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Porins in the Genus *Borrelia*

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1. Introduction

1.1 The cell envelope of *Borrelia* compared to other gram-negative bacteria

Borreliae belong to the spirochete phylum, an ancient evolutionary branch only remotely related to Gram-negative and Gram-positive bacteria, although their envelope is regarded to be Gram-negative. Spirochetes of the genus *Borrelia* exhibit a complex life style, characterized by the ability to shuttle between hematophagous arthropods and various vertebrates, which exposes them to a variety of niches that are different in nutritional composition as well as immunological pressure. Survival, transmission and ultimately pathogenesis during infection obviously require a large degree of adaptive biological capacity. Thus, the interactions between the host and *Borrelia* pathogens need to be optimized for interaction with different environments such as the tick midgut, and various mammalian tissues. The different surface-exposed proteins play an important role in this process by providing ligands for receptor-mediated adhesion, mechanisms of host immune response avoidance, as well as pathways for the acquisition of nutrients.

Borreliae are considered to be Gram-negative bacteria because they possess two membranes and a periplasmic peptidoglycan layer, which in *Borrelia* is anchored to the inner membrane [1]. In spite of this, if *Borrelia* are compared to typical Gram-negative bacteria such as *Escherichia coli*, their cell envelope differs in many aspects (Fig. 1). While flagella in typical Gram-negative bacteria are placed on the surface of the outer membrane, the flagella in *Borreliae* are found in the periplasmic space between inner and outer membranes. This peculiar characteristic is shared among the spirochetes. Another large difference between the common Gram-negative eubacteria and *Borreliae* spirochetes is the absence of lipopolysaccharides [2, 3]. Instead, the outer membrane in *Borreliae* is very rich in lipoproteins, which represent the primary interface between the bacterium and the host [4]. The borrelial outer membranes are considerably more fluid than those of other Gram negative bacteria. They also possess a lower density of membrane spanning proteins [5, 6]. Porins, the focus of this review, are also found in the outer membrane of spirochetes with remarkably unique characteristics.

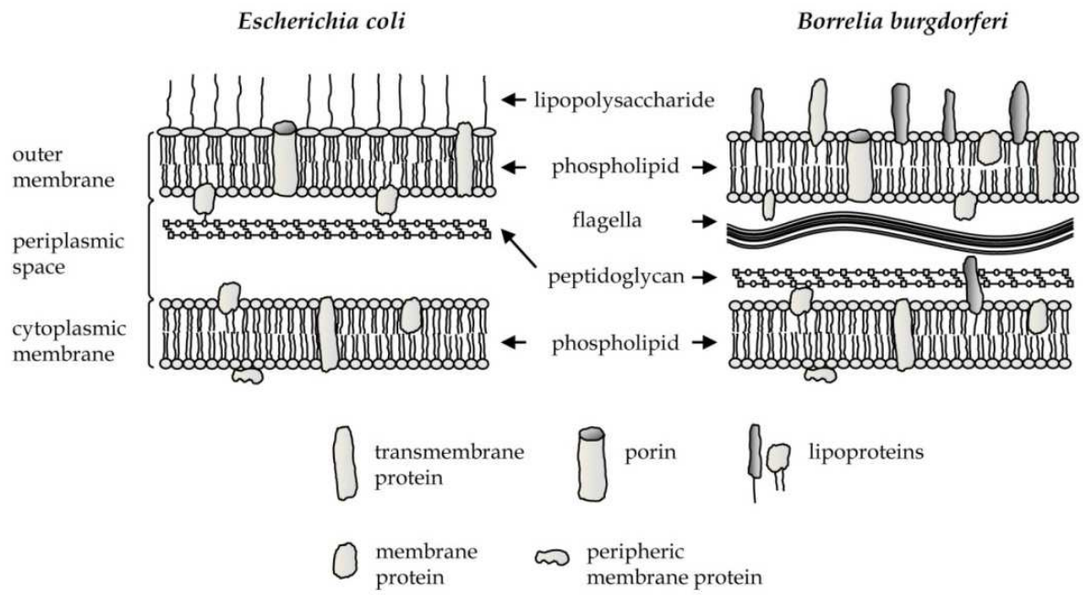

Fig. 1. Comparison of the cell envelope of a typical Gram negative bacterium such as *Escherichia coli* and *Borrelia burgdorferi*. While *E. coli* has a high content of lipopolysaccharides in its outer membrane, *B. burgdorferi* has a high amount of lipoproteins. Periplasmic flagella and a lower amount of external membrane spanning proteins are also typical *Borrelia* features.

