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Mutant OmpF porins of *Yersinia pseudotuberculosis* with deletions of external loops: Structure–functional and immunochemical properties

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ABSTRACT

Recombinant mutant OmpF porins from *Yersinia pseudotuberculosis* outer membrane were obtained using site-directed mutagenesis. Here we used four OmpF mutants where single extracellular loops **L1**, **L4, L6,** and **L8** were deleted one at a time. The proteins were expressed in *Escherichia coli* at levels comparable to full-sized recombinant OmpF porin and isolated from the inclusion bodies. Purified trimers of the mutant porins were obtained after dialysis and consequent ion-exchange chromatography. Changes in molecular and spatial structure of the mutants obtained were studied using SDS–PAGE and optical spectroscopy (circular dichroism and intrinsic protein fluorescence). Secondary and tertiary structure of the mutant proteins was found to have some features in comparison with that of the full-sized recombinant OmpF. As shown by bilayer lipid membrane technique, the pore-forming activity of purified mutant porins was identical to OmpF porin isolated from the bacterial outer membrane. Lacking of the external loops mentioned above influenced significantly upon the antigenic structure of the porin as demonstrated using ELISA.

1. Introduction

Pore-forming proteins (porins) of Gram-negative bacteria outer membrane (OM) belong to the β-structured integral membrane proteins that form hydrophilic pores for passive diffusion of low molecular weight compounds [1]. In the native membrane porins exist as homotrimers which monomeric units formed by antiparallel β-strand cylinder surrounding water-filled channels. Regions of porin polypeptide chain connecting β-strands, so-called loops, are rather short if located on the periplasm side and are much longer if located on the outer side of the membrane [2 l]. The outer loops form more complicated spatial structures than periplasmic ones. The regions corresponding to the outer loops of porins are shown to coincide with hydrophilic maxima and to form antigenic determinants [1]. In addition, according to some data the conformation changes in the regions of the loops L3, L5, L7, and L8 have significant impact on the efficiency of the pore diffusion [2]. Loop L2 plays an important role in stabilizing of the trimer structure of porin in the native OM [1]. However, experimental data on the relationship between the fine protein structure and functional and biological properties of porins are only known for a limited number of pore-forming proteins [3]. In this regard, the definition of the role of external loops of *Yersinia pseudotuberculosis* OmpF porin in protein structure and function can expand our knowledge about participation of the individual loops in the relationship between the pseudotuberculosis microbe and a host. The purpose of this study is to reveal an effect of the external loop deletions on the spatial and antigenic structure and pore-forming activity of OmpF porin from OM of *Y. pseudotuberculosis*.

2. Materials and methods

2.1. Enzymes

DNA polymerase Pfu Ultra II (Stratagene, USA); GoTaq-DNA polymerase (Promega, USA), restriction endonucleases NdeI, BamHI and DpnI (Fermentas, Lithuania).

2.2. Primers

Mutagenic primers were synthesized by "Evrogen", Russia.

2.3. Site-directed mutagenesis

To obtain mutant proteins with deletions of loops **L1**, **L4, L6,** and **L8** site-directed mutagenesis of the recombinant plasmid

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pET41a (+)-m55 carrying *ompF* gene was performed as described in [4]. Then expression constructions were designed. Target mutant proteins were expressed in Rosetta cells of *Escherichia coli* in amount comparable to the mature (hereinafter-full-sized) recombinant OmpF porin [4].

2.4. Expression and isolation of the porins

Expression, isolation and purification of recombinant (full-sized and mutant) OmpF porins were described in [4,5].

2.5. Change in molecular structure of the porins under temperature

Porin samples were aliquoted in amount of 0.5 μM, incubated for 15 min at corresponding temperature and analyzed by SDS–PAGE.

2.6. Methods of optical spectroscopy

All absorbtion, CD and fluorescence measurements were performed on the porin samples in 30 mM Tris–HCl buffer, pH 7.8, 0.1% SDS at 25 °C.

2.6.1. UV spectra

UV spectra were recorded with a Cecil CE 7200 spectrophotometer (England) in quartz cuvettes with a 1-cm layer. The correction for light scattering of the protein solution was carried out as described in [6]. Specific absorbtion factor $A^{0.1}/_{1\ cm}$ of porin was taken equal to 1.00.

