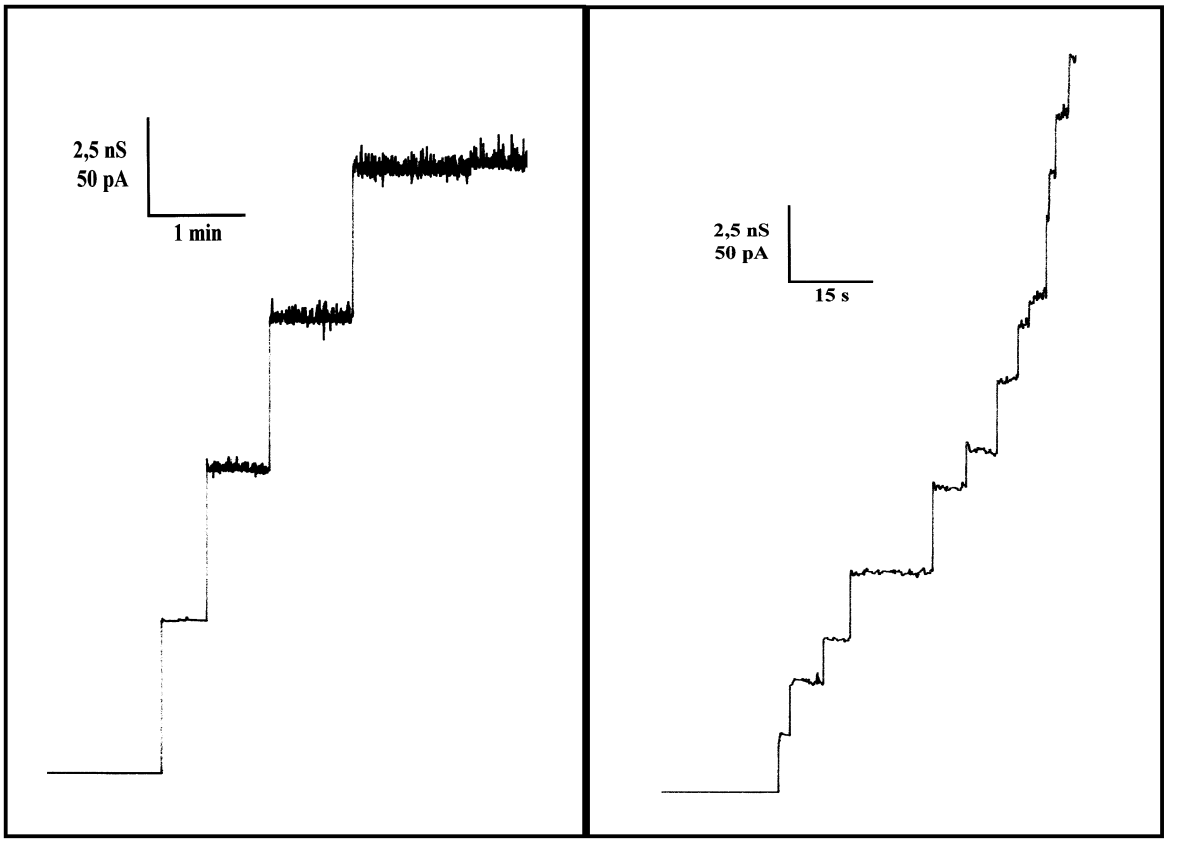

Fig. 5. Conductance recordings of 1% diphytanoyl phosphatidylcholine/*n*-decane membranes after the addition of porin from *Serratia marcescens*. The aqueous phase contained 1 M KCl and approximately 100 ng/ml protein. The membrane potential was 20 mV; $T = 20$ °C.

A The porin was added to a freshly formed membrane.

B The channel recording shows the current steps observed after reforming the membrane under otherwise identical conditions. Note that the time resolution was increased by a factor of four as compared to Fig a and that the conductance resolution of the two recordings A and B differ by a factor of 10.

high voltages up to 150 mV, which means that the Omp1 channels of *S. marcescens* were not voltage-gated as OmpF of *E. coli* and the conductance of a single channel in a trimer could not be evaluated [31]. The Omp1 channel conductance of 2 nS in 1 M KCl suggests that the diameters of the three Omp1-channels in a trimer are presumably very similar to those of the OmpF trimers, which are close to 1 nm [6,9,22,30].

