FOR THE RECORD

Prediction of the membrane-spanning β-strands of the major outer membrane protein of *Chlamydia*

MARÍA JOSÉ RODRÍGUEZ-MARAÑÓN,[1] ROBIN M. BUSH,[2] ELLENA M. PETERSON,[1] TILMAN SCHIRMER,[3] AND LUIS M. DE LA MAZA[1]

[1]Department of Pathology, University of California, Irvine, Irvine, California 92697, USA
[2]Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, California 92697, USA
[3]Division of Structural Biology, Biozentrum, University of Basel, Basel, Switzerland

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Abstract

There is preliminary experimental evidence indicating that the major outer-membrane protein (MOMP) of *Chlamydia* is a porin. We tested this hypothesis for the MOMP of the mouse pneumonitis serovar of *Chlamydia trachomatis* using two secondary structure prediction methods. First, an algorithm that calculates the mean hydrophobicity of one side of putative β-strands predicted the positions of 16 transmembrane segments, a structure common to known porins. Second, outer loops typical of porins were assigned using an artificial neural network trained to predict the topology of bacterial outer-membrane proteins with a predominance of β-strands. A topology model based on these results locates the four variable domains (VDs) of the MOMP on the outer loops and the five constant domains on β-strands and the periplasmic turns. This model is consistent with genetic analysis and immunological and biochemical data that indicate the VDs are surface exposed. Furthermore, it shows significant homology with the consensus porin model of the program FORESST, which contrasts a proposed secondary structure against a data set of 349 proteins of known structure. Analysis of the MOMP of other chlamydial species corroborated our predicted model.

Keywords: *Chlamydia*; major outer-membrane protein (MOMP); porin; topology model

The genus *Chlamydia* includes three species that infect humans: *Chlamydia trachomatis*, *Chlamydia psittaci*, and *Chlamydia pneumoniae* (Schachter and Dawson 1978; Moulder et al. 1984; Stephens 1999). Using serological techniques, the 15 major *C. trachomatis* human serovars are divided into two groups, a C complex that includes serovars A, C, H, I, and J, and the B-complex that includes the B, Ba, D, E, L1, and L2 serovars. Serovars F and G are related to the B-complex, while K and L3 are more related to the C complex than to the B-group (Wang and Grayston 1984). In addition, the *C. trachomatis* mouse pneumonitis (MoPn) serovar, also known as *Chlamydia muridarum* (Everett et al. 1999), was isolated from a mouse following inoculation with human tissues (Nigg 1942).

Chlamydia are characterized by a unique developmental cycle involving a metabolically inactive infectious form, the elementary body (EB), that after invading the eukaryotic cell differentiates into a metabolically active form called the reticulate body (RB), which is responsible for intracellular replication (Moulder et al. 1984). The MOMP accounts for 60% of the mass of the outer membrane of the EBs (Caldwell et al. 1981; Hatch et al. 1981). Alignment and comparison of MOMP sequences from all the *C. trachomatis* serovars and *C. psittaci* have revealed the existence of five constant and four variable domains (VDs) (Stephens et al. 1987; Fitch et al. 1993).

Preliminary evidence suggests that MOMP may have a porin-like function (Bavoil et al. 1984; Wyllie et al. 1998). Bacterial porins are an unorthodox class among integral

Reprint requests to: Dr. Luis M. de la Maza, Department of Pathology, University of California, Irvine, Irvine, CA 92697–4800, USA; e-mail: lmdelama@uci.edu; fax: (949) 824-2160.

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membrane proteins because their primary sequences are abundant in charged residues, and no hydrophobic stretches long enough to span the membrane as α-helices can be identified (for review, see Schirmer 1998). Structural studies have shown that bacterial porins are folded into a closed barrel-like β-pleated sheet structure delimiting a pore. Consequently, conventional hydropathy methods that predict membrane-spanning segments based on α-helix structures are inadequate for this class of membrane protein (Jeanteur et al. 1991, 1994).

