Immuno-detection of the virulence determinant OmpX at the cell surface of Enterobacter cloacae

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Received 23 September 1997; revised 7 November 1997; accepted 9 November 1997

Abstract

A model for the topology of the Enterobacter cloacae outer membrane protein OmpX has been proposed, based on the primary sequence and on analogy to homologous proteins. According to this model the membrane embedded part of the protein consists of eight antiparallel $\alpha$-strands. Four random coil loops are located at the bacterial surface and three $\alpha$-turns at the periplasmic side of the membrane. Antibodies were raised against synthetic peptides representing five OmpX domains, four of which are putative peripheral and one located in the membrane. The accessibilities of OmpX to these antibodies were tested in intact cells by immuno-gold electron microscopy. This study showed that OmpX is indeed an outer membrane protein, the N-proximal loop of which forms an IgG-accessible epitope at the cell surface.

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Keywords: Outer membrane; OmpX; Topology; Virulence; Immuno-gold labelling

1. Introduction

The Enterobacter cloacae outer membrane protein OmpX (16.5 kDa) is involved in resistance to $\beta$-lactam antibiotics. Overexpression of this protein causes a down regulation of the expression of the major pore proteins OmpF and OmpC, resulting in decreased susceptibility to $\beta$-lactams [1].

Recent studies showed that invasiveness of E. cloacae for rabbit ileal epithelial cells in situ varied with the concentration of OmpX in the outer membrane [2]. This feature provides a functional link between OmpX and a group of virulence related proteins: Ail from Yersinia enterocolitica [3], and PagC [4] and Rck [5] both from Salmonella typhimurium. There is also a highly significant amino acid homology of the membrane embedded domains of these proteins and OmpX [5].

Rules governing folding of bacterial outer membrane proteins [6] predict a characteristic $\alpha$-sheet structure for the topology of OmpX [7]. According to this model the polypeptide chain traverses the outer membrane eight times as amphipathic $\alpha$-strands, thereby creating four loops at the bacterial surface. In this study the accessibility of OmpX to specific antibodies raised against five epitopes of this protein was examined by immuno-gold electron microscopy (immuno-EM) of E. cloacae cells. In particular, we sought evidence for the exposure of the four loops at the bacterial surface.

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Since interactions of antibodies with surface exposed epitopes can be obstructed by O-antigens of lipopolysaccharide (LPS), we also included in our studies experiments with *Escherichia coli* PC2277 which is an O-antigen deficient strain.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pPK03-1 was constructed by subcloning of a 2.6-kb *Eco*RI-*Hind*III fragment from pPK00 containing ompX [8], in pKK223-3 [9].

2.2. Protein alignment

The method described by Ferenci [10] was used to compare the proteins belonging to the OmpX family.

2.3. Synthetic peptides

Peptides representing parts of the OmpX protein were synthesized with 7-fluorenylmethoxycarbonyl chemistry on an automated Abimed AMS 422 multiple peptide synthesizer. Peptides were analyzed by reverse-phase high-performance liquid chromatography (HPLC). Sequences of the synthetic peptides were as follows: peptide A, Q-S-D-M-Q-G-V-M-N-K-T-N; peptide B, K-D-R-T-E-N-G-S-Y-N-K; peptide C, Q-Q-T-E-N-Q-G-L-N-R-T; peptide D, Q-S-R-I-R-N-V-D-V-G-T-W; and, peptide β8, D-V-G-T-W-I-A-G-V-G-Y-R-F.

2.4. Coupling of synthetic peptides to bovine serum albumin

In a single-step coupling method [11], free NH₂ groups were cross-linked using a limiting amount of glutaraldehyde. A solution was prepared containing 5 μmol of synthetic peptide and 0.4 μmol bovine serum albumin (BSA) in 2 ml phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄ (pH 7.4)). An equal volume of 0.2% glutaraldehyde in PBS was added carefully, and the reaction mixture was incubated at room temperature for 1 h. The pH was readjusted to 7.4 with 200 μl 1 M glycine in PBS and incubation was continued for 1 h. BSA-peptide conjugates were dialysed overnight against PBS.

2.5. Immunization of rabbits

New Zealand White rabbits (2.5-3.0 kg body weight) were immunized subcutaneously with 2 ml of a mixture consisting of 1 ml PBS containing 100 μg synthetic BSA-peptide conjugate mixed thoroughly with an equal volume of Freund’s incomplete adjuvant (FIA; Life Technologies, Breda, The Netherlands). Animals were boosted a further 2 to 5 times with 100 μg BSA-peptide conjugate at two-week intervals.