Omp1 porin from the *S. marcescens* outer membrane was only moderately cation-selective, as inferred from single-channel experiments, in which KCl was replaced by LiCl or KAcetate, i.e. the mobile ions K^+ and Cl^- were replaced by the less mobile ions Li^+ and $Acetate^-$ (Table 2). The single-channel conductance in 1 M LiCl and 1 M K Acetate decreased on average by about a factor of two compared with the conductance in 1 M KCl when the reconstitution of monomeric and dimeric conductive units is considered. However, the single-channel conductance was higher in KAc than in LiCl, suggesting that the channel was cation-selective. This result also indicated that the single-channel conductance followed the conductivity of the bulk aqueous salt solutions of KAc and LiCl, which was about half that of 1 M KCl

[32]. These results are expected if the outer membrane porin Omp1 of *S. marcescens* is wide and water-filled. It is noteworthy that similar results were obtained for general diffusion porin of *E. coli* and other enteric bacteria [22]. Measurements at various KCl concentrations support this hypothesis, because the single-channel conductance was a linear function of the bulk aqueous conductance (Table 2), which also characterises enteric general diffusion porins [7].

Table 2
Average single-channel conductance G, of the *S. marcescens* porin as a function of different salt solutions of concentration c. The membranes were formed from 1% diphytanoyl phosphatidylcoline dissolved in *n*-decane; $T=20$ °C. The pH of the aqueous salt solutions was approximately 6 unless otherwise indicated. G is given as the mean of at least 100 single steps similar to those shown in

Salt	Concentration c [M]	Left-side maximum	Right-side maximum
		G (nS)	
LiCl	1.0	0.75	1.5
KCl	0.1	0.30	0.60
	0.3	0.70	1.3
	1.0	2.0	4.0
	3.0	4.5	9.5
Potassium acetate pH 7 (KAc)	1.0	1.2	2.0

3.4. Zero-current membrane potential measurements

The results of the single-channel experiments agree with zero-current membrane potential measurements in the presence of salt gradients. After incorporation of a large number of channels in membranes bathed in 100 mM KCl, five-fold salt gradient were established across the membranes by addition of small amounts of concentrated KCl solution to one side of the membrane. For KCl, the more diluted side of the membrane became slightly positive with an average potential of 22 mV for the five-fold gradient. This points to the preferential movement of potassium through the channel, i.e. the channel is slightly cation-selective, as suggested above by the single-channel data. Analysis of the zero-current membrane potential using the Goldman–Hodgkin–Katz equation [23] revealed a ratio of 3.9 for the permeabilities P_K and P_{Cl}.These values differ from that of the anion-selective protein PhoE, which is anion-selective ($P_K/P_{Cl}=0.3$) [22]. These results suggest, simi-

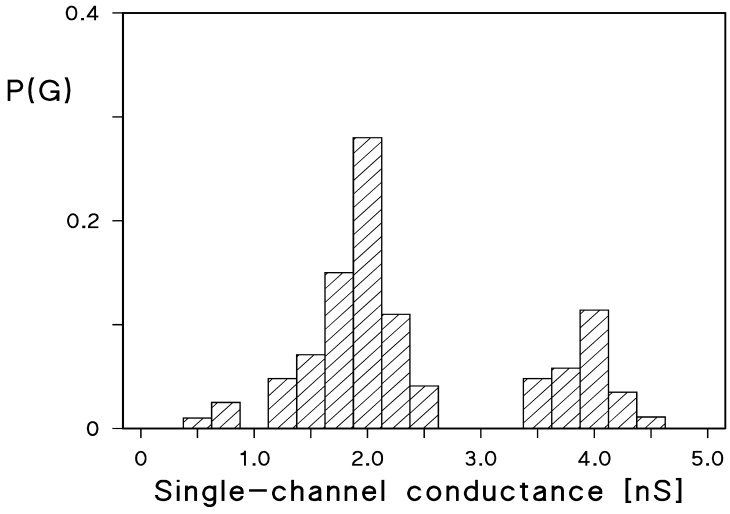

Fig. 6. Histogram of the average conductance steps measured after reconstitution of the porin from *Serratia marcescens* into black lipid bilayer membranes made of diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl and 100 ng/ml protein. The membrane voltage was 20 mV; $T=20$ °C. The average channel conductance was 2.0 nS for the left-side maximum and approximately 4 nS for the right-side maximum. The total number of steps was 288 steps from six different membranes.