Here, we have predicted the topology of *C. trachomatis* MOMP using the hydrophobicity algorithm of Schirmer and Cowan (1993) and an artificial neural network (Diederichs et al. 1998). The combination of both methods, the results of which were in excellent agreement, allowed us to propose the topology of MOMP.

Results

Hydrophobicity algorithm

Applying the β-side hydrophobicity algorithm of Schirmer and Cowan (1993) to the sequence of *C. trachomatis* MoPn MOMP yielded the plot shown in Figure 1A. This method analyzes the sequence for transmembrane β-strands by computing the mean hydrophobicity of each face of a putative β-strand. Therefore, values for even and odd residues have been plotted in separate curves. Peaks with Hs'≥0.6 are likely to indicate membrane-spanning β-strands. As observed with other porins, they occur in pairs as two strands joined by a short loop at the periplasmic end of the barrel, well separated by long loops that are exposed at the cell surface.

Very similar plots were obtained for four other chlamydial MOMPs from different species and strains: *C. trachomatis*, serovars E and L3, *C. pneumoniae*, strain AR39, and *C. psittaci*, strain A22/M, which show at least 66% amino-acid identity amongst themselves (data not shown). It can, therefore, be concluded that the predicted topology also is valid for these sequences.

Artificial neural network

To corroborate the above results, we employed an artificial neural network (ANN) that had previously been trained with a set of seven outer-membrane porins with known high-resolution structures (Diederichs et al. 1998). The ANN predicts the *z*-coordinates of Cα atoms with regard to the outer membrane in the *xy*-plane. Thus, high *z*-values indicate long extracellular loops, while low *z*-values correspond to periplasmic loops. Applying this ANN to MoPn MOMP gave the graph shown in Figure 1B. By design, the ANN will fail to identify short extracellular segments such as loop L4. In MoPn MOMP, the sixth extracellular loop, which corresponds to VD4, contains a nonapeptide conserved among chlamydial species. Because of its proximity to the outer membrane, the ANN identifies this region as a transmembrane segment, nevertheless some immunological studies have shown this conserved region is neither a transmembrane nor surface exposed. Thus, we propose that this loop is comprised of two well-defined segments protruding outside the cell, termed for clarity L6 and L6a in Figure 1B and 1C, separated by the conserved nonapeptide in close proximity to the outer membrane.

Two-dimensional model

The two-dimensional (2-D) model of Figure 1C shows the proposed topology of *C. trachomatis* MoPn MOMP. We used four criteria to assign residues to transmembrane segments: (1) A β-side hydrophobicity index Hs'>0.6; (2) Seven to nine residues are needed to span the core of the membrane with every second residue being hydrophobic and thus, positive and negative Hs' alternate; (3) A *z*-value Cα between 0.5 and 0.3 given by the ANN; and (4) Segments with high sequence variability (VD) are excluded by negative inference based on immunological, biochemical, and evolutionary evidence, as discussed below.

The model depicts the protein as an unrolled β-barrel viewed from the outside. The 16 β-sheet membrane-spanning segments are represented by tilted squares and account for approximately one-third of all the amino-acid residues. The residues facing away from the pore are represented in bold, while nonbold, tilted squares indicate the residues with side chains that face the pore. All of the residues facing away from the pore are hydrophobic or aromatic, with the exception of D34, D108, H244, D311, and K322. The model also reveals an abundance of aromatic residues along the upper and lower ends of the β-strands, located at the junction between hydrophobic and hydrophilic environments. The barrel edge in contact with the periplasmic space exhibits a pattern of alternating Phe and Tyr. Aliphatic residues in the middle of the β-barrel form a nonpolar ribbon in contact with the membrane lipids. The side of the MOMP facing the outside of the cell predominantly exhibits long loops, while the turns facing the periplasmic space are short. The external loops contain numerous charged side chains, mostly Asp, and to a lesser extent, Glu.

