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# Molecular Characteristics of OmpF-Like Porins from Pathogenic *Yersinia*

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**Abstract**—Nonspecific pore-forming proteins (porins) are the major proteins of the outer membrane of Gram-negative bacteria responsible for diffusion of low-molecular-weight compounds. Nucleotide sequences of the OmpF-like porins from the pathogenic bacteria *Yersinia pseudotuberculosis* (YPS) and *Yersinia enterocolitica* (YE) were cloned and determined. Values of molecular weights (MW) and isoelectric points (IEP) calculated for these proteins (for OmpF-YPS: MW 37.7 kD, IEP 4.45; for OmpF-YE: MW 39.5 kD, IEP 4.34) are in good agreement with experimental data. The OmpF-like *Yersinia* porins are highly homologous to each other (83-92%) and also to the OmpF protein from *Serratia marcescens* (70%); the homology to the OmpF porin from *E. coli* is significantly lower (52-58%). Multiple alignment of the amino acid sequences of mature OmpF proteins provided the distribution of conservative amino acid residues typical for porins. Moreover, the OmpF-like porins from *Yersinia* are characterized by the presence of extended regions with high and low homologies, which coincide with the transmembrane domains and "external" loops, respectively, of the topological model of the OmpF porin from *E. coli*. By predictive methods, the secondary structure of the OmpF-like porins from *Yersinia* was obtained. This structure is represented by 16 β-strands connected by short "periplasmic" and longer "external" loops with unordered structure.

*Key words*: porin, OmpF, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*

Permeability of the outer membrane (OM) of bacteria is provided by certain proteins that form channels with varied specificity. The so-called nonspecific porins, such as OmpF, OmpC, and PhoE proteins, are prevalent among pore-forming proteins of the bacterial membrane. Unlike other channel formers responsible for a specific function (e.g., passage of maltose-like oligosaccharides or nucleosides), the above-mentioned porins form water-filled pores poorly selective to cations; they provide the diffusion of low-molecular-weight compounds (not over 600 daltons). Porins belong to the class of β-structured proteins, and they mainly exist as homotrimers, both in the OM of microorganisms or isolated [1]. According to X-ray crystallography data on the OmpF protein from *E. coli* [2], the basic structural element of the porin monomer is an ellipsoidal in section cylinder consisting of 16 transmembrane β-strands (the so-called β-barrel) connected by short periplasmic and the longer "external" loops. The OmpF and OmpC porins are known to play a tremendous role in adaptation of bacteria to the environmental conditions, such as temperature, osmotic pressure, and pH of the medium [3]. Thus, reciprocal regulation of the biosynthesis of OmpF/OmpC proteins increases the expression level of the OmpF porin at low temperature, decreased osmotic pressure, and low content of nutrients in the medium. The OmpC protein is expressed under conditions of increased content of nutrients, increased temperature, and high osmotic pressure. It should be also noted that porins greatly contribute to development of the resistance of bacteria to antibiotics by decreasing the level of their expression and/or the number of open pores [4, 5].

The *Yersinia* genus includes 11 species, among which three are pathogenic for humans (*Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*); they are the causative agents of plague, pseudotuberculosis, and intestinal yersiniosis, respectively. As a rule, pathogenic bacteria have a variety of features, so-called pathogenicity factors, which promote their penetration into and survival inside the host's body. These factors include nonspecific porins, which are involved in adhesion, invasion, and intracellular parasitism of microorganisms. Nonspecific porins from *Yersinia* have been shown to be β-structured proteins resistant to high temperature, proteases, and detergents [6]. Immunogenic properties of porins suggest an important role of these proteins in development of infection and

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immune response of the host [7]. A test-system elaborated on the base of porin from *Y. pseudotuberculosis* is successfully used for diagnostics of pseudotuberculosis and in differentiating outbreaks of intestinal infections [8].

*Yersinia* are psychrophilic bacteria that can survive over a wide temperature range, from 4 to 45°C. We think that such wide adaptive abilities of these microorganisms are caused by differences in functional properties of the pore-forming proteins that are synthesized when these bacteria are exposed to varied temperatures. Thus, porins from "cold" (cultured at 4-6°C) and "warm" (cultured at 37°C) *Yersinia* are different in the pore size, and the channels formed by proteins from different *Yersinia* species have different electrophysiological characteristics that are obvious when the porins are reconstructed into model membranes [9]. Thus, nonspecific porins from *Yersinia* are a promising object for establishing correlations between the fine molecular structure and biological properties of the proteins and/or the microorganism as a whole. No doubt, the difference in virulence of pathogenic and nonpathogenic *Yersinia* species, as well as specific features of their inhabitancy, is associated with differences in properties of the pore-forming proteins of these microorganisms.

This work presents results of determination of nucleotide sequences of cloned genes, which encode the synthesis of OmpF-like porins in the bacteria *Y. pseudotuberculosis* and *Y. enterocolitica*. Based on these data, the amino acid sequences of these proteins were compared to each other and to those of similar proteins of other enterobacteria. Some physicochemical parameters and the secondary structure of the porins under study were determined by predictive methods.

## MATERIALS AND METHODS

Encoding sequences of the *OmpF*-like porin genes were obtained by polymerase chain reaction (PCR) of chromosomal DNA isolated from *Y. pseudotuberculosis* (serovar 1B, strain KS 3058) and *Y. enterocolitica* (serovar 0:3, strain 164) using the direct and inverse primers: 5′-CAGTAGTAATCCCAGCT-3′ and 5′-CTTAGAACTGATAAACCAAG-3′, respectively. PCR was performed in 60 mM Tris-HCl buffer (pH 8.5) containing 1.5 mM $MgCl_2$, 25 mM KCl, 10 mM 2-mercaptoethanol, 0.1% Triton X-100, 200 μM dNTP, the direct and inverse primers (50 pM each), and TaqSE-DNA polymerase (Sibenzim, Russia) (2.5 units). PCR was performed as follows: 1) predenaturation of chromosomal DNA at 95°C for 3 min; 2) 30 cycles including denaturation at 94°C for 20 sec, annealing at 55°C for 30 sec, the polymerase reaction at 72°C for 1 min 10 sec; 3) completion of the PCR fragments at 72°C for 5 min. Results of the reaction were analyzed by electrophoresis in 1% agarose gel. The PCR fragments were cloned into pBluescript SK(−). The *E. coli* strain DH5a was transformed with recombinant plasmids (pBS5 for *OmpF* from *Y. pseudotuberculosis* and pBS2 for *OmpF* from *Y. enterocolitica*). The nucleotide sequences of the recombinant plasmid insertions were determined by the Sanger's method with an automated DNA sequencer (ABI 310, Applied Biosystems, USA) using the direct and inverse M13 primers (Sibenzim) and an ABI Prism BigDye Terminator sequencing set (Applied Biosystems).

The type-specific primers were designed using PRIMER PREMIER5 and GENERUNNER programs. The nucleotide sequences were analyzed and their structures were tested using CHROMAS and GENERUNNER programs. Homologous sequences were searched for and analyzed using the BLAST program on the following servers: NCBI (USA), KEGG (Japan), and EMBL (Germany). The multiple alignment of the sequences was performed with the ClustalW algorithm on the EMBL server. The protein hydrophobicity was analyzed on the server of the Weizman Institute of Science (Israel) using the Kyte–Doolittle algorithms (at window length of 17). The secondary structure and transmembrane topology were predicted using the following programs: GOR (Southampton Bioinformatics Data Server), Tmpred (ISREC, Switzerland), and PredictProtein (USA).

## RESULTS AND DISCUSSION

**Designing type-specific *OmpF*-primers.** To create type-specific primers for amplification of nucleotide sequences of the *OmpF*-like porin genes, we analyzed signal peptides of the known OmpF-like proteins from enterobacteria (*E. coli*, *Serratia marcescens*, *Salmonella typhimurium*, *Salmonella typhi*, *Xenorhabdus nematophilus*). Moreover, porin genes were also searched for in the genome of *Y. pestis* [10] using the software supported on the server http://www.sanger.ac.uk/Projects/ Y_pestis/ blast_server.shtml (the Sanger Institute, Great Britain). The nucleotide sequence of the *OmpF* gene of *S. marcescens* (NCBI, U81967) was probed because this bacterium is the phylogenetically closest to the *Yersinia* genus [11]. As a result, the gene sequence (YO1411) was found which was named by the authors as *putative OmpC porin*, and this name suggested that the sequence was not of the definite type of nonspecific porins. The further analysis of the promotor region of the YO1411 gene revealed motifs specific for promotors of the *OmpF*-like genes of enterobacteria, i.e., the definite location of the OmpR-binding sites and the presence of a sequence complementary to 4.5S *micF* RNAs. Based on these data, the YO1411 gene was identified as an *OmpF*-like gene and used for designing the *OmpF*-primers of *Yersinia*. The resulting amino acid sequence of the putative OmpC porin was included into the list of the OmpF porins.

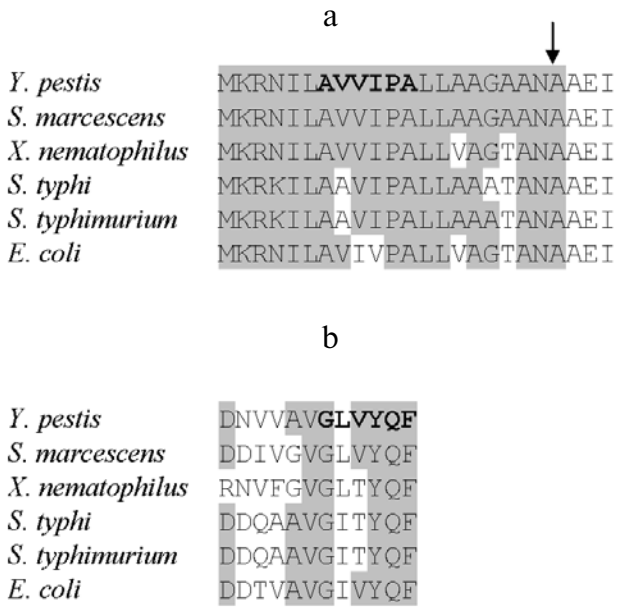


**Fig. 1.** Comparison of sequences of enterobacterial OmpF-like proteins. a) Sequences of signal peptides; b) C-terminal sequences. Identical amino acid residues are shown by gray background. Sequences chosen for creating the primers are in bold print; the upright arrow points to the site of proteolysis of the protein.

Figure 1 presents results of the multiple alignment of terminal sequences, and the regions chosen by us as the most suitable for designing the primers are shown. The primers were designed based on regions of the YO1411 gene (*Y. pestis*) encoding the peptides AVVIPA and GLVYQF stop-codon and tested with programs for primer designing. The specificity of the primers to *Yersinia* DNA was tested with the chromosomal DNA from *E. coli*. According to electrophoresis, no specific PCR-fragment was produced.

**Cloning encoding sequences of the OmpF-like protein genes.** The PCR resulted in PCR-fragments of both *Yersinia* species of about 1.1 kb which were cloned into the pBluescript SK(−) vector. The resulting plasmids, pBS5 for *OmpF* of *Y. pseudotuberculosis* and pBS2 for *OmpF* of *Y. enterocolitica*, were sequenced using the universal direct and reverse M13 primers. The translated sequences were named OmpF-YPS and OmpF-YE. Further analysis was performed using amino acid sequences of the corresponding mature proteins from three *Yersinia* species, including *Y. pestis*.

**Secondary structure and transmembrane topology of the OmpF-like *Yersinia* proteins.** The primary structures of three *Yersinia* porins were compared in amino acid composition (Table 1). Data for *Y. pestis* were taken from the Swiss-Prot bank (Q8ZG94). Table 1 shows that the proteins under study have virtually the same contents of the same amino acid residues. Similarly to other enterobacterial porins, they have a high content of glycine (11-12% of the total content of amino acids), asparagine (9.5%), aspartic acid (10%), and tyrosine (7.5%). For the *Yersinia* porins under study, isoelectric points (IEP) and molecular weights (MW) were calculated: for OmpF from *Y. pseudotuberculosis* IEP was 4.45 and MW was 37.7 kD, and for OmpF from *Y. enterocolitica* IEP was 4.34 and MW was 39.5 kD. These MW values are in agreement with experimental data obtained earlier by SDS-electrophoresis in polyacrylamide gel [6] and MALDI TOF Mass Spectrometry [12].

The OmpF-like porins from *Yersinia* displayed high homology to each other (83-92%) and also to the OmpF porins from *S. marcescens* (70%). The homology of the proteins under study to the OmpF porins from *E. coli* was significantly lower (52-58%) (Table 2). The homology level found by us on comparison of the primary sequences of the OmpF-like proteins from various *Yersinia* species is in agreement with results obtained by comparing the 16S rDNA sequences for the same strains of the bacteria [11].

By multiple alignment of amino acid sequences of the mature OmpF proteins from enterobacteria (Fig. 2) the following features were found. First, extended regions with high and low homology were found, and the conservative regions coincided with the transmembrane domains and highly variable regions fell onto the "external" loops of the OmpF from *E. coli* [2]. Second, the distribution of hydrophobic and aromatic amino acid residues, first of all, glycine, tyrosine, and phenylalanine residues, was obviously regular. Aromatic amino acids are known to form in porins two so-called aromatic "belts" located at the membrane boundaries with the periplasmic and extracellular spaces. The function of these "belts" is retaining the homotrimer in the lipid layer of the membrane [2].

Figure 3 presents the hydrophobicity profiles of the OmpF from *Y. pseudotuberculosis* and the OmpF from *Y. enterocolitica* (Kyte–Doolittle, window 17), and the two sequences are virtually inside the hydrophobic area of the plot. The comparative analysis was performed by superimposing the profiles of two proteins, which corresponded to the OmpF from *S. marcescens* (Swiss-Prot, O33980) and one of the *Yersinia* porins under study. Obviously (Fig. 3), these profiles are alike in the prevalence and distribution of hydrophobic (transmembrane) domains and small number of hydrophilic (extramembrane) domains.

The secondary structures of *Yersinia* porins were determined using programs for predicting secondary structure of proteins (GOR, PredictProtein, Tmpred) with consideration of X-ray crystallography data on the OmpF from *E. coli* [2]. As shown in Fig. 2, the OmpF-like *Yersinia* porins consist of 16 β-strands and 8 "external" loops with unordered structure. As expected, the β-strands coincide with the highly conservative regions and the regions with unordered structure correspond to highly variable regions. According to the topological model for the OmpF from *E. coli*, the β-strands form transmembrane regions of the protein and the sequences with

**Table 1.** Amino acid composition of the OmpF-like porins from *Yersinia*

| Amino acid residue | *Y. pseudotuberculosis* | | *Y. pestis* | | *Y. enterocolitica* | |
|---|---|---|---|---|---|---|
| | Number of amino acid residues | % | Number of amino acid residues | % | Number of amino acid residues | % |
| Ala (A) | 24 | 7.10 | 26 | 7.69 | 26 | 7.4 |
| Arg (R) | 11 | 3.25 | 10 | 2.69 | 11 | 3.2 |
| **Asn** (N) | 33 | 9.76 | 34 | 10.06 | 36 | 10.3 |
| **Asp** (D) | 32 | 9.47 | 32 | 9.47 | 32 | 9.2 |
| Cys (C) | 0 | 0 | 0 | 0 | 0 | 0 |
| Glu (E) | 11 | 3.25 | 12 | 3.55 | 11 | 3.2 |
| Gln (Q) | 15 | 4.44 | 15 | 4.44 | 19 | 5.4 |
| **Gly** (G) | 38 | 11.24 | 39 | 11.54 | 42 | 12.0 |
| His (H) | 2 | 0.59 | 2 | 0.59 | 2 | 0.6 |
| Ile (I) | 12 | 3.55 | 13 | 3.85 | 10 | 2.9 |
| Leu (L) | 21 | 6.21 | 22 | 6.51 | 22 | 6.3 |
| Lys (K) | 19 | 5.62 | 18 | 5.33 | 18 | 5.2 |
| Met (M) | 6 | 1.78 | 4 | 1.18 | 5 | 1.4 |
| Phe (F) | 22 | 6.51 | 21 | 6.21 | 19 | 5.4 |
| Pro (P) | 3 | 0.89 | 3 | 0.89 | 3 | 0.9 |
| Ser (S) | 19 | 5.62 | 20 | 5.92 | 21 | 6.0 |
| Thr (T) | 20 | 5.92 | 20 | 5.92 | 21 | 6.0 |
| Trp (W) | 3 | 0.89 | 3 | 0.89 | 3 | 0.9 |
| **Tyr** (Y) | 26 | 7.69 | 25 | 7.40 | 27 | 7.7 |
| Val (V) | 21 | 6.21 | 19 | 5.62 | 21 | 6.0 |
| Charged | 188 | | 188 | | 197 | |
| Uncharged | 150 | | 150 | | 152 | |
| Molecular weight, daltons | 37724.4 | | 37424.9 | | 38569.8 | |
| Total number of amino acids | 338 | | 338 | | 349 | |

**Table 2.** Homology between OmpF and the OmpF-like porins (degree of homology is expressed in %)

| | *Y. pseudotuberculosis* | *Y. pestis* | *Y. enterocolitica* | *S. marcensens* | *E. coli* |
|---|---|---|---|---|---|
| *Y. pseudotuberculosis* | 100 | 92 | 83 | 70 | 55 |
| *Y. pestis* | | 100 | 82 | 70 | 58 |
| *Y. enterocolitica* | | | 100 | 70 | 52 |
| *S. marcensens* | | | | 100 | 57 |
| *E. coli* | | | | | 100 |

unordered structure correspond to the "external" loops. The secondary structures of the porins under study have virtually no α-helical regions (Fig. 2). These results are consistent with calculations of the secondary structure based on circular dichroism spectra of the porin from *Y. pseudotuberculosis* isolated by digestion of the peptidoglycan layer with lysozyme [13]. This method for isolation of nonspecific porins results in protein preparations with spatial structure most similar to the protein structure in the OM of bacteria [14].

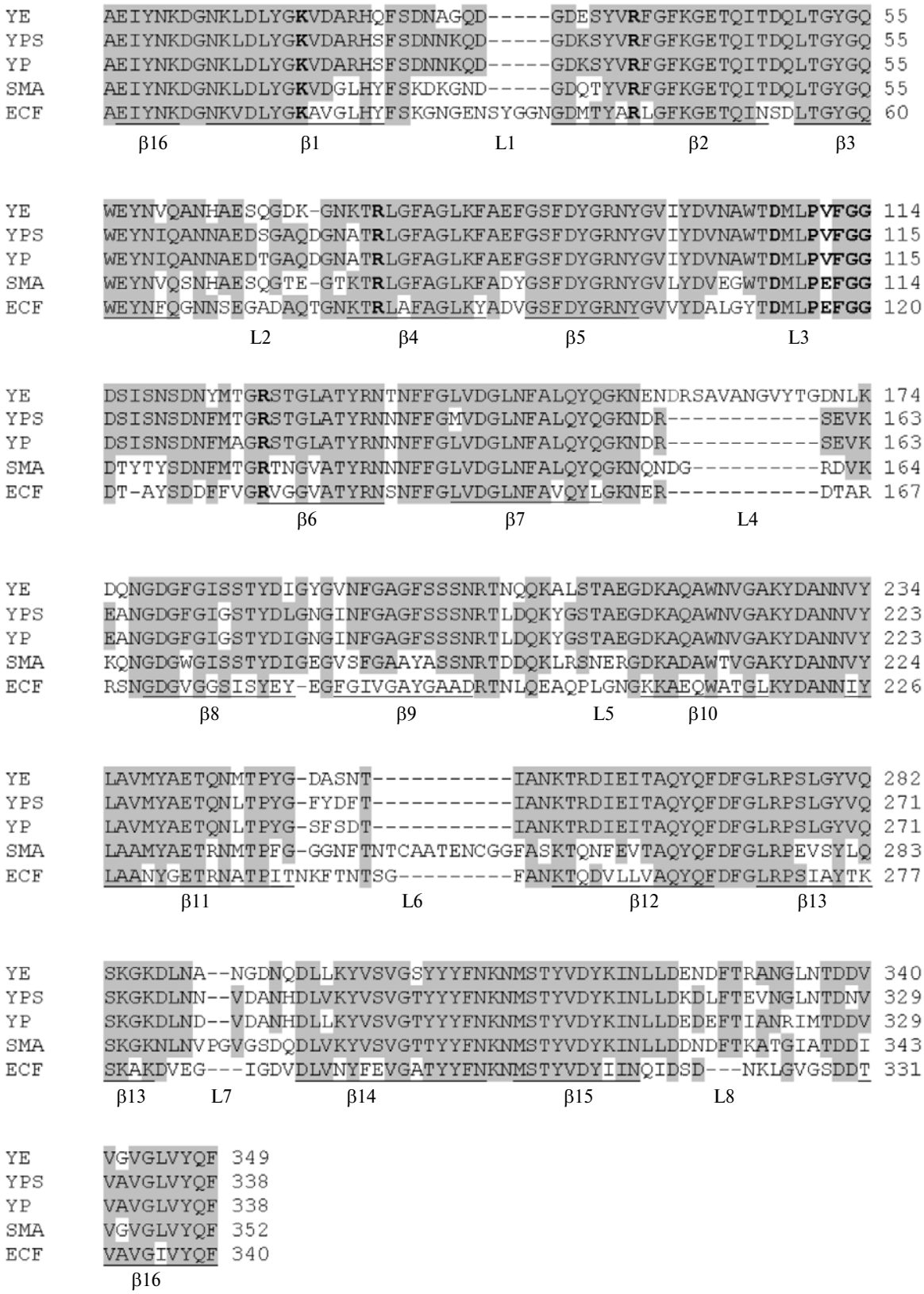


**Fig. 2.** Multiple alignment of amino acid sequences of the mature OmpF proteins. Identical amino acid residues are shown with gray background. Functionally important amino acid residues significant for the pore size are in bold print. YPS, OmpF *Y. pseudotuberculosis*; YE, OmpF *Y. enterocolitica*; YP, OmpF *Y. pestis* (Swiss-Prot Q8ZG94); SMA, OmpF *S. marcescens* (Swiss-Prot O33980); ECF, OmpF *E. coli* (Swiss-Prot Po2931).

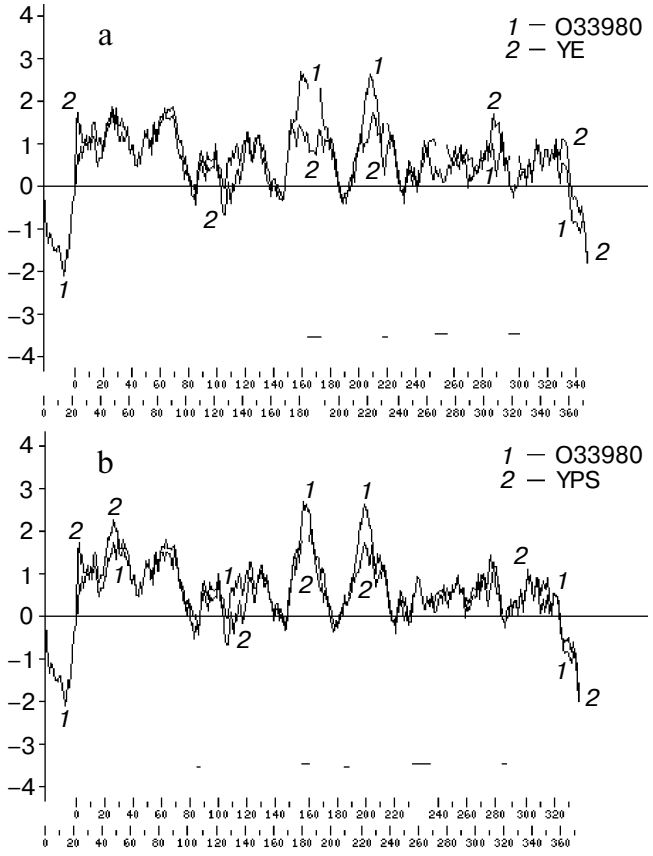


**Fig. 3.** Kyte–Doolittle hydrophobicity profiles of the OmpF-like *Yersinia* proteins obtained with software supported on the server http://bioinformatics.weizmann.ac.il/hydroph/cmp_hydph.html. For comparison, the hydrophobicity profile of the OmpF from *S. marcescens* was used (OmpF_SERMA, O33980). a) The hydrophobicity profile of YE – OmpF *Y. enterocolitica*; b) the hydrophobicity profile of YPS – OmpF *Y. pseudotuberculosis*. Amino acid residue numbers are given along the abscissa axis and hydrophobicity indices are given along the ordinate axis.

Regions of the sequences in the porin loops are different not only in the primary structure but also in length (Fig. 2). The most extended loops are L1 of OmpF from *E. coli*, L4 of OmpF from *Y. enterocolitica*, and L6 of OmpF from *S. marcescens*. Among all known porins, only the OmpF porin from *S. marcescens* contains cysteines, which are located in L6 loop [16]. It seems that the disulfide bond, which as a rule is produced between cysteines, provides for an additional element of the tertiary structure in the loop and determines some features that are not yet determined.

The most interesting are results of comparative analysis of sequences in the "external" loops of *Yersinia* porins. Thus, the conservativeness for the transmembrane regions of the proteins (β-strands) is 84% (β8)-100% (β5, β6, β9, β10, β13, β15) and for the region of loops it is 21% (L4 loop)-100% (L3 loop). The OmpF porins from the causative agents of plague and pseudotuberculosis are mainly different in loops L6 and L8. Just these loops seem to be responsible for the main antigenic differences of these proteins.

L3 loop is known to enter into the pore and be involved in the mechanism of the channel "opening-closing". During interaction of this loop with the internal wall of the β-barrel, the key amino acids are K-16, R-42, R-82, R-132 (the β-barrel) and D-116, E-117 (loop 3) [2, 15]. It is interesting that the three *Yersinia* porins have the same substituent in the conservative pentapeptide PEFGG where the E residue is substituted with V. Other functionally important amino acid residues are similar in *Yersinia* (Fig. 2).

Porins display a tendency for association. In the native membrane and in isolated state in detergent solutions they usually exist as trimers [17]. The L2 loop is extremely important for stabilizing the oligomeric structure. The amino acid sequences of the L2 loops (Fig. 2) of the porins from *Y. enterocolitica* and *S. marcescens* are significantly more similar (67% identity) than the proteins from *Y. enterocolitica* and other *Yersinia* (54% identity). These results suggest a phylogenetic relationship of the microorganisms *Y. enterocolitica* and *S. marcescens*.

The low homology of the amino acid sequence in the L2 loops of the *Yersinia* porins under study is interesting from the standpoint of our previous experimental data [12] on the markedly lower thermostability of the porin trimers from *Y. enterocolitica* as compared to the protein trimers from *Y. pseudotuberculosis*.

The porin loops are plausible targets for adhesion of other cells and for binding bactericidal compounds to the surface of Gram-negative bacteria. Note that the functional properties of the loops are still not clearly understood. Most often they act as binding sites for bacteriophages, bactericides, the complement subcomponents, and antibodies [18]. Thus, the L7 loop of the OprD from *P. aeruginosa* is involved in regulation of the OM permeability for β-lactam antibiotics [19]. The L5, L6, L7 loops of the OmpF from *E. coli* are involved in the interaction with K-20 phages [20], and substitution of the amino acid residue 119 in the L3 loop influences the transport of antibiotics [21].

Thus, variations in the structure of the "external" loops and the associated modulation of porin properties seems to be a mechanism used by bacteria for adaptation to their environment and withstanding the host's immune system.

Our findings have shown that the basic differences in the primary structures of porins from pathogenic *Yersinia* occur in the "external" loops of the β-barrel. We will continue the search for correlations of these differences with functional properties of the *Yersinia* porins.

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