2.6.2. Circular dichroism

CD spectra were recorded in a Jasco-500A spectropolarimeter (Japan) in quartz cuvettes with 0.1- and 1-cm layers for the far UV (180–250 nm) and near UV (250–350 nm) regions, respectively. In the peptide region, ellipticity (θ) was calculated as the mean residue ellipticity taking the molecular weight of amino acid residue as 110 Da, in the units of deg cm^2/dmole by the formula:

$$[\theta] = [\theta]_{obs} \cdot S \cdot 110/10 \cdot C \cdot l,$$

where S is sensitivity; C is the protein concentration, mg/ml; and l is the optical path length, mm.

In the aromatic spectral region, ellipticity $[\theta]_M$ was calculated as molar ellipticity taking the molecular weights of porin monomer and trimer as 40 and 120 kDa, respectively. The spectropolarimeter was calibrated using 0.06% aqueous solution of 10-sulfonate-*D*-camphoric acid ammonium salt. Ellipticity ratio of the bands at 191 and 290 nm was 2.05. The content of the secondary structure elements of the proteins were calculated with CD Pro [7].

Monitoring of changes in the second structure of the recombinant (full-sized and mutant) porins at θ_{210} was performed under samples heating in interval of 30–90 °C with step of 5 °C. Protein samples were incubated for 15 min at each temperature.

A minimum of four consecutive scans were accumulated, and the average spectra stored.

2.6.3. Intrinsic protein fluorescence

Fluorescence spectra of the porins were measured on Hitachi 850 spectrofluorimeter (Japan) with a 1 cm pathlength cell. Samples were prepared in 30 mM Tris–HCl, pH 7.8, 0.1% SDS and the protein concentration was 0.5 μM. Fluorescence was excited by the light with wavelength of 280 and 296 nm. The excitation and emission slits were set at 5 nm. Fluorescence spectra corrected by rhodamide B (Wako Pure Chemical Industries, Japan) were recorded by subtracking the Raman band of the buffer solution. The fluorescence spectra were intensity-normalized at the emission maximum. Decomposition of the tryptophan emission spectra into the components [8] was carried out using an optimization software based on the Marquardt method [9].

2.7. Pore-forming activity assay

The method of bilayer lipid membrane (BLM) formation, instrument description, and the methods for measuring the electric parameters of BLM are presented in [10].

The experiments on the recombinant (full-sized and mutant) OmpF porins reconstitution into BLM were performed at room temperature. The aqueous phase contained 0.1 M NaCl, 30 mM Tris–HCl buffer, pH 7.8 and the porins at concentrations of 10–20 ng/ml. The aliquots of the porin stock solutions in 30 mM Tris–HCl buffer, pH 7.8, 0.1% SDS were added to the aqueous phase before BLM formation. Thus, porin molecules were present on both side of BLM. The fluctuations of BLM current in the presence of porin were measured in the mode of membrane potential fixation and recorded at a membrane potential of 20–50 mV. Measuring path possible to record the current fluctuations was up to 10^{-13} A.

2.8. Immunological methods

All experiments involving animals were carried out in accordance with EU Directive 2010/63/EU.

2.8.1. Animal immunization

Mice of the BALB/c line were kept under standard conditions with free access to food and water. The animals at the age of 4–8 weeks (20 ± 2 g) were devided into six groups (five mice in each) and immunized with the recombinant (full-sized and mutant) porins. The protein samples were dissolved in a 0.9% NaCl in a concentration of 1 mg/ml and intraperitoneally administred to the animals at a dose of 100 μg per mouse three times at an interval of 7 days. Blood sera were obtained in a month after 3rd injection.

2.8.2. ELISA

To study blood sera indirect ELISA [11] was carried out using Costar microplates (USA). Antibodies binding to the antigens were detected using anti-mouse IgG conjugated with HRP at 1:2000 dilutions (Invitrogen, USA). The results were measured on μQuant, Bio-Tek Instruments. Inc. (USA) spectrophotometer at 492 nm, 0.04% solution of ortho-phenylenediamine was used as chromogen.