Analysis using the FORESST program

The FORESST program found MOMP to more closely fit its porin model than other possible structural models. The homology between the porin consensus model and our predicted secondary structure (Fig. 1D) was statistically significant (Z = 3.30). The primary difference between the two was that the MOMP contained additional residues in the VDs. These segments are on the large external loops in our

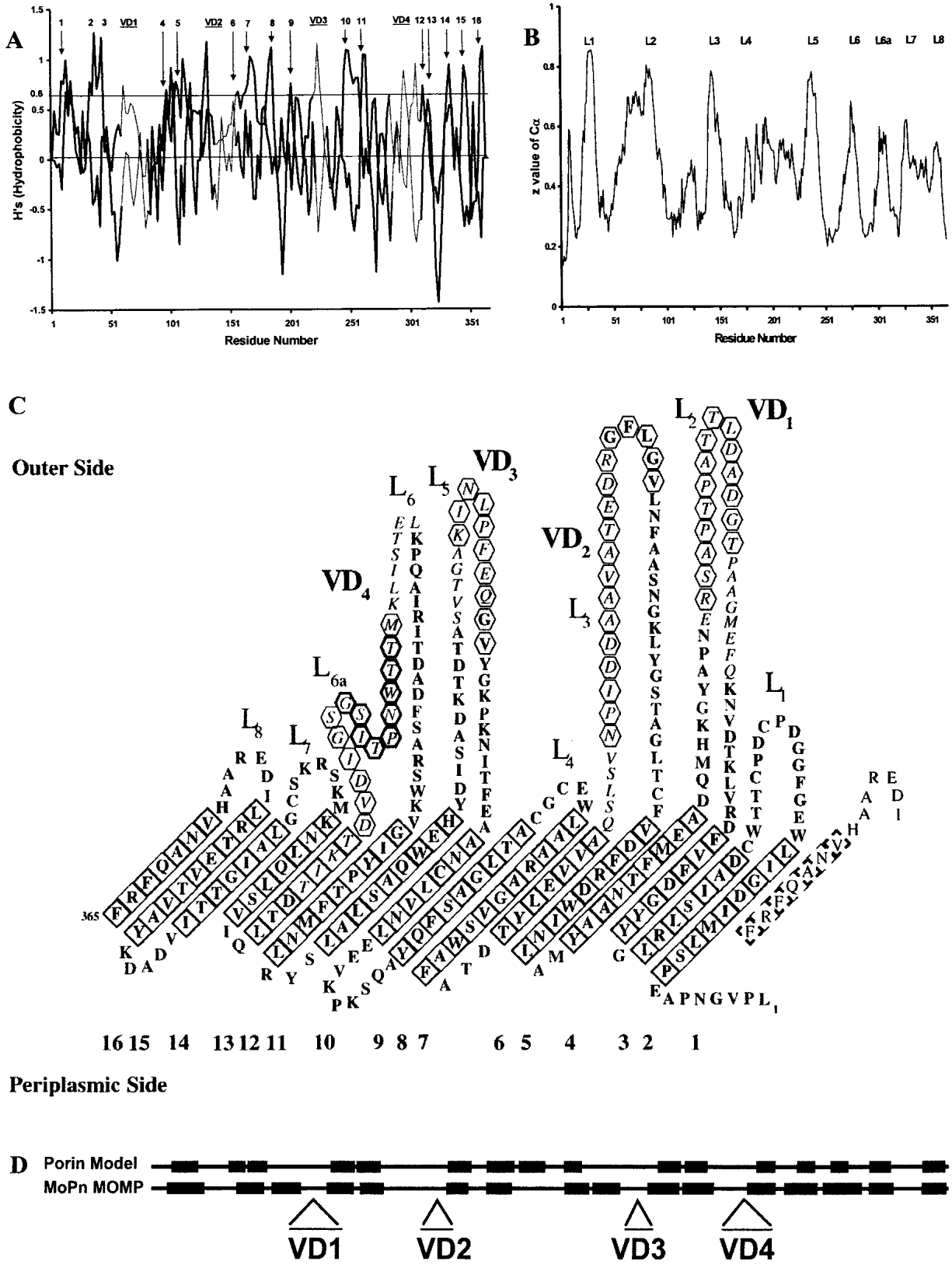
A
H's (Hydrophobicity)
Residue Number
B
z value of Cα
Residue Number
L1
L2
L3
L4
L5
L6
L6a
L7
L8
C
Outer Side
Periplasmic Side
VD1
VD2
VD3
VD4
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
D
Porin Model
MoPn MOMP
VD1
VD2
VD3
VD4

Fig. 1.

model (Fig. 1C). When we shortened the length of the large loops by removing the VDs, the FORESST Z-score increased from 3.30 to 4.04. This supports the hypothesis that MOMP is a porin and that the VDs lie on large external loops.