larly to the single-channel experiments, that there is not much difference in the permeability between anions and cations through Omp1, which represents another indication that the outer membrane channel Omp1 of *S. marcescens* is a general diffusion pore.

3.5. Structural model of Omp1

The sequence of Omp1 has a high homology to other general diffusion pores from *E. coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* (Fig. 7). The sequence identity with outer membrane proteins from other species is between 94 and 60%. The closest homology (96%) was seen with the *S. marcescens* reported by Hutsul and Worobec [19] the reason for this difference could be normal diversity within strains of one species. In *S. marcescens* 2170 Omp1 and Omp3 proteins showed a similar osmoregulation, Omp1 seems to be expressed at a basal level and is induced (with Omp3) at low osmolarity conditions. On the contrary, Omp2 was induced under high osmolarity conditions (data not shown).

A topology model of the structure of the Omp1 is proposed based on its homology to *E. coli* PhoE. The primary structure fits well to the template structure and is thus likely to show the structural features found in the monomers of OmpF and PhoE of *E. coli* [9]. The alignment already shows that the parts of the sequence, which form β-strands in PhoE from *E. coli* are highly conserved. The highest variability was found in regions forming the extracellular loops. Here also gaps and insertion could be seen within the porin-family. The insight II modelling software (Accelrys) was used to build the model (Fig. 8a). The coordinates of regions of high conservation were directly transferred from PhoE to the Omp1 model. Region of higher variability, mainly the extracellular loops were designed by the loops search or loops generate command of the homology module, followed by an energy minimization routine to prevent unfavorable folding (further details see Section 2). A loop structure was chosen, which was compact and did not protrude far away from neighbouring loops. A critical part of the sequence is shown in Fig. 8b in detail.

The third loop is not part of the extracellular protrusion but folds inwards and constricts the channel diameter. Residues located at this part of the structure lining the channel lumen and could influence the channel characteristic. It is visible that there is an asymmetric distribution of charges within the constriction zone. Positive charged amino acids (K16, R37, K74, R76, R127) are located on the side of the barrel wall and negatively charged residues (E111, D107 and D115) are located on the opposite side at the constricting loop L3. This establishes a strong electric field across the channel, which works as an electric sieve for sorting components, which diffuse through the channel and subsequently is responsible for the ion selectivity observed. Fig. 8b (middle and right side) shows the same region of the structures of PhoE (which exhibits weak selectivity for anions [22]) and OmpF (preferring cations over anions in a factor of about four). It should be noted that L3 of PhoE and Omp1 is one residue longer than that of *E. coli* OmpF. The additional amino acid, Tyr119 (in Omp1) does not point towards the channel lumen and is located in a way that it does not constrict the channel diameter. The comparison of the structures show that except one additional negatively charged residue (Asp18) the composition of charged residues within the constriction site in Omp1 is identical to OmpF of *E. coli*. This additional negative charge compared to OmpF of *E. coli* could be responsible for the slightly higher cation selectivity of the Omp1 protein. However, voltage gating has been described in most of the porins. When the membrane potential increased pores tend to become close at certain threshold potential, 135 mV in PhoE and 145 mV en OmpF of *E. coli* [33]. The physiological significance of this behaviour remains unclear since Donnan potential existing across the membrane is not enough to close pores [34]. Furthermore, other conditions such as pH, pressure and the presence of membrane derived oligosaccharides or polyamines affect pore opening/closing in vitro and may contribute to pore gating events under physiological conditions [35].

