Enterobacter aerogenes OmpX, a cation-selective channel *mar*- and osmo-regulated

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Abstract The *ompX* gene of *Enterobacter aerogenes* was cloned. Its overexpression induced a decrease in the major porin Omp36 production and consequently a β-lactam resistance was noted. Purified outer membrane protein X (OmpX) was reconstituted into artificial membranes and formed ion channels with a conductance of 20 pS in 1 M NaCl and a cationic selectivity. Both MarA expression and high osmolarity induced a noticeable increase of the OmpX synthesis in the *E. aerogenes* ATCC 13048 strain. In addition, OmpX synthesis increased under conditions in which the expression of the *E. aerogenes* major non-specific porins, Omp36 and Omp35, decreased.

Keywords: Outer membrane protein; Membrane channel; OmpX; Regulation

1. Introduction

In *Enterobacteriaceae*, resistance to β-lactam antibiotics is frequently associated with the production of hydrolytic enzymes and membrane alterations, leading to a reduction in the intracellular concentration of active antibiotics [1]. Among the emerging resistant bacteria, *Enterobacter aerogenes* is now a leading cause of nosocomial respiratory tract infections [2,3].

Outer membrane protein X (OmpX), was first identified in *Enterobacter cloacae* and its overexpression in *Escherichia coli* causes a reduced expression of the major porins, resulting in decreased susceptibility to β-lactams [4,5]. *E. cloacae* OmpX belongs to a family of outer membrane proteins that includes *E. coli* OmpX, *Yersinia enterocolitica* Ail, *Salmonella typhimurium* PagC and Rck, and *Klebsiella pneumoniae* OmpK17 [6–11]. Most of these proteins are involved in bacterial virulence, but the precise function and regulation of *E. coli* OmpX is still unclear. Regarding the crystal structure of OmpX from *E. coli* determined by X-ray crystallography and by NMR, the family forms an eight-stranded antiparallel β-barrel [12,13]. The structure shows no continuous pathway between the periplasmic and the external end of the barrel [12]. OmpX is also closely related to the eight-β strand OmpA, a major outer membrane protein involved in the stabilization of the bacterial envelope [14].

Furthermore, a recent study reported that an *E. aerogenes* clinical isolate exhibiting a phenotype of multidrug resistance shows a decrease of the major porin expression associated with an overproduction of OmpX [15]. The aim of this study was to characterize functional and regulation aspects of *E. aerogenes* OmpX.

2. Materials and methods

2.1. Bacterial strains, growth conditions and antibiotic susceptibility test

The *E. aerogenes* type strain ATCC 13048 was used as reference strain. Transformations with the plasmid pMD01 encoding the *ompX* gene or with the plasmid pRC1 encoding the *marA* gene from *E. aerogenes* [16] were performed. Bacteria were grown routinely in Luria–Bertani broth or in Nutrient broth (NB) supplemented or not with 20% sorbitol, at 37 °C. Kanamycin (Km), 30 μg/ml and clavulanic acid, 4 μg/ml were added if necessary. The determination of minimal inhibitory concentrations (MICs) was carried out as previously described [15].

2.2. Cloning and sequencing

X1 and X2 primers were designed from conserved sequence regions between *E. coli* and *K. pneumoniae ompX* genes: X1 5′-TTAGGAATAATCTGGATG-3′ and X2 5′-CGAAAGTGATTAGAAGCGGTA-3′. Amplification was made on the genomic DNA of *E. aerogenes* purified by using the Wizard Genomic DNA Purification Kit (Promega). PCR amplifications were performed in a GeneAmp PCR System 2400 thermocycler (Perkin–Elmer) with an initial 4-min denaturation at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, a 1-min annealing at 52 °C and a 1-min extension at 72 °C. The 30 cycles were followed by a 7-min extension at 72 °C. The sequence of the PCR product was determined with an ABI Prism 377 DNA sequencer with Dye fluorescent terminators and the X1–X2 primers. The nucleotide sequence obtained was deposited to the NCBI server under the Accession No. AY267336. X3 primer (5′-CTTACGGACTTATTAGAAAC-3′) was determined before the Shine–Dalgarno (SD) of *ompX*. X2–X3 PCR amplification was made under the same conditions. The 568-pb PCR fragment obtained was cloned in the pDRIVE® vector (PCR Cloning Kit, Qiagen) under the *lac* promotor (pMD01).