Discussion

The structural characterization of membrane proteins from microorganisms has provided unique insights into their pathogenesis and has aided in the development of therapeutic and preventive measures (Destenaves and Thomas 2000; Klebe 2000). In the case of the genus *Chlamydia*, the MOMP is considered to be a major target for the development of vaccines and therapeutic strategies (Caldwell and Perry 1982; Ward 1992; Fitch et al. 1993; de la Maza and de la Maza 1995; Brunham et al. 2000; Stephens, 2000). In contrast to other gram-negative bacteria, peptidoglycan has not been found in the outer membrane of *Chlamydia* (Moulder et al. 1984). To account for this observation, it has been proposed that the MOMP, along with the 60 kDa and the 12 kDa cysteine-rich proteins, forms a supramolecular structure supported by disulfide bonds that may be responsible for the integrity of the outer membrane (Bavoil et al. 1984; Raulston 1995; Hatch 1996; Wyllie et al. 1998). This supramolecular structure should provide enough plasticity for the structural changes that occur from the time the organism has the compact conformation of the infectious 300 nm EB, to the metabolically active RB that can have a diameter up to 1500 nm. MOMP probably functions as a porin during replication of the RB (Bavoil et al. 1984). However, the MOMP also may act as an adhesin (Su et al. 1990), both at the time the EB infects the host cell and when the RB adheres to the inclusion membrane to interact with the host mitochondria and endoplasmic reticulum (Peterson and de la Maza 1988; Hackstadt et al. 1995).

Crystallization of membrane proteins poses significant technical challenges and, as a result, only a handful of tertiary structures have been solved (Schulz 2000). In the absence of the tertiary structure of the *Chlamydia* MOMP, we have built a model of the topography of this protein using the structural prediction algorithm of Schirmer and Cowan (1993) and the artificial neural network of Diederichs et al. (1998).

Our model exhibits several structural features that have been identified as common to nonspecific bacterial porins (Schulz 2000). The *Chlamydia* MOMP has 16 β-sheets spanning the depth of the membrane forming a barrel-like structure with long loops protruding out of the cell, while short turns face the periplasmic space. Its barrel-forming amino acids have hydrophilic and hydrophobic characteristics alternating in a way such that the residues facing the pore are in contact with water, while those facing away from the pore are in contact with the membrane lipids. The model also shows the relative abundance of aromatic residues along the upper and lower ends of the β-strands, located at the interface between the hydrophobic and the hydrophilic environment. This "girdle" of aromatic residues lining the boundaries of the β-barrel has been described in other bacterial porins (Schulz 1994). Schulz (1994) proposed that the function of the Tyr-Phe girdle was to prevent conformational damage of the porin during mechanical movements in the membrane. Like other bacterial porins, the chlamydial MOMP also has a band of middle-sized, nonpolar residues located in the mid-section of the β-barrel (Schulz 1994). Only the first eight N-terminal amino acids were not assigned by our prediction.

An alternative assignment of the transmembrane segments of the MoPn MOMP suggests the presence of 18 β-strands. In this model, the two extra strands would occupy positions five and six, which would be located between loops L2 and L3, and correspond to residues assigned to loop L3 on the 16-strand model of Figure 1c. Even though the topology predicted by the hydrophobicity algorithm does not allow the ruling out of the existence of 18 transmembrane segments, the prediction of the z-coordinates by the ANN algorithm does not support the presence of neither transmembrane segments nor external loops. Therefore, an 18 β-strand model most likely can be excluded based on the combined results of these two prediction methods.