2.9. Statistical analysis

The results were statistically processed on Windows XP using the Microsoft Excel software. The confidence of the results was confirmed by the determination of the standard deviation (σ) and standard error (by the equation for limited sample).The confidence of the differences of the mean values was evaluated using Student's criterion ($p < 0.05$).

3. Results and discussion

3.1. Site-directed mutagenesis of ompF gene and expression of the recombinant porins

Site-directed mutagenesis and expression of recombinant (full-sized and mutant) OmpF porins were performed according to the procedure described in [4].

3.2. Isolation and refolding of the recombinant porins

Isolation and refolding of mutant porin monomers with deletions of the loops **L1**, **L4, L6,** and **L8** (hereinafter - **del1**, **del4**, **del6,** and **del8**) was performed according to the procedure developed earlier for the full-sized recombinant porin monomer, **RP-1** [5], except that in the case of mutant proteins isolation we used double amount of lysozyme and DNAase. Trimers of the full-sized (**RP-2)** and mutant (**del1**, **del4**, **del6,** and **del8**) porins were obtained using exhaustive dialysis and consequent ion-exchange chromatography as described in [4,5].

3.3. Molecular and spatial structure of the mutant porins

3.3.1. Change in molecular structure of the recombinant porins under temperature

As we can see in Fig. 1, the mutant porin **del4** had the highest thermal stablility (Fig. 1D) in comparison with the rest mutant porins. Dissociation of **del4** occurred in the same temperature interval as dissociation of **RP-2** (75–90 °C) (Fig. 1E). Samples **del1** и **del6** were less resistant to the action of temperature: porin monomers were observed on electrophoregrams at 60–70 °C and 70–80 °C, respectively (Fig. 1A and B). Trimer dissociation of **del 8** began at 70 °C and finished at 90 °C, but in this case polypeptide bands in oligomeric zone were yet observed at 80 °C (Fig. 1C). Thus, any outer loop lacking induced some differences in thermostability of porin molecule.

3.4. CD analysis

To reveal an influence of the loop deletions upon changes of the secondary as well as the higher order of the porin structure, CD spectra were recorded in the far and near UV, respectively. The near UV CD spectrum is determined by the three-dimensional arrangement and flexibility of aromatic amino acids, mainly tyrosine and tryptophan, and thus is sensitive to alterations of the spatial structure of the protein. CD spectra of the mutant porins in the aromatic region (Fig. 2B) have a lower amplitude (**del4, del6**, and **del8**) and a lower resolution (**del1**, **del6**, and **del8**) of the bands in comparison with that of the **RP-2** indicating looser tertiary structure of the mutant proteins. The CD spectra of the mutant porins in the region of peptide bonds absorption (Fig. 2A) were typical for β-structured protein of (α + β) type [12]. However, the proteins with deletions of various loops differ in the shape and the intensity of negative and positive bands of the spectra.

Using CDPro software [7] the content of secondary structure elements of the mutant porins was determined. The most significant increasing in content of α-helix has been revealed in the case of **del1** and **del6** (Table 1**).** As we can see in the Table 1, the mutant porins with deletions of the loops **L1** and **L6** contained more α-helix regions (1.8 and 2.7 times, respectively) in comparison with **RP-2**. Porin mutant with the loop **L6** deletion, despite the high content of α-helix (the characteristic feature of denaturated porins) contained the least amount of disordered structure. Simultaneously, this pattern as soon as **del4** had the similar to **RP-2** ratio of the two types of β-structure (β-disordered/β-ordered): 0.5 and 0.55, respectively. For other mutant proteins (**del1** and **del8**) these relations were of great value equal to 0.66 and 0.70, respectively. Thus, any outer loop lacking induced changes in the content and ratio of elements of secondary structure of the porins.

Nevertheless, as shown by CD monitoring at 222 nm the changes of the secondary structure of the mutant porins under heating in the interval of 30–90 °C occurred by similar way. The midpoint of all sigmoid plots obtained corresponded to 60 °C similar to that of the full-sized protein (data not shown).