1.1.1 Lipoproteins in the outer membrane of *Borrelia*

The protein content of the borrelial outer membrane is represented by a huge quantity of lipoproteins. As an indication of their importance in *Borrelia*, lipoproteins are the most abundant proteins in these species [7]. These proteins are located on the surface of the bacterial cell and anchored in the outer leaflet of the outer membrane. Borrelial lipoproteins play a major role in the activation of the innate immune response of the host as well as in the adaptive responses and pathogenicity of *Borrelia* spirochetes. Thus, this class of proteins is responsible for the activation of host inflammatory response as tissue inflammation which is a unifying feature during the manifestation of Lyme disease. Lipoproteins dominate pathogenicity mechanisms exploited by *B. burgdorferi*, including antigenic variation [8, 9], evasion of complement killing [10-12] and adhesion mechanisms [13].

The most abundant and best described outer membrane lipoproteins in the Lyme disease species *B. burgdorferi* are the so-called outer surface proteins (Osps). The most prominent lipoprotein species is OspA. OspA tertiary structure contains a single-layer β-sheet connecting the N- and C-terminal globular domains [14]. OspA acts as an adhesin in the tick environment [15] and it could have a protective function for P66 against proteases [16]. OspC, another well-studied *B. burgdorferi* lipoprotein, is homologous to variable small proteins (Vsps) of relapsing fever *Borrelia* [17]. The tertiary structure of OspC is also known and differs completely from OspA/B. It basically consists of five parallel α-helices and two short β-strands [18, 19]. This lipoprotein seems to be required to infect mice [20]. Interestingly, the OspA/B and OspC expression is reciprocally regulated and undergoes a switch during spirochete transition from the tick vector to the mammalian host. Thus, the

OspA expression is up-regulated in unfed ticks while OspC is expressed in large amounts in feeding ticks and in the mammalian host [21].

Beside the Osps, there is a wide variety of lipoproteins with different functionalities present in *B. burgdorferi*, such as the multicopy lipoproteins Mlps [22], Lp6.6, a small lipoprotein of 6.6 kDa [23], the decorin-binding proteins DbpA/B [24], the glycosaminoglycan-binding protein Bgp [25], P35 comprising two lipoproteins [26], the Vmp-like-sequence Vls [27], the Bmp family [28], P22 [29], the OspE/F related proteins Erps [30], the Elps containing OspE/F-like leader peptides [30] and the complement regulator acquiring surface proteins CRASPs [10].

In contrast to Lyme disease species, the most abundant proteins in the relapsing fever species' outer membranes are the variable major proteins Vmp. These lipoproteins are expressed with different surface epitopes through antigenic variation [31-33]. The lipoproteins of relapsing fever agents are subdivided into two families: the variable large proteins (Vlps) and the variable small proteins (Vsps) [7, 34, 35]. Interestingly, the expression of the single Vsp species is correlated to the pattern and the state of the disease. Vsps undergo antigenic variation and at least three mechanisms are known for surface alteration of relapsing fever lipoproteins [36, 37].

1.2 Porins in gram-negative bacteria

Porins are a group of proteins located in the outer membrane of Gram negative bacteria that facilitate the transfer of substances in both directions between the surrounding environment and the periplasmic space. Porins are also found in mycobacteria, chloroplasts and mitochondria [38, 39].

Porins form water-filled channels that allow a passive transport of molecules down their concentration gradients [40, 41]. This transport through the outer membrane does not require energy in contrast to the uphill substrate translocation via transporters in the inner membrane.