On the MOMP external loops, we found a large number of Asp (18) and Glu (9) residues. The side chains of Asp and Glu have been reported to participate in the binding to the carboxylate groups of lipopolysaccharide (LPS) through di-

Fig. 1. (*A*) β-side hydrophobicity plot of *Chlamydia trachomatis* mouse pneumonitis (MoPn) MOMP. Values for even- and odd-numbered residues are plotted as two separate curves. Peaks that have been assigned to transmembrane β-strands in panel *C* are labeled 1 to 16. The locations of the four variable domains (VD1 through VD4) are indicated by bars at the top of the plot, and the residues corresponding to those regions are joined by thin lines. (*B*) Topology plot from an artificial neural network program trained on bacterial outer-membrane proteins: predicted *z*-values are plotted against the *C. trachomatis* MoPn MOMP amino-acid sequence. The extracellular loops are marked L1 through L8 as assigned in panel *C*. (*C*) Predicted topology of the MOMP of *C. trachomatis* MoPn. The 16-stranded β-barrel has been unrolled and the view is from its outer surface. Italics highlight amino acids in the VDs, while tilted squares indicate residues in transmembrane β-strands, with a bold outline if the side chains are facing away from the pore. Antibodies have been mapped to the segments indicated by hexagons. The conserved peptide in VD4 is indicated by bold hexagons. (The amino-acid sequence is in one-letter code.) (*D*) Secondary-structure homology using FORESST. The predicted membrane-spanning β-sheets (black boxes) of *C. trachomatis* MoPn MOMP more closely align with those of the FORESST general porin model after MOMP segments containing the four VDs were deleted.

valent cations such as calcium and magnesium (Cowan et al. 1992). Nevertheless, not all the negatively charged residues are located on the external loops. Three Asp residues and two Glu are part of the small turns facing the periplasmic side of the membrane. Negatively charged residues also can be seen in the periplasmic turns of the porins from *Rodopseudomonas blastica* and *Escherichia coli* OmpF (Cowan et al. 1992; Kreusch et al. 1994). Furthermore, as expected, residues with the highest bend potential, Gly, Pro, Asp, Tyr, Lys, and Ser, are well represented in the periplasmic turns of the MoPn MOMP (Chou and Fasman 1978).

Chlamydial MOMPs differ from other porins of gram-negative bacteria in that they possess a large number (7 to 10) of Cys residues (Stephens et al. 1987; Stephens, 1999). The ability of MOMP to form intra- and intermolecular disulfide bonds with outer-membrane, cysteine-rich proteins is thought to contribute to the rigidity of the EB, which lacks the peptidoglycan layer found in other gram-negative bacteria (Bavoil et al. 1984; Moulder et al. 1984; Hatch 1996). Our predicted topology of the MoPn MOMP assigns seven of the eight Cys residues to the external loops, while the only Cys crossing the membrane (C204) is poorly conserved among serovars. Interestingly, six of the seven Cys located in the external loops are in L1, L4, and L7, three of the four shorter loops that do not have sequence variation. These three loops are in close proximity to L2, the loop that may act as a latch to stabilize the formation of the porin trimers (see below). Thus, it is possible that loops L1, L4, and L7, by being in or close to the trimer interface, may provide rigidity to the membrane of the EB by way of disulfide bonds, while at the same time allowing the necessary plasticity to the membrane for the RB to form following reduction of the disulfide bridges (Hackstadt et al. 1985). Furthermore, the location of the Cys residues in this model suggests that the MOMP cannot form disulfide bonds with other proteins unless they are surface exposed. In this respect, the N terminus of the 60-kDa, cysteine-rich protein is thought to extend to the surface of the bacterial cell (Everett and Hatch 1995; Mygind et al. 1998).