Peptide specificities and titres of antisera were determined by ELISA. The ELISA test was quantified

<table>
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<th>Bacterial strains and plasmids used in this study</th>
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<td><strong>Bacterial strains</strong></td>
<td><strong>Relevant characteristics</strong></td>
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<tr>
<td><em>E. cloacae</em> 2249-1</td>
<td>clinical isolate</td>
</tr>
<tr>
<td><em>E. cloacae</em> JS101</td>
<td>2249-1, ΔompX::aphA; Km'</td>
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<tr>
<td><em>E. coli</em> PC2277</td>
<td>K12, thi Δ(pro-tfrA) lacY galK xyl ara mtl rpsL supE fla; heptose deficient LPS</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pACYC184</td>
<td>Cm', Tc'</td>
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<td>pJS04</td>
<td>pACYC184::ompX; Cm'</td>
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<tr>
<td>pKK223-3</td>
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<tr>
<td>pPK00</td>
<td>pBR322::ompX</td>
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<tr>
<td>pPK03-1</td>
<td>pKK223-3::ompX; Ap'</td>
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*Km', kanamycin resistance; Cm', chloramphenicol resistance; Tc', tetracycline resistance; Ap', ampicillin resistance.

bPhabagen Collection, Utrecht, The Netherlands.
as $\Delta A_{495\text{nm}}$, which is the absorbance in wells coated with 125 mg of BSA-peptide conjugate minus the absorbance in wells coated with 125 mg BSA. Empirically, the criterion chosen for bleeding rabbits was $\Delta A_{495\text{nm}} \geq 2.3$.

2.6. Purification of OmpX-specific antibodies

The IgG fraction was isolated by affinity chromatography on a protein A-Sepharose 4A column (Pharmacia, Uppsala, Sweden), according to the manufacturer’s instructions. They were further purified by adsorption to, and elution from OmpX which had been immobilized on nitrocellulose (Schleicher and Schüll, Den Bosch, The Netherlands) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Titres and specificities were determined by ELISAs and Western blots of outer membrane protein preparations.

2.7. Electron microscopy

Bacteria were taken from frozen stocks and grown overnight at 37°C in Luria Bertani medium (LB). Cultures were diluted (1:10 000) in fresh LB and incubated at 37°C till OD$_{450\text{nm}}$ was 0.4. The suspension was adjusted with PBS to OD$_{450\text{nm}} = 0.1$. One ml of suspension was mixed with an equal volume of fixative (2% v/v glutaraldehyde and 0.2% w/v OsO$_4$ in PBS), and incubated at 37°C for 1 h. Fixed bacteria were applied to a Parlodion film by the agar filtration technique [12]. This film was transferred to EM-grids (copper, 3.05 mm, 400 mesh), which were washed three times for 10 min by floating on PBS. Grids were then floated on drops of PBS containing anti-OmpX IgG (approx. 200 mg l$^{-1}$), supplemented with 1% (w/v) powdered cow milk (PCM; Campina, Eindhoven, The Netherlands) and incubated overnight at 4°C. The grids were washed five times with PBS/PCM and incubated with goat-anti-rabbit IgG pre-conjugated with gold particles (GAR-Au) for 1 h at room temperature. Initially 15 nm colloidal gold particles (EM GAR G15, Amersham) were used and later on 10 nm particles (GAR EM 10 nm, Aurion). The washing procedure was repeated, after which the grids were rinsed with distilled water, dried overnight at 37°C, and examined with a Philips EM201S microscope (80 kV).

3. Results

3.1. Protein alignment

The topological model of OmpX proposed by Stoorvogel et al. [7] is shown in Fig. 1. To corroborate the model the multiple alignment method [10] was applied to the OmpX family of proteins, containing Ail from Yersinia enterocolitica [3], PagC [4], and Rck [5] both from Salmonella typhimurium, Lom...
from lysogenic bacteriophage Lambda [13], and OmpX. The results are shown in Fig. 2. The putative transmembrane regions show high $I_{\text{max}}$ values, whereas in the proposed external loops these values are low. Peptides representing these four putative loops (A, B, C and D) were synthesized and used to raise antisera. In addition, one membrane spanning segment ($\beta_8$) was included.

### 3.2. Synthetic peptides

The amino acid sequences of the synthetic peptides are indicated in Fig. 1. HPLC patterns (not shown) of synthetic peptides A, B, C and $\beta_8$ revealed that they were synthesized in high yields and degrees of purity (> 80%). Peptide D on the other hand, contained multiple side products. In a second effort to synthesize this peptide the results were improved, but still not quite satisfactory (> 50%). Nevertheless, this preparation was also used for immunization.

### 3.3. Immunization and purification of anti-OmpX IgG

For each peptide two rabbits were immunized. From sera with the highest $\Delta A_{495\text{nm}}$ in the ELISA test, ($A_1$, $B_2$, $C_2$, $D_2$, and $\beta_8$) the IgG fraction was isolated and analyzed by Western blotting (see Fig. 3). Panel I shows the results for anti- $A_1$ IgG. Although intense OmpX-specific bands (16.5 kDa) were observed in lanes 2 and 3, cross-reactions with proteins of 40 kDa were present in all lanes. After sequential adsorption of anti- $A_1$ IgG to, and elution from filter-immobilized OmpX, mono-specific antibodies were obtained (Fig. 3, panel II). This procedure was applied also to antisera $B_2$, $C_2$, $D_2$, and $\beta_8$ with similar results (not shown).