The structure of loop L6 of Omp1 is the most speculative one since it is formed by 25 residues

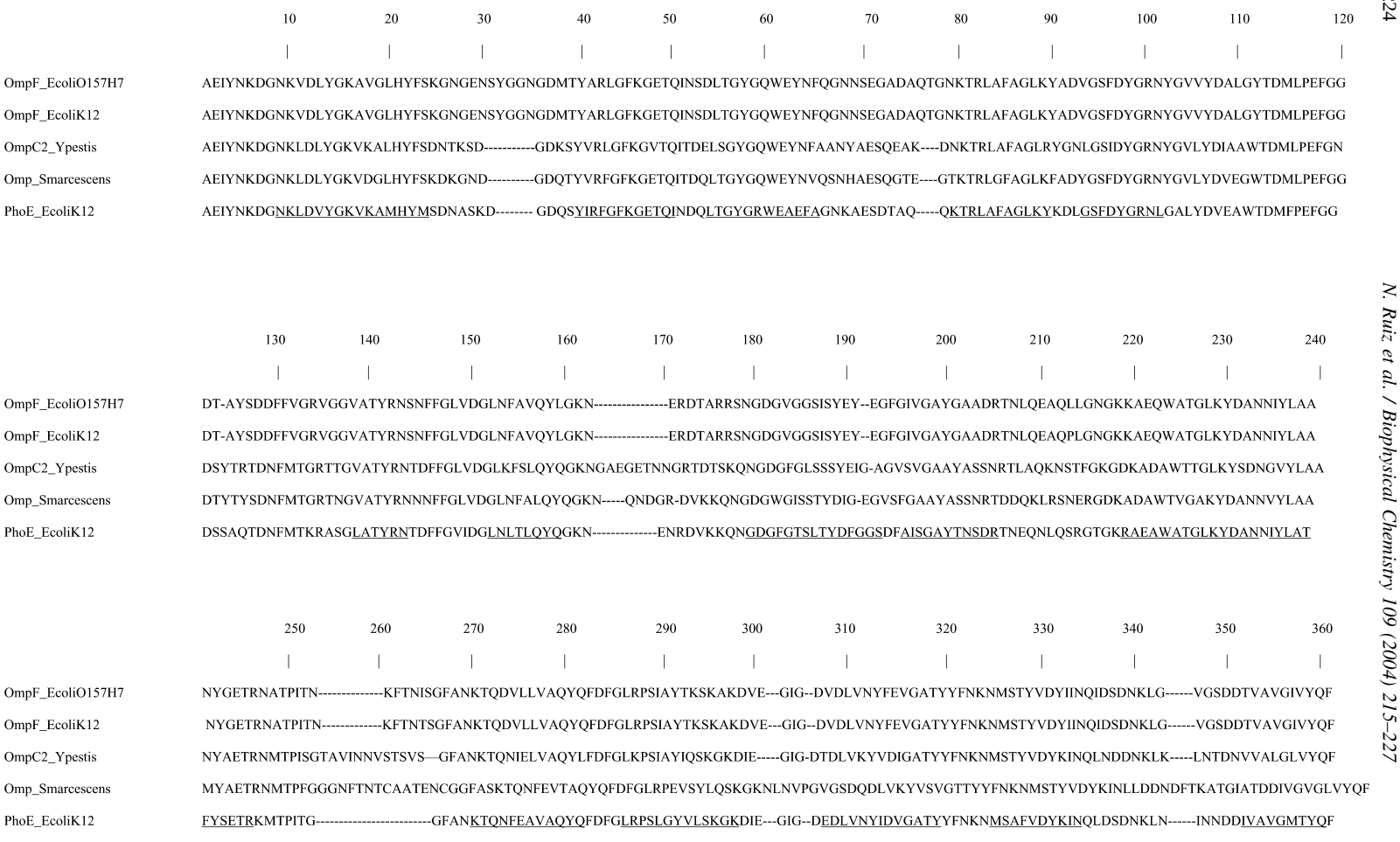

Fig. 7. Amino acid sequence alignment of Omp1 of *S. marcescens*, OmpF of *E. coli*, OmpC of Yersinia pestis and PhoE of *E. coli*. Underlined amino acids from PhoE were used for the modelling of Omp1.