3.5. Intrinsic protein fluorescence

Changes in the tertiary structure of the mutant porins in comparison with **RP-2** were observed using intrinsic protein fluorescence. The total emission spectra of the full-sized and mutant proteins and approximation of tryptophan fluorescence spectra by spectral emission forms of tryptophan residues [8] allowed us to estimate changes in the microenvironment of aromatic fluorophores by deleting some external loops (Table 2). Lacking of the loops **del**, **del4**, and **del6** strongly affected the parameters of the spectra of both total and tryptophan emission (data not shown).

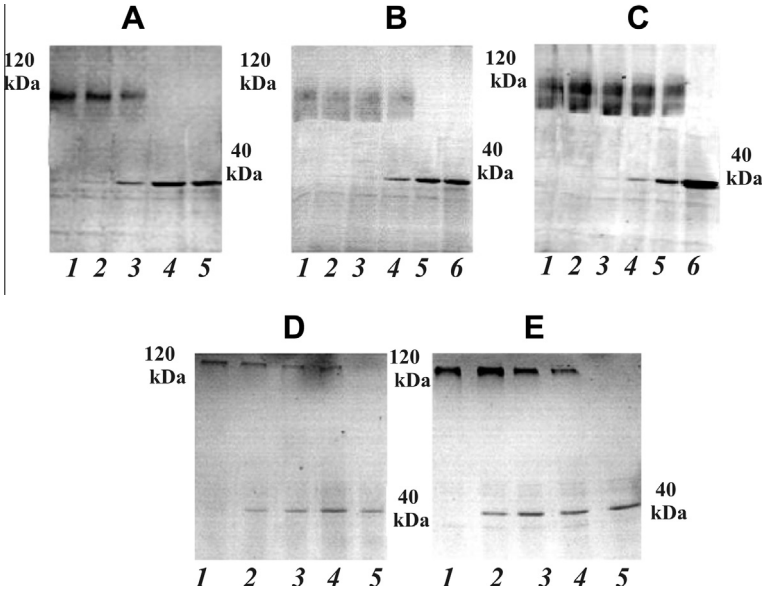

Fig. 1. Electrophoregrams of temperature denaturation of mutant porins with deletions of external loops **L1** (A), **L6** (B), **L8** (C), **L4** (D) and of full-sized recombinant OmpF porin, **RP-2**, (E) from *Y. pseudotuberculosis*. (A) 1 – original **del 1;** 2, 3, 4, 5 – samples heated at 50, 60, 70, and 90 °C, respectively; (B) 1 – original **del 6;** 2, 3, 4, 5, 6 – samples heated at 50, 60, 70, 80, and 90 °C, respectively; (C) 1 – original **del 8;** 2, 3, 4, 5, 6 – samples heated at 50, 60, 70, 80, and 90 °C, respectively; (D) 1 – original **del 4;** 2, 3, 4, 5 – samples heated at 70, 75, 85, and 90 °C, respectively; (E) 1 – original **RP-2;** 2, 3, 4, 5 – samples heated at 70, 75, 85, and 90 °C, respectively.

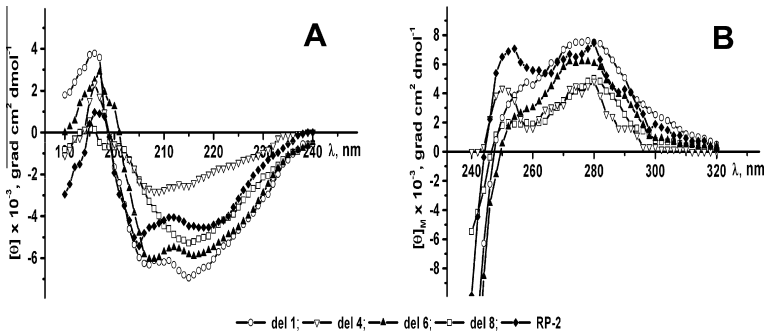

Fig. 2. CD spectra of recombinant, full-sized (**RP-2**) and mutant (**del1, del4, del6,** and **del8**) OmpF porins from *Y. pseudotuberculosis* in the peptide (A) and aromatic (B) region. The porin samples (1.0 μM and 6.0 μM for peptide and aromatic regions, respectively) were dissolved in 30 mM Tris–HCl buffer, pH 7.8, 0.1% SDS. CD spectra were recorded at 25 °C.