2.3. Outer membrane isolation and immunodetection

Outer membrane preparation was carried out as previously described [15]. Outer membrane proteins were separated on a SDS–polyacrylamide gel, then electro-transferred onto nitrocellulose sheets. The incubation with specific antisera directed against denatured *E. coli* porins, OmpA or OmpX and the immunodetection with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies

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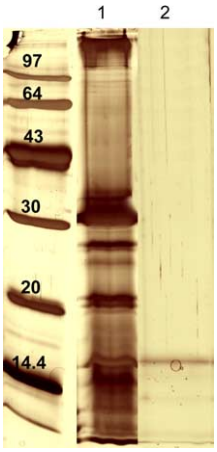

Fig. 1. Analysis of the purity form of electroeluted OmpX 18 kDa, by silver staining. Lane 1, outer membrane unheated of ATCC 13048, pMD01 overexpressing OmpX and lane 2, electroeluted OmpX.

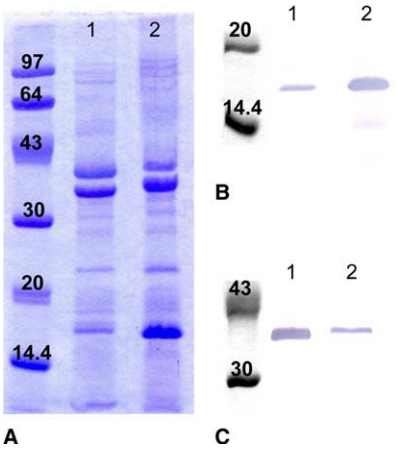

Fig. 2. Analysis of outer membrane fractions from *E. aerogenes* strains: by Coomassie blue (A), by immunodetection for OmpX production (B) and for the major porin Omp36 expression (C). SDS–PAGE gel (12%), outer membrane samples were heated for 10 min at 96 °C. Lane 1, ATCC 13048 and lane 2, ATCC 13048, pMD01 overexpressing OmpX.

(Jackson ImmunoResearch), was carried out as previously described [17].

2.4. OmpX purification and reconstitution in planar lipid bilayers

Outer membrane fraction from ATCC 13048 pMD01 strain overexpressing OmpX was loaded on a polyacrylamide gel (15%). After migration and staining by Coomassie blue, the band corresponding to OmpX was excised and electroeluted by using a Biotrap system (Schleicher & Schuell) with 25 mM Tris-base, 192 mM glycine and 0.025% SDS. The purity of the electroeluted sample was checked out by analytical SDS–PAGE with silver staining (Fig. 1).

Virtually solvent-free planar lipid bilayers were formed by the Montal and Mueller technique as described in [18]. Currents and amplitude histogram were obtained from the stored signals using SATORI software from Intracell (UK). 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) or a mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (POPC/DOPE, 7/3, w/w) were used as the lipids (Avanti Polar). The electrolyte solutions were 1 M NaCl buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2 ethanesulfonic acid (pH 7.4). For reconstitution experiments performed at room temperature, OmpX was diluted in 1% octyl-polyoxyethylen (octyl-POE) and 1 ng was added to the *cis* side of the membrane. For selectivity experiments, a NaCl gradient was set up across the lipid bilayer: 0.2 M on the *cis* side and 1 M on the *trans* side by adding small amounts of concentrated solution to one side of the bilayer, while the other remained at the low concentration. The zero-current potential was corrected by deducting the asymmetric potential due to the salt gradient.