The functional characterization of the external loops presents a challenge because of their exceptionally high sequence variability (Welte et al. 1991; Schulz 2000). In the *E. coli* OmpF, of the eight surface exposed loops, the second (L2) connects one subunit with its neighbor by latching into its pore and stabilizing the trimers that are common among bacterial porins (Phale et al. 1998). Residue E71 on L2 forms salt bridges and hydrogen bonds with R100 and R132 on the channel wall in the adjacent subunit. In the case of the chlamydial MOMP, formation of trimers has been suggested although not yet proven (McCafferty et al. 1995; Wyllie et al. 1998). If the second loop (L2), containing VD1, is also the latching loop in chlamydial MOMP, the trimer interface could approximately correspond to the constant domains CD1 and CD5. If a similar latching mechanism is taking place in *Chlamydia*, a negatively charged residue (E82 in MoPn MOMP) at the end of VD1 conserved in 53 of the 58 MOMP sequences analyzed by Bush and Everett (2001) stands out as a likely candidate. Alternatively, an adjacent, highly conserved (in 55 out of 58 sequences aligned) Arg residue (R81 in MoPn MOMP) also could be involved.

In porins, the third loop (L3) extends inside the pore and is attached to the barrel wall, whereby considerably constricting the lumen of the pore (Cowan et al. 1992). An asymmetrical arrangement of positive and negative charges at the constriction site causes a strong electrostatic field parallel to the plane of the membrane that spreads along the channel governing ion selectivity. In the *E. coli* OmpF, a positively charged cluster formed by three Arg (R42, R82, and R132) protrudes from the barrel wall to face two negatively charged acidic side chains (D113 and E117) located on L3 (Saint et al. 1996; Phale et al. 2001). We have examined the topology predicted for *C. trachomatis* MoPn MOMP in search of a similar asymmetrical charge distribution. Loop L3 of the MoPn MOMP is negatively charged with four acidic residues (3 Asp and 1 Glu) and two basic residues (Arg and Lys). Basic residues are, by comparison, more abundant in the transmembrane segments: R39, R50, R109, and R173 located in or close to strands 2, 3, 5, and 7, as it was found in *E. coli* OmpF (Phale et al. 2001).

As can be seen in Figures 1A and 1B, VD3 and part of VD4 are predicted as transmembrane segments. This assignment has to be rejected on immunological, biochemical, and evolutionary evidence. Already in the original paper by Schirmer and Cowan (1993), it was shown that the Hs' algorithm can produce false positives. Immunological studies and immunoelectronmicroscopy, for example, have shown that mice inoculated with *Chlamydia* produce antibodies against all four VDs of the MOMP (Kuo and Chi 1987; Baehr et al. 1988; Baghian et al. 1990; Andersen 1991; Peterson et al. 1991; Pal et al. 1993, 1996, 1997a,b, 2000; Qu et al. 1993; Villeneuve et al. 1994). Like with *Neisseria gonorrhoeae* only the long loops L2, L3, L5, and L6 of *C. trachomatis* and *C. psittaci* have VDs probably reflecting the fact that they are more immunoaccessible (van der Ley et al. 1991). Loops L1, L4, and L7 are not only shorter than the loops containing the VDs but, as previously discussed, also have numerous Cys residues probably involved in disulfide bonding and thus, imposing a significant restriction on structural changes (see above). In the case of *C. pneumoniae* however, the lack of sequence variability suggests that the MOMP is immunorecessive, or it is not surface accessible (Christiansen et al. 1999; Wolf et al. 2001).

Alignment of MOMP sequences from different chlamydial species and serovars identified a conserved nonapeptide within the highly variable VD4: TTWNPTISG (indicated in bold hexagons in Fig. 1C and corresponding to the sequence

TTLNPTIAG in the MOMP of the human *C. trachomatis* serovars (Stephens et al. 1987; Fitch et al. 1993). The algorithms employed here suggest that this invariant region is a putative transmembrane segment. However, the assignment of loops L6 and L6a immediately before and after this nonapeptide argues otherwise. Su et al. (1990) suggested that this conserved region functions as a cryptic hydrophobic binding site responsible for *Chlamydia*-host cell interactions. Epitope mapping supports the assumption that this region is not a transmembrane segment. The monoclonal antibody MoPn 13–2 binds to this region of the MoPn MOMP (Pal et al. 1997b). Also, the subspecies-specific monoclonal antibody E4 binds to this invariant region TLNPTIA within the VD4 of all the human chlamydial serovars (Peterson et al. 1988). In vitro, this antibody reacted by immunofluorescence and dot-blot ELISA with native EB of the B- and C-related complexes, but it reacted only with heat-treated EB of the C complex. The fact that a mild treatment exposes this binding site suggests that, even in the members of the C complex, this epitope is not buried in the membrane.