Table 1
Content of the secondary structure elements of the mutant and full-sized recombinant porins* from *Y. pseudotuberculosis*.

Sample	α-Helix		β-Structure		β-Turn	Irregular structure
	A**	B**	A**	B**		
Del1	0.056	0.052	0.174	0.115	0.232	0.371
Del4	0.003	0.034	0.232	0.131	0.222	0.351
Del6	0.064	0.095	0.212	0.104	0.206	0.319
Del8	0.024	0.042	0.156	0.109	0.227	0.441
RP-2	0.015	0.044	0.218	0.121	0.231	0.370

* Calculated according CONTIN/CONTINLL (CDPro package) [7].
** Ordered (A) and disordered (B) protein secondary structure.

Table 2
Contribution of tryptophan spectral forms (classes) in fluorescence spectra (excited at 296 nm) of the mutant and full-sized recombinant porins from *Y. pseudotuberculosis*.

Sample	S*	I	II	III
del1	0.000	0.491	0.405	0.104
del4	0.169	0.000	0.000	0.831
del6	0.005	0.616	0.000	0.379
del8	0.102	0.258	0.285	0.355
RP-2	0.099	0.201	0.269	0.431

* λ_{max} of S-form 315–317 nm; λ_{max} of form I 330–332 nm; λ_{max} of form II 340–343 nm, λ_{max} of form III 350–353 nm. Resolution of experimental spectra of the protein studied into the components corresponding to spectral classes of tryptophan residues was performed using an optimization program based on the Marquardt method [9].

Protein sample **del4** had a longest wavelength maximum and the smallest amplitude of tryptophan spectrum.

This can be the result of the highest content of spectral form III in **del4.** On the contrary, in the case of **del1** and **del6** the significant portion of aromatic fluorophores located in a hydrophobic environment: tryptophan residues of spectral form I increased (2.4 and 3.1 times, respectively) in comparison with **RP-2.** For **del1** an increase of the contribution of tryptophan residues of spectral form II was also observed. Thus, deletion of the outer loops studied except **L8** induce the significant changes in microenvironment of aromatic fluorophores in the porin molecule.

3.6. Pore-forming activity of the mutant porins

Functional activity of the mutant porins in comparison with **RP-2** was characterized by BLM technique. After adding the porin sample (10–20 ng) into the aqueous phase stepwise membrane conductance changes typical for the pore-forming proteins were observed. Analysis of the current records through the BLM in the presence of the proteins studied showed that the most probable conductance levels of the **RP-2** and mutant porins had similar values about 300 ± 60 pS. This value corresponded to the conductivity of trimer channels of isolated OmpF porins of *Yersinia* genus [13] and to calculated conductivity of *Y. pseudotuberculosis* OmpF porin channels [14]. In addition, the mutations did not significantly affect upon voltage dependence of OmpF channel and closure potential. Thus, lacking of the loops **L1**, **L4**, **L6**, and **L8** had no effect on pore-forming properties of OmpF protein from outer membrane of *Y. pseudotuberculosis*. The results obtained were consistent with the data published earlier for OmpF porin mutants from *E. coli* [15]. Deletions of the external loops of *E. coli* OmpF were shown not to influence an efficiency of the mutant proteins reconstitution into BLM and voltage dependence of the channel [15].

3.7. Immunochemical characteristics of the mutant porins

Specific antisera to recombinant porins studied were used for comparative immunochemical characteristics of the proteins. Triple immunization with **RP-2** and the mutant proteins in amount of 100 μg per mouse let us obtain the immune sera with titer in the range of 3.5–4.7 (-lg). In order to find out whether the regions corresponding to the external loops of the porin studied include B- and T-epitopes, we used the obtained specific antisera.

ELISA results of interaction of the recombinant OmpF porins (the full-sized and mutant proteins) with antibodies against **RP-2** have shown that the lacking of the loop regions impact differently on the effectiveness of the interaction of antigens and antibodies (Fig. 3).