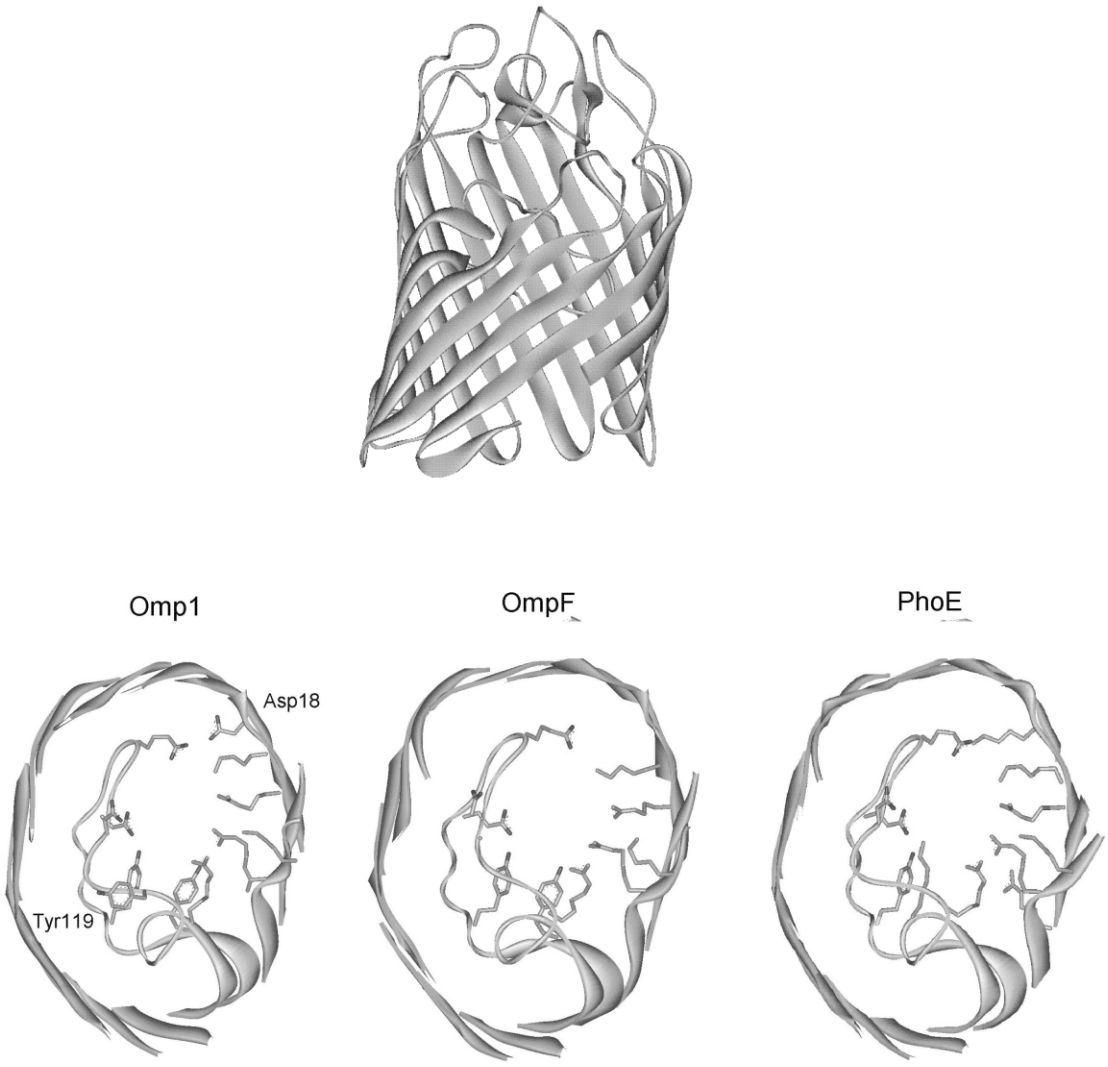

Fig. 8. (a) Structure of Omp1 of *S. marcescens* from the side. (b) Structure of Omp1 of S. marcescens and OmpF and PhoE of *E. coli* from the top with the charged residues highlighted.