Most of the porins described to date form β barrels composed of antiparallel β-sheets. Frequently, they are associated in oligomers that confer a higher stability to the whole complex (Fig. 2). In the outer membrane of Gram negative bacteria, channel-tunnel proteins like TolC in *Escherichia* and BesC in *Borrelia* are also found. They form pores in the outer membrane and are part of bigger protein complexes involved in drug-resistance known as efflux pumps (Fig.2).

Porins can be classified in two groups depending on whether they are selective for a class of molecules or not [42]:

General diffusion porins allow the transport of small molecules, ions and water through a membrane. The permeability of general diffusion pores for substrates is dependent only on their size and charge. Examples for this kind of porins are OmpF and OmpC of *E. coli* [40].

Substrate-specific porins represent pores or channels that are specialized for the transport of certain molecules. These molecules bind to the channel interior and are guided to the periplasm. Tsx is an example of a substrate specific porin in *E. coli* that is specialized in the nucleoside transport from the surrounding media to the periplasmic space (Fig. 2) [43, 44]. Other examples include porins with specificity for carbohydrates [45], phosphate [46] and antibiotics [47].

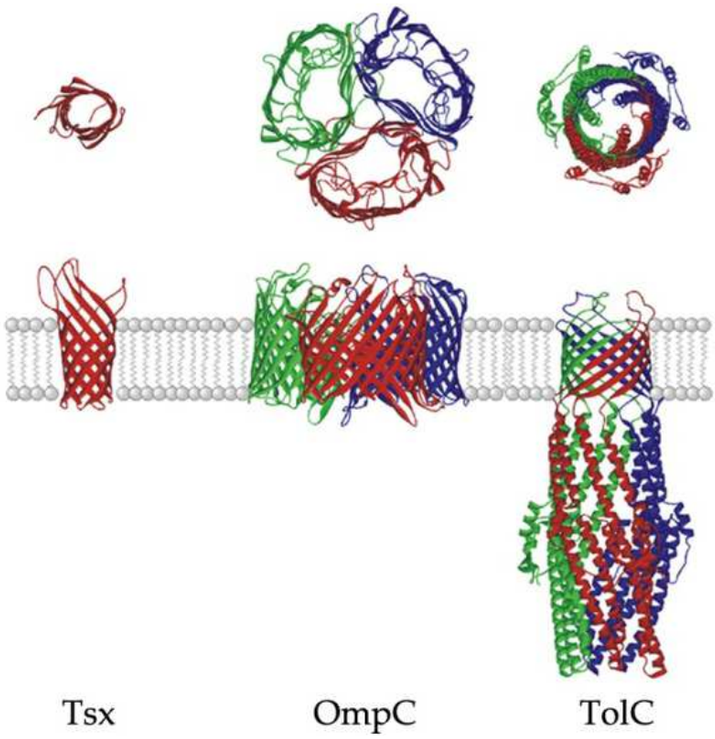

Fig. 2. Bacterial outer-membrane pore-forming proteins. Diffusion channels have usually a β-barrel tertiary structure with longer loops to the outside and short turns towards the periplasmic space. In *E. coli*, Tsx is a substrate specific porin for nucleosides while OmpC is a general diffusion porin. Usually porins form trimers like OmpC although other associations are also possible like in the case of Tsx. TolC is a channel-tunnel that together with an inner membrane transport system and adaptor proteins forms an efflux pump for export of drugs and other substances. These channel-tunnels are also formed by trimers but they only form a single pore.

2. Porins in the genus *Borrelia*

Borrelia species possess a small genome composed of a small linear chromosome complemented with a set of up to 21 linear and circular plasmids [48]. Many of their genes are related to bacterial virulence and the adaptation of bacteria to the different hosts and vectors. The absence of genes in *Borrelia* involved in the biosynthesis of amino acids, fatty acids or nucleotides is very remarkable. This metabolic deficiency makes *Borrelia* species strictly dependent on substances provided by their host. Therefore, the role of the *Borrelia* porins in the acquisition of nutrients is of extreme importance.

Up to now, five porins and a channel-tunnel as part of a multi-drug efflux system have been described in the outer membrane of different *Borreliae*. P66, P13, BBA01 (a P13 paralogue) and BBA74 have been described in *Borrelia burgdorferi*. Oms38 has been described in relapsing fever species, and possess a homologue in *Borrelia burgdorferi* designated DipA. Another outer membrane pore-forming protein is BesC. BesC is a channel-tunnel which is part of a bigger complex spanning the whole cell envelope which is involved in secretion of toxic compounds for *Borrelia*, like for example antibiotics.

2.1 P66: A porin with an unusual high single channel conductance

P66 exhibits a dual function. P66 is a well-studied pore-forming outer membrane protein present in Lyme disease and relapsing fever *Borreliae* [49-55]. Interestingly, P66 exhibits dual functions. It was shown to act as an adhesin which can bind to β(1)- and β(3)-chain integrins [51-53]. And strikingly, it acts as a porin in the outer membrane with the capability of forming pores with an extremely high single-channel conductance. This could be demonstrated by studies in planar lipid membranes [50, 54, 55]. In addition, P66 is also of special interest because of the presence of surface-exposed loops [49, 56] exhibiting a certain immunogenic potential [57], a property of interest in the search of vaccine candidates against European LD *Borreliae*.

P66 is present in both Lyme disease and relapsing fever agents. Comparison of the deduced P66 amino acid sequences from published genomes of the Lyme disease species *B. burgdorferi* B31, *B. afzelii* pKo, *B. garinii* PBi and the relapsing fever species *B. duttonii* Ly, *B. recurrentis* A1 and *B. hermsii* HS1 revealed 41% inter-species identity with partially highly conserved domains. The three LD species' P66 share the high sequence identity of 90%. P66 has according to its name a molecular weight of 66 kDa as can be seen after SDS-PAGE of purified P66.

P66 exhibits an unusually high single channel conductance. The pore-forming properties of P66 channels from the LD species *B. burgdorferi*, *B. afzelii* and *B. garinii* have been studied in detail by different groups [50, 54, 55]. P66 is able to form pores in planar lipid bilayers with the enormously high single-channel conductance of 11 nS in 1 M KCl [55]. Thereby, the conductance for P66 of the LD species *B. burgdorferi*, *B. afzelii*, *B. garinii* in 1 M KCl were all similarly high, in a range between 9 and 11 nS [55]. Measurements in different KCl concentrations from 0.1 to 3 M revealed that the single channel conductance was approximately a linear function of the electrolyte concentration which indicates a lack of binding sites for ions.

The P66 channels are nonselective for small anions and cations [50, 55]. This was demonstrated by single-channel experiments with salts containing cations and anions of different size such as LiCl and KCH_3COO and by the analysis of the membrane potential using the Goldman-Hodgin-Katz equation [58]. P66 channels are permeable for both anions and cations, because the calculated values of the permeability ratio of cations over anions (P_c/P_a) in KCl were in the range between 0.8 and 1.0 for all measured P66 homologues [55]. Values close to one indicate equal pore permeability for anions and cations.

P66 channels exhibit voltage-dependent closure. This was demonstrated in multi-channel experiments. *B. burgdorferi* P66 started to show voltage-dependent closure at potentials as high as + 70 and -60 mV reaching a maximal conductance decrease of approximately 20%. P66 homologues of different LD species all showed similar voltage-dependent behavior with slight species-specific differences of the potential for the start of conductance decrease. For example, for P66 of *B. afzelii* and *B. garinii*, the voltage-dependent closure started at potentials as high as +/- 40 mV and the conductance decreased by approximately 30% at applied potentials of +/-80 mV and higher [55].

The pore diameter of P66 is small related to the huge single-channel conductance. The above mentioned outstanding high single-channel conductance of 11 nS (in 1 M KCl) is atypical and rare for Gram-negative bacterial porins. However, certain spirochetal porins exhibit also an extremely high single-channel conductance, such as the one of the major outer membrane

protein of *Spirochaeta aurantia*, which forms pores with a conductance of 7.7 nS in 1 M KCl [59] and the one of the major surface protein of *Treponema denticola*, Msp, a 53 kDa species (forming 1.8 nS pores in 0.1 M KCl) [60]. So far, beside selectivity and estimated pore diameters, very little is understood about the pore size and the structure of these outer membrane channels. In terms of P66, the channel diameter was calculated to be about 2.6 nm based on the assumption that the conductance of the channel is equal to the conductivity of a simple cylinder of aqueous salt solution [50]. This would be a rather large diameter compared to other pore-forming outer membrane proteins [61]. Anyway, the calculated value of the P66 diameter appears to be rather preliminary and theoretical.

Hence, the question arises what is the apparent channel diameter of P66 and its molecular organization in the outer membrane of *Borrelia* species. This question was answered by conductance measurements of the P66 channel reconstituted in planar lipid membranes in the presence of non-charged molecules, so-called nonelectrolytes (NEs), and the conductance was studied as a function of the size of the spherical NEs (Thein et al. unpublished). When the NEs can enter the P66 channel they will reduce its conductance as has been shown previously [62]. This means that the molecular mass cut-off for NEs and their hydrodynamic radii could provide a measure of the pore diameter [62].

Single-channel measurements with P66 in the presence of NEs revealed that NEs with a hydrodynamic radius (r) ≤ 0.8 nm enter the pore whereas NEs with r ≥ 0.94 nm are not permeable and cannot enter the P66 channel (Thein et al. unpublished). Evaluation of these data obtained with a method described by Krasilnikov et al. 1998 [63] indicated that the precise value of the effective P66 radius is about 0.94 ± 0.1 nm (Thein et al. unpublished). This means that P66 has an effective pore diameter of less than 1.9 nm, a value which is significantly smaller than the prediction of 2.6 nm [50]. Thus, P66 has an apparent channel diameter close to the one of several other Gram-negative bacterial porins and other membrane channels [63-65], that were characterized by the use of NEs. But one has to consider that the P66 single-channel conductance is up to 60-fold higher than the single-channel conductance of those channels with a comparable channel diameter (Thein et al. unpublished).

The molecular organization of P66 seems to be peculiar. This discrepancy between single-channel conductance and effective diameter suggests that the channel-forming domain of P66 is based on an outstanding molecular organization. And indeed, when P66 is subjected to a Blue Native PAGE, a 440 kDa protein band can be observed. This means that P66 can indeed form a hexamer or a heptamer in its membrane-active configuration because seven 66 kDa monomers could form an oligomer with a calculated molecular mass of approximately 460 kDa. This finding is supported by conductance measurements of one single P66 unit. After reconstitution of one single 11 nS unit, the defined single-channel conductance of P66, in the membrane, the ionic current through the channel can be substantially blocked by the addition of 90 mM PEG 400. And strikingly, this blockage occurs stepwise in seven closing substates (Fig. 3) (Thein et al. unpublished). The conductance of all those substates is fairly homogenous and is on average about 1.5 nS. The substate conductance of 1.5 nS confirms the Blue Native PAGE result, because seven 1.5 nS substates yield a conductance in the range of 11 nS. This means that the individual P66 molecules are forming a high molecular mass protein complex: The individual channels in the oligomer act like molecular sieves with an exclusion size of approximately 1.9 nm. These findings have not been observed for typical porins of Gram-negative bacteria (Thein et al. unpublished).

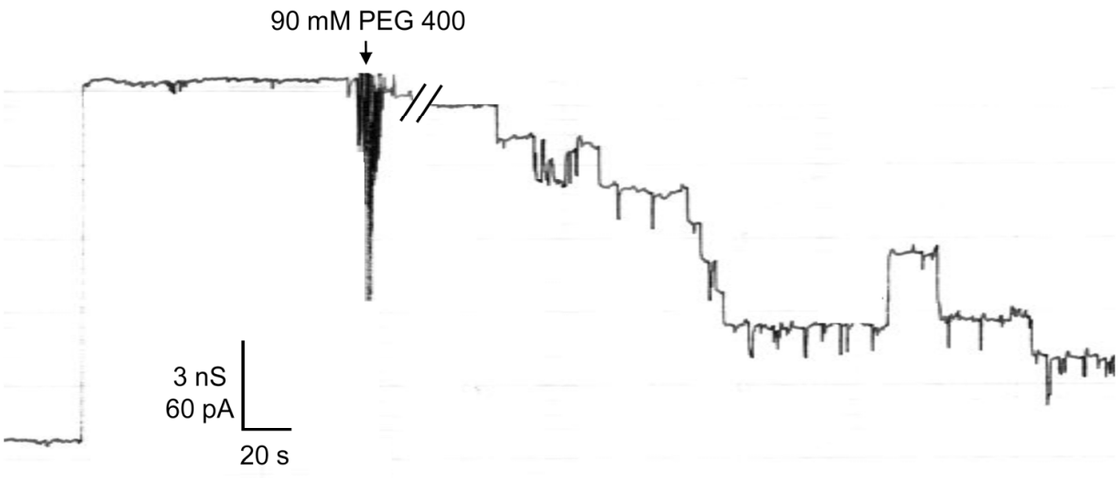

Fig. 3. Blockage steps of a single P66 channel after addition of PEG 400. A single P66 channel was reconstituted in a diphytanoyl phosphatidylcholine artificial membrane embedded in a 1M KCl salt solution. Some time after addition of PEG 400 the channel was blocked in several steps, each one corresponding probably to an independent channel subunit.

2.2 P13: An atypical α-helical porin

P13 is a surface-exposed epitope. In 1995 Sadziene et al. [66] described an accessible protein in a *B. burgdorferi* strain called *B. burgdorferi* B313 lacking several lipoproteins. In this study the inactivation of four lipoproteins, OspA, OspB, OspC and OspD, in *Borrelia burgdorferi* B31 and its posterior inoculation in a rodent triggered an immune response against a surface exposed 13 kDa protein [66]. This antibody had no influx in the growth of a native strain highlighting a possible P13 protective function of these mutated lipoproteins. To study the effect of this antibody on cell growth, the B313 mutant was compared with another mutant, B311, where only two lipoproteins, OspC and OspD, were mutated. The MIC of this antibody for the B311 stain was more than 600-fold higher than for the B313, revealing a possible P13 protective function of OspA and/or OspB against the immune system [66]. This protective function of some lipoproteins for transmembrane proteins has also been described for other *Borrelia* proteins [16].

Other experiments treating *Borrelia* cells with proteinase K and trypsin confirmed the exposure of P13 to the surrounding media [67]. Further immunofluorescence assay and immunogold-labeling confirmed again the homogeneous distribution of P13 in the outer membrane of *Borrelia burgdorferi* [67]. The surface exposure of P13 and the lack of any other known homologue in other living organisms makes this protein a perfect candidate to be used in new diagnosis and therapeutic strategies [67].

P13 permeabilized lipid membranes similarly as bacterial porins. Purification to homogeneity followed by black lipid bilayer experiments revealed a pore forming activity for P13. Single channel insertions in artificial membranes displayed a conductance of 3.5 nS in 1 M KCl [68]. Further analysis of the P13 pore forming activity revealed that the channel was slightly cation selective and voltage independent [68]. No specific substrate has been found for P13 although titration experiments had been carried out with different sugars,

amino acids and other relevant substrates (Bárcena-Uribarri et al. unpublished data). For these reasons P13 is considered to be a general diffusion porin with a quite stable structure.

P13 is posttranslationally processed and modified on its N- and C-termini. Mass spectrometry analysis of mature P13 located in the outer membrane revealed a molecular mass of 13,968 ±1 Da. This fact was consistent with the molecular mass estimations by SDS-Page and Tricine-SDS-Page. N-terminal and C-terminal sequencing of the protein revealed a blocked N-terminus and a processed C-terminus that lacked the last 28 amino acids [67, 69]. A pyroglutamic acid modification was later proven to be present at the N- terminus [69]. Computer predictions for a Signal Peptidase I cleavage site after amino acid 19 in the N-terminus were consistent with the predicted molecular mass for mature P13.

P13 is processed at the C-terminal end in the periplasmic space. Another remarkable peculiarity of P13 is the C-terminal peptide that is cleaved in the periplasmic space by CtpA [70]. This kind of caboxyl-terminal proteases have also been identified in other bacteria [71] including an homologue in *E. coli* [72] and in chloroplasts of algae and higher plants [73, 74]. In *Borrelia burgdorferi*, the CtpA is the responsible for the cleavage of 28 amino acids in the C-terminal end of P13. The CtpA protease has also an influence on the expression of several other proteins, such as BB0323 and Oms28. CtpA has a signal sequence for the transport to the periplasmic space and therefore the processing of the C-terminus of P13 is believed to happen in the periplasmic space [70].

The aim of the C-terminal processing and a possible function of the C-terminal peptide of P13 is unknown. However, a mutant of the CtpA protease showed that this processing is not required to localize P13 in the outer membrane of *Borrelia* [70]. Whether the P13 has to be processed to form a protein complex or if the P13 peptide has its own function has not been clarified yet.

No lipidation or glycosylation was found to occur in P13. A potential leader peptidase II cleavage-lipid modification was found in the P13 sequence. However, experiments with radio-labeled fatty acids or immunoblots for glycoprotein detection showed no apparent modification of P13 by fatty acids or carbohydrates [67]

P13 is an α-helical transmembrane protein. Initial experiments predicted three α-helical transmembrane domains for P13 based on computer modeling. When the model was compared to other strain sequences it could be observed that the hypothetical transmembrane domains were highly conserved while the exposed loops regions were more variable [67]. This fact is in agreement with a surface exposure of the loops that undergo a higher immune pressure. Later on, this hypothesis was tested with an experimental approach where three fragments of mature P13 were designed and produced recombinant. Based on the computer model two were transmembrane domains and one corresponded to the external loop. Only the segment though to be the external loop was recognized by an antibody that strictly recognizes the natural epitope of the P13 protein confirming this hypothesis (Fig. 4) [66, 75].

The P13 oligomeric structure does not follow the typical model of a bacterial porin. Most of the 3D-structures of porins show β-barrel cylinders often organized as trimers [76]. Each monomer forms an individual channel that acquires a higher stability in association with two other identical units. In the case of P13 its small size makes the formation of a channel by itself improbable and an oligomeric quaternary structure is required to form a channel in

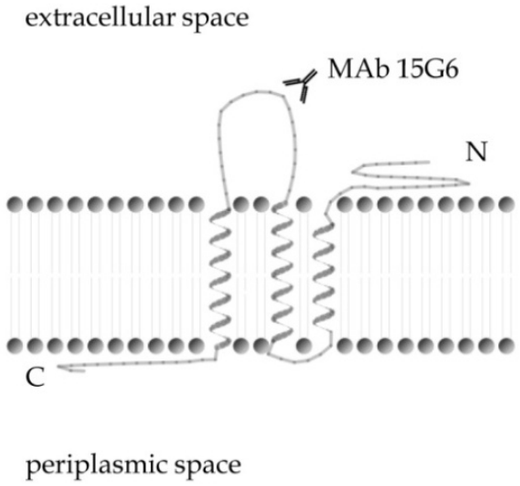

Fig. 4. P13 predicted secondary structure. Computer modeling and some experimental approaches disclose three α-helical transmembrane domains. An external loop is placed to the outside and it is the antigenic determinant for the Mab 15G6 antibody, which recognizes the surface-exposed region of P13 in "*in vivo*" immunolabeling.

the outer membrane of *Borrelia*. Some indications of P13 oligomerization have been previously described but the exact number of monomers involved in