Enzyme digestion, biochemical analysis, and substitution-rate studies also support the location of the VDs outside the membrane. For example, the infectivity of serovar L2 was resistant to treatment with trypsin, while infectivity of the B serovar was significantly reduced following digestion with this enzyme (Su et al. 1988). Comparison of the VD2 and VD4 showed a trypsin-sensitive Lys residue in each of these two sites on the B serovar, while the MOMP of the L2 serovar was cleaved only at the Lys in VD4. Based on these findings, it was concluded that VD2 and VD4 are surface exposed and play a critical role on chlamydial infectivity. Recently, Hughes et al. (2001) showed that a recombinant MOMP in which the VD4 was deleted retained its ability to form pore channels, indicating VD4 is not directly involved in the pore function.

The assignment of VDs to surface-exposed loops also is consistent with the results of Brunham et al. (1994), who found a higher rate of nonsynonymous than synonymous substitutions in the MOMP VDs. This pattern suggests the action of positive selection. Very few genes show evidence of positive selection. The major class of such genes is pathogen surface proteins (van der Ley et al. 1991; Smith et al. 1995; Endo et al. 1996; Bush et al. 1999; Yamaguchi-Kabata and Gojobori 2000; Bush 2001). Here, the hypervariability of the VDs probably helps the pathogen to evade recognition by the immune system of the host.

In summary, we are proposing a topology scheme for the MOMP of *Chlamydia* consistent with immunological, biochemical, and evolutionary data. The model should help to understand the role of MOMP in the pathogenesis of this microorganism, to develop preventive and therapeutic measures, and to assist in planning further experiments to advance our knowledge of the structure of this protein.

Materials and methods

Prediction of membrane-spanning β-strands

The topology of the MOMP from *C. trachomatis*, serovars MoPn, E and L3, *C. pneumoniae*, strain AR39, *C. psittaci*, and strain A22/M (Accession numbers JT0947, MMCWTE, JE0413, A43587, and A60109, respectively) was predicted using an algorithm developed by Schirmer and Cowan (1993). This algorithm calculates the mean hydrophobicity of one side of a putative β-strand by taking the average of the hydrophobic indices of every second residue within a sliding window of four.

$$Hs'(i) = 1/4[h(i-2) + h(i) + h(i+2) + h(i+4)].$$

To account for the observed preponderance of aromatic residues in flanking positions, the method assigns an arbitrarily increased "hydrophobicity index" of 1.6 to aromatic residues when found in positions $i-2$ or $i+4$.

Topology prediction using an artificial neural network

An ANN trained to predict the topology of bacterial outer-membrane proteins with a predominance of β-strands was applied to the MOMP sequences (Diederichs et al. 1998). This ANN, available online (http://strucbio.biologie.uni-konstanz.de), predicts the z-coordinate of Cα atoms in a coordinate frame with the outer membrane in the xy-plane, such that low z-values (<0.3) indicate periplasmic turns, medium z-values indicate transmembrane β-strands, and high z-values (≥0.6) indicate extracellular loops.

Secondary structure comparisons with known porins

We used the computer program FORESST (Fold Recognition from Secondary Structures, Release 1.0, http://absalpha.cit.nih.gov/) to determine the degree of homology between our predicted secondary structure model and a data set of 349 proteins of known structure (Di Francesco et al. 1999; Geetha et al. 1999). Our query sequence consisted of a string of 365 letters, each representing a position in the MoPn MOMP protein sequence. FORESST provides results as Z-scores, which are the number of standard deviations by which the hidden Markov score for the query sequence was above the average score for the family model using a random protein (Cowan et al. 1992; Kreusch et al. 1994). Comparisons with z-scores of 3.0 and higher are considered significantly homologous.

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