3. Results

3.1. Overexpression of OmpX causes major porin decrease and a β-lactam resistance

SDS–PAGE of the outer membrane showed the overexpression of a protein migrating to about 18 kDa in the transformed strain and immunoblotting identified OmpX (Fig. 2A and B). Conjointly, the analysis of the outer-membrane proteins indicated a low expression of the Omp36 major porin. This result was confirmed by porin immunodetection (Fig. 2C). In contrast, no variation of OmpA expression was immunodetected (data not shown).

The cephalosporin susceptibility of the OmpX overproducing strain was studied (Table 1). The presence of the plasmid pMD01 carrying *ompX* resulted in a 4- to 8-fold increase of MICs for cefepime, cefpirome and ceftazidime. The use of clavulanic acid, a well-known β-lactamase inhibitor [15], indicated that β-lactamase activity was not involved in this resistance profile.

Table 1
β-lactam antibiotic susceptibility of *E. aerogenes* strains

	MIC (μg/ml)		
	Cefepime	Ceftazidime	Cefpirome
ATCC13048	0.25	1	0.25
*ATCC13048, pDRIVE®	0.5	1	0.5
*ATCC13048, pMD01	2	4	4

Values are means of three independent determinations. *MICs were determined in MH broth supplemented with 30 μg/ml kanamycin and 4 μg/ml clavulanic acid. pDRIVE®, vector alone and pMD01, plasmid ensuring OmpX overexpression.

3.2. OmpX functional analysis

Purified OmpX diluted in 1% octyl-POE was introduced in the *cis* side of a POPC/DOPE bilayer bathed by a 1 M NaCl solution. After constant potential application, discrete current fluctuations were observed demonstrating the formation of channels by OmpX (Fig. 3A). Control experiments were performed where the same amount of a protein-free 1% octyl-POE solution was added into measurement compartment and no channel activity was detected. OmpX typical single-channel recordings show no step-like incorporation but a single level of current. This can be compared to the ionophore behaviour of monomeric porins like *E. coli* OmpA and *Pseudomonas aeruginosa* OprF [19,20]. The major conductance value measured by the different histograms associated to the recordings was small: 20 ± 3 pS (mean value calculated from $n = 7$ independent experiments). However, higher minor conductance values (40 ± 5 pS, Fig. 3A, and 130 ± 7 pS), which may result from the incorporation of aggregates, were also detected during the different reconstitution experiments. Reincorporation of OmpX was also performed in DPhPC lipid bilayers without modifying the single-channel conductance value (data not shown).