compared to 11 of the PhoE structure. Two cysteine residues were identified, which may be located in this external loop. Cysteine residues have been described in OprF and OprB porins from *Pseudomonas aeruginosa* and LamB from *E. coli* [36]. The four-cysteine residues of the monomeric OprF porin are involved in the switching between its two functional pore sizes [30]. In LamB the replacement of the two cysteines by serine does not affect maltodextrin binding. Based on the models, one can speculate that loop 6 should be close to the loop 3; if this is so, they would contribute to some of the permeability properties of the channel, particularly in the constriction region.

Acknowledgments

This work was supported by grants of both DGICYT PM98-0189 and SAF2002-00698 (Span-

ish Ministry of Science and Technology/ FEDER) to Miguel Viñas, and of both the Deutsche Forschungsgemeinschaft and the fonds der Chemischen industrie to Roland Benz. Roland Benz and Miguel Viñas gratefully acknowledge an Acciones Integradas Spanish-German exchange grant.

References

[1] H. Nikaido, Transport across the outer membrane, J Bioenerg. Biomembr. 25 (1993) 581–589.
[2] H. Nikaido, M. Vaara, Molecular basis of bacterial outer membrane permeability, Microbiol. Rev. 49 (1985) 1–32.
[3] W. Achouak, T. Heulin, J.M. Pagès, Multiple facets of bacterial porins, FEMS Microbiol. Lett. 199 (2001) 1–7.
[4] M.H. Saier, Families of proteins forming transmembrane channels, J. Membrane Biol. 175 (2000) 165–180.
[5] M. Luckey, H. Nikaido, Diffusion of solutes through channels produced by phage lambda receptor protein of *Escherichia coli*: inhibition by higher oligosaccharides of maltose series, Biochem. Biophys. Res. Commun. 93 (1980) 166–171.
[6] R. Benz, Structure and function of porins from Gram-negative bacteria, Ann. Rev. Microbiol. 42 (1988) 359–393.
[7] R. Benz, in: J.M. Ghuysen, R. Hakenbeck (Eds.), Bacterial cell wall. Solute uptake through bacterial outer membrane, Elsevier Science B.V, Amsterdam, 1994, pp. 397–423.
[8] H. Nikaido, Porin and specific channels of bacterial outer membranes, Mol. Microbiol. 6 (1992) 435–442.
[9] S. W. Cowan, P. Schirmer, C. Rummel, M. Steiert, R. Ghosh, R.A. Pauptit, et al., Crystal structures explain functional properties of two *Escherichia coli* porins, Nature 358 (1992) 727–733.
[10] T. Schirmer, T.A. Keller, Y.F. Wang, J.P. Rosenbusch, Structural basis for sugar translocation through maltoporin channels at 3.1 Å resolution, Science 267 (1995) 512–514.
[11] M.S. Weiss, U. Abele, J. Weckesser, W. Welte, E. Schiltz, G.E. Schulz, Molecular architecture and electrostatic properties of a bacterial porin, Science 254 (1991) 1627–1630.
[12] A. Hejazi, F.R. Falkiner, *Serratia marcescens*, J. Med. Microbiol. 46 (1997) 903–912.
[13] J.J. Farrar, in: PR Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, R.H. Yolken (Eds.), A Manual of Clinical Microbiology, Enterobacteriaceae: Introduction and identification, ADS Press, Washington DC, 1995, pp. 438–447.
[14] U. Hechler, M. Van der Weghe, H.H. Martín, J.M. GuFiére, Overproduced β-lactamase and the other outer membrane barrier as resistance factors in *Serratia marcescens* highly resistant to β-lactamase stable β-lactam antibiotics, J. Gen. Microbiol. 135 (1989) 1275–1290.
[15] M. Puig, C. Fusté, M. Viñas, Outer membrane proteins from *Serratia marcescens*, Can. J. Microbiol. 39 (1993) 108–111.
[16] M. Regué, C. Fabregat, M. Viñas, Isolation and partial characterization of a generalized transducing bacteriophage specific for *Serratia marcescens*, Res. Microbiol. 142 (1991) 23–27.
[17] S.T. Cole, I. Sonntag, U. Henning, Cloning and expression in *Escherichia coli* k-12 of the genes for major outer membrane protein OmpA from *Shigella dysenteriae*, *Enterobacter aerogenes* and *Serratia marcescens*, J. Bacteriol. 149 (1982) 145–150.
[18] A.M. Leranoz, M.C. Fusté, R.A. Hull, R.P. Williams, M. Viñas, Cloning and expression in *Escherichia coli* of a gene encoding proline oxidase of *Serratia marcescens*, Microbios 67 (1991) 87–94.
[19] J.A. Hutsul, E. Worobec, Molecular characterization of *Serratia marcescens* OmpF porin, and analysis of *S. marcescens* OmpF and OmpC osmoregulation, Microbiology 143 (1997) 2797–2806.
[20] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T2, Nature 227 (1970) 680–785.
[21] R. Benz, K. Janko, W. Boos, P. Läuger, Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*, Biochim. Biophys. Acta 511 (1978) 305–319.
[22] R. Benz, A. Schmid, R.E.W. Hancock, Ion selectivity of Gram-negative bacterial porins, J. Bacteriol. 162 (1985) 722–727.
[23] R. Benz, K. Janko, P. Läuger, Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli*, Biochim. Biophys. Acta 551 (1979) 238–247.
[24] P.G.S. Mortimer, L.V.J. Piddock, A comparison of methods used for measuring the accumulation of quinolones by *Enterobacteriae*, *Pseudomonas* and *Staphylococcus aureus*, J. Antimicrob. Chemother. 28 (1991) 639–653.
[25] M. Berlanga, N. Ruiz, J. Hernández-Borrell, T. Montero, M. Viñas, Role of the outer membrane in the accumulation of quinolones by *Serratia marcescens*, Can. J. Microbiol. 46 (2000) 716–722.
[26] H. de Cock, M. Struyre, M. Kleerebezem, T. van der Krift, J. Tommassen, Role of the carboxy-terminal phenylalanine in the biogenesis ot outer membrane protein PhoE of *Escherichia coli* K-12, J. Mol. Biol. 269 (1997) 473–478.
[27] M. Stryvé, M. Moons, J. Tommanssen, Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein, J. Mol. Biol. 218 (1991) 141–148.
[28] H. Nikaido, Outer membrane barrier as a mechanism of antimicrobial resistance, Antimicrob. Agents Chemother. 33 (1989) 1831–1836.
[29] L.V.J. Piddock, Mechanism of quinolone uptake into bacterial cells, J. Antimicrob Chemother. 27 (1991) 399–403.
[30] R.E.W. Hancock, Role of porins in outer membrane permeability, J. Bacteriol. 169 (1987) 929–933.