### 3.4. Immuno-gold electron microscopy

Bacteria were labelled with hyper-immune IgGs and processed with GAR-Au for immuno-EM. The results obtained for epitope A are shown in Fig. 4. Gold particles could not be detected at the cell surface of E. cloacae JS101 (pACYC184), a OmpX-minus strain (panel I). On the other hand E. cloacae 2249-1 (pJS04), an OmpX-overproducing strain, carried about 150 gold particles per cell (panel II). No differences were found using either 15 nm or 10 nm gold particles.

Antibodies recognizing epitopes B, C, D, and $\beta_8$ in Western blots, did not confer the ability to bind...
GAR-Au particles. To eliminate any steric hindrance caused by O-antigens, the experiment was repeated with *E. coli* PC2277, which lacks the side chain moiety of LPS. Again, cells carrying the vector plasmids (pACYC184; pKK223-3) did not bind gold particles (not shown). *E. coli* strains PC2277 (pJS04) and PC2277 (pPK03-1) which produce various amounts of OmpX, adsorbed GAR-Au after reaction with anti-A$_1$-IgG (panels III and IV, respectively). The amounts of gold particles correlated with the concentrations of OmpX in their outer membranes as determined by SDS-PAGE (not shown). The average number of gold particles bound to *E. coli* PC2277 (pJS04) was about 300 per cell, which was twice as high as that bound to *E. cloacae* 2249-1 (pJS04). Despite LPS mutations, sera raised to peptides representing loops B, C, D, and β8 did not react with O-antigen deficient, OmpX-producing *E. coli* PC2277.

4. Discussion

All hyper-immune sera reacted well with BSA-peptide conjugates in ELISA tests. In Western blots two bands were observed, an OmpX-specific one at 16.5 kDa, indicating that the antibodies recognized their respective linear epitopes in denatured OmpX, and another at 40 kDa. This latter band, seen with all sera tested, is probably due to antibodies raised against the BSA carrier protein. It was eliminated by absorption to, and elution from filter-immobilized OmpX.

Immuno-gold labelling of bacteria showed that epitope A is accessible to IgG and thus is located at the bacterial surface. Furthermore, more gold particles were sequestered by *E. coli* cells lacking the O-antigen moiety of LPS, than by *E. cloacae* cells with a similar OmpX content. This demonstrates that the polysaccharide chains of LPS, protruding from *E. cloacae* cells interfere to some extent with the binding of antibodies to epitope A. The amounts of gold particles bound by bacteria with different expression vectors correlated well with the apparent level of OmpX produced by these cells. No differences were observed when either 15 nm or 10 nm gold particles were used in the assays. The gold particles seem to be located at some distance from the bacterial surface, a phenomenon inherent to this method [14].

Antibodies specific for epitopes B, C, D, and β8 did not result in immuno-gold labelling. For purely practical reasons peptide D was synthesized as a dodecamer, while the putative external loop is an octamer. Consequently, parts of epitope D are buried in the membrane, and this may have inhibited binding of anti-D-specific IgG. Peptide β8 contains the C-terminal X-Y-X-F motif, which is highly conserved among outer membrane proteins and is required for proper insertion of these proteins in the outer membrane [6]. Antibodies specific to the β8 domain did not give rise to immuno-gold labelling, which is conform the topological model.

Epitopes B and C did not react with their specific IgGs. This could imply that the model is not fully accurate with respect to these loops, but other explanations are feasible. It could be that these peptides are part of a conformational epitope and thus do not react with antibodies raised against linear peptides. An alternative explanation is that these loops are folded into the interior of the protein in a manner analogous to the ‘eyelet’ structures found in the *Rhodobacter capsulatus* pore [15] and in PhoE [16] from *E. coli*. The exposure of epitope A at the bacterial surface may be significant for the virulence properties of OmpX [2].

Acknowledgments

We are indebted to Dr. John Stephen (School of Biological Sciences, The University of Birmingham, UK) for critical reading and correcting the English text, and to the staff of the test animal facility of the Leiden University Hospital (Centraal Proefdier Verblijf AZL), especially Jan van Elk, for housing and handling the rabbits used in our experiments. We thank Jan Wouter Drijfhout (Department of Immuno-Haematology, Leiden University) for his assistance with the selection and synthesis of the peptides. We are obliged to Evelien Pas and Professor Nanninga (Department of Electron Microscopy and Molecular Cytology, University of Amsterdam), and Piet Cambier and Lambert Verschragen (Department of Electron Microscopy, Leiden University) for their invaluable advice.

This work was financially supported by the Netherlands Organization for the Advancement of Pure
References