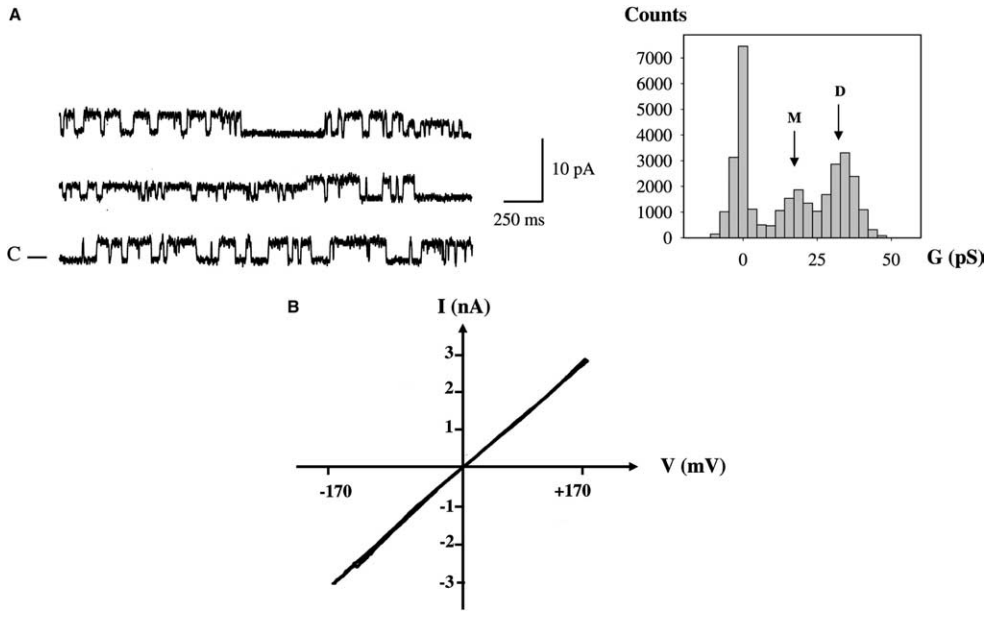

Fig. 3. Ionophore behaviour of *E. aerogenes* OmpX reconstituted in POPC/DOPE membranes. (A) Single-channel activity in 1 M NaCl. Recordings are shown at an applied voltage of +80 mV, gain: 10, filter: 300 Hz and digitization rate: 3000 Hz. The associated amplitude histogram measures two single conductance levels: 22 pS (M for monomer) and 38 pS (D for dimer). (B) Macroscopic current–voltage (*I–V*) curve for OmpX added to either side of the membrane. Voltage ramps (±170 mV at 2 mV/s).

Current–voltage relationship of the OmpX channels was also examined by macroscopic experiments with a 10-fold increase of the protein concentration. Fig. 3B shows that OmpX presents an ohmic response to the voltage applied and no voltage gating, a characteristic of trimeric porins [21]. The ionic selectivity of the channel was determined by installing asymmetrical conditions (salt concentration gradient of 5) and the resulting zero current potential allowed the estimation from the Goldman–Hodgkin–Katz equation [22] of the P_{Na}/P_{Cl} ratio of 9.4 ± 1.2. This demonstrated a markedly cationic selectivity for the OmpX channel.

3.3. Regulation of OmpX expression

By using macroarrays, Barbosa and Levy [23] reported a large number of *E. coli* genes, including *ompX*, differentially regulated by MarA. To highlight an overproduction of OmpX when MarA is overexpressed, we transformed *E. aerogenes* ATCC 13048 with pRC1 encoding *E. aerogenes marA*. A noticeable increase of immunodetected OmpX was observed in the MarA overproducing strain (Fig. 4A).

In *E. coli*, high osmolarity conditions induce a decrease of the OmpF expression concomitantly to the increase of OmpC expression via the *ompB* locus and its two components EnvZ and OmpR [24]. With the characterization of pore-forming properties of OmpX, we studied the effect of osmolarity on the expression of OmpX: *E. aerogenes* ATCC 13048 was grown in NB broth supplemented or not with sorbitol and the production of OmpX was confirmed by immunodetection. OmpX was overproduced in the high osmolarity medium (Fig. 4B), suggesting an osmo-regulation control on the gene.

4. Discussion

OmpX expression is regulated by the environmental osmolarity: when the osmolarity of the medium increases, the

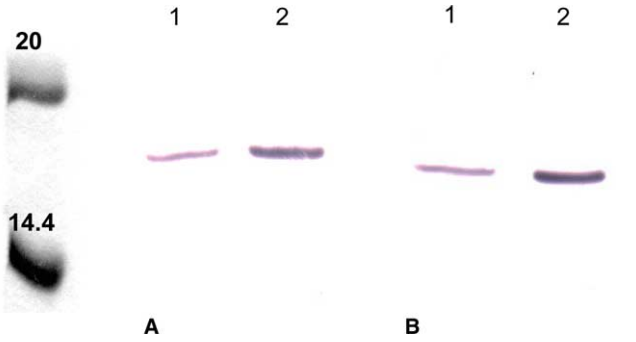

Fig. 4. Western blot analysis of the regulation of OmpX expression by MarA and by osmolarity. SDS–PAGE gel (12%), whole cells sample heated for 10 min at 96 °C. (A) Lane 1, ATCC 13048 and lane 2, ATCC 13048, pRC1. (B) Lane 1, ATCC 13048 in NB broth and lane 2, ATCC 13048 in NB broth with 20% sorbitol.

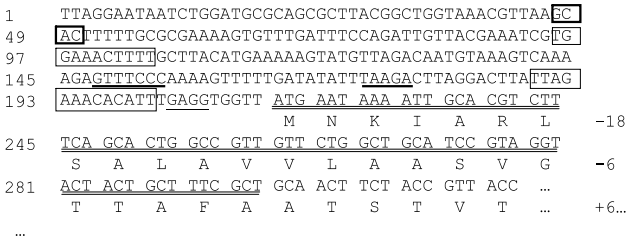

Fig. 5. Promotor region of *ompX*. Putative −35 and −10 regions are underlined in bold. The SD sequence is underlined. The bold box indicates the putative marbox and the simple boxes indicate the putative OmpR binding sites. The DNA sequence of the open reading frame is divided into codons with the corresponding amino acid indicated under each codon. The signal sequence is underlined twice. Numbers on the left refer to the nucleotide sequence and on the right to the amino acid sequence, with +1 as the first amino acid of the mature peptide (for complete sequence see Accession No. AY267336).

protein expression increases. Regarding the three OmpR binding sites described in *ompC* promoter [25], we only identified two sites in the *ompX* promoter region (Fig. 5). The OmpX overproduction results in a strong decrease of *E. aerogenes* Omp36. This could reflect a step by step regulation of outer membrane permeability: (i) at high osmolarity, *E. aerogenes* preferentially synthetizes Omp36 in place of Omp35 which exhibits a noticeable higher conductance, e.g., 1000 and 1430 pS, respectively [26] and (ii) the simultaneous overproduction of OmpX decreases the Omp36 expression. This conjoint double down-regulation induces a restriction of membrane permeability which is responsible for the decrease of β-lactam susceptibility, e.g., cefepime, ceftazidime and cefpirome. Moreover, we clearly show an increase of OmpX synthesis when MarA is overexpressed. Using the consensus sequence, AYnGCACnnWWnnRYYAAAYn [27], we found a putative marbox, weakly degenerated compared to that of *E. coli* OmpX, at −161 pb from the ATG (Fig. 5).

Both *E. coli* OmpX and OmpA were crystallized and their structures resolved to 1.9 and 1.65 Å, respectively [12,28]. Structure determinations were also performed by NMR measurements [13,29]. Comparison of the OmpX channel with the channel of the *E. coli* OmpA N-terminal domain is interesting because they share an eight-stranded antiparallel β-barrel. The β-barrel interior was described as polar with a hydrogen-bonding network including several charged residues, with water filled cavities but no pathway for water or ions between the periplasmic and the external side [12,28,30]. Different studies showed unambiguously the channel activity of the N-terminal domain of OmpA after reconstitution in planar lipid bilayers [19,20]. This discrepancy can be explained by NMR measurements or by molecular dynamics calculations which hypothesised the formation of a central pore involving conformational flips of polar and charged residues located in the mid-section of the barrel [29,31,32]. Based on these data, one may postulate that OmpX forms a pore as suggested by Beckstein et al. [33], and the present study supports this hypothesis. Comparison between OmpX and OmpA β-barrel domain shows different shear number, +8 and +10 for OmpX and OmpA, respectively, which is probably related to a smaller and more ellipsoidal cross-section for OmpX [13,28]. This may explain the smaller conductance obtained. Regarding the selectivity of OmpX, the ratio of permeability P_{Na}/P_{Cl} suggested a preferential cation movement through the channel. This cation-selectivity was higher than the one of general diffusion porins from *E. aerogenes* [18,26].

E. aerogenes OmpX forms a cation-selective channel, its expression is up-regulated by MarA and by the osmolarity. The overexpression of this protein in resistant strains could balance the decreased expression of non-specific porins by favouring the passage of small solutes. Recent studies showed that the expression of OmpX is regulated also by the pH, the global regulatory gene *hns* and during *E. coli* biofilm formation [34,35]. It would be interesting to further investigate the regulation of *ompX* to elucidate the role of this outer membrane protein in *E. aerogenes* adaptability.

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