[31] H. Schindler, JP. Rosenbusch, Matrix protein from *Escherichia coli* outer membranes forms voltage-controlled channels in lipid bilayers, Proc. Natl. Acad. Sci. USA 75 (1978) 3751–3755.

[32] G.W. Castellan, in: G.W. Castellan (Ed.), Physical chemistry. The ionic current in aqueous solutions, Addison-Wesley, Reading, Mass, 1983, pp. 769–780.

[33] P. van Gelder, N. Saint, P. Phale, E.F. Eppens, A. Prilipov, et al., Voltage sensing in the PhoE and OmpF outer membrane porins of *Escherichia coli*: role of charged residues, J. Mol. Biol. 269 (1997) 468–472.

[34] K. Sen, J. Hellman, H. Nikaido, Porin channels in intact cells of *Escherichia coli* are not affected by Donnan potentials across the outer membrane, J. Biol. Chem. 263 (1988) 1182–1187.

[35] H. Samartzidou, A.H. Delcour, Escherichia coli porin has an opposite voltage-dependence to the homologous OmpF, The EMBO J. 17 (1998) 93–100.

[36] T. Ference, S. Stretton, Cysteine-22 and cysteine-38 are not essential for the functions of maltoporin (LamB) protein, FEMS Microbiol. Lett. 61 (1989) 335–340.