As can be seen in Fig. 3, the fullness of interaction between antibodies to **RP-2** and the mutant porins was about of 56–73%

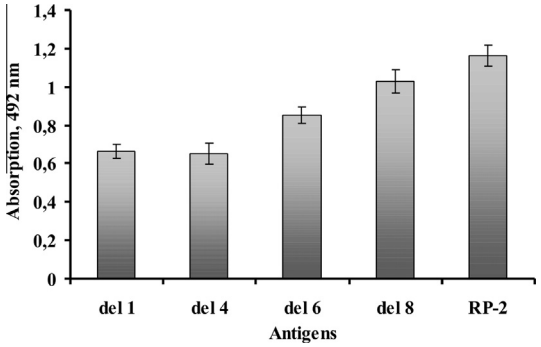

Fig. 3. ELISA results of interaction of the recombinant (the full-sized and mutant) OmpF porins from *Y. pseudotuberculosis* with mice specific antibodies against **RP-2** (antiserum dilution 1:800). Data of this figure were means of three replicates ±SD. Significance of differences $p < 0.05$.

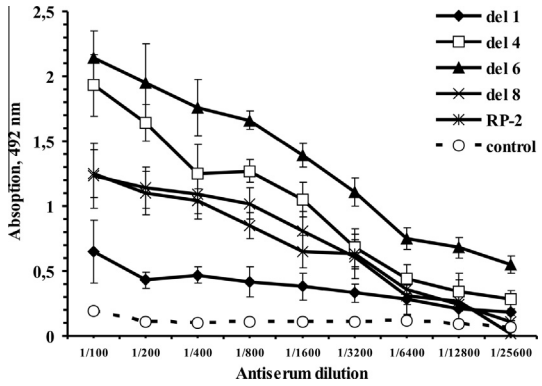

Fig. 4. ELISA results of interaction of mice specific antibodies against the recombinant (the full-sized and mutant) OmpF porins from *Y. pseudotuberculosis* with homologous antigens. Data of this figure were means of three replicates ±SD. Significance of differences $p < 0.05$.

compared to that of **RP-2** and homological antiserum. Based on these results, it can be assumed that the outer loop **L1**, **L4**, **L6**, and **L8** are involved in the formation of B-cell antigenic epitopes in various degree. The greatest number of B-epitopes was lost following the deletion of loops **L1** and **L4**. This is consistent with results of inhibition of OmpF porin interaction with the homologous antiserum by synthetic peptides[1] corresponding to sequences of external loops. Peptides coincided to **L1** and **L4**. were found to exhibit maximal inhibitory activity (data not shown). At the same time the lack of **L8** had a minimal effect on the efficiency of interaction of **RP-2** with specific antibodies. Thus, this loop apparently not involved in the formation of B-cell epitopes of porin (Fig. 3).

Analysis of ELISA results shown in Fig. 4 allowed to suggest that lacking of any of the loops had a various impact on the immunogenicity of the porin. So, completeness of interaction between **del8** and homological antiserum was comparable to that of **RP-2**. Obviously, lacking of **L8** did not affect on immunogenic activity of OmpF porin. So, the lack of the loop did not affect the completeness of interaction between **del8** and homological antiserum and was comparable to that of **RP-2**. However, the immunogenicity of the sample **del1** was considerably lower. As known, antibody production is induced by interaction of specific regions in the primary protein structure with the T-cell receptors of lymphocytes (T-epitopes). Thus, **L8** region includes the least amount of T-epitopes, whereas **L1** participates in the formation of T-epitopes of OmpF porin.

Interestingly, immunization of mice by mutant porins **del4** and **del6** induced the highest level of specific antibodies production. Apparently, this fact due to the lack of loops **L4** and **L6** makes some "internal" T-epitopes of the protein (perhaps, localized in the β-strand regions) more accessible to the antigen presenting cells. This assumption is based on theoretical calculations of the localization of antigenic determinants we performed using computer programs [16–18]. According to the results obtained, significant portion of accounted antigenic determinants regions corresponded to β-strands.

Thus, mutations in recombinant porins, leading to significant changes in the spatial structure of the protein molecule has a remarkable effect on the immunochemical characteristics of the OmpF porin. Analysis of the data obtained indicates the least impact of the loop **L8** deletion in changes of both spatial structure and immunobiological properties of OmpF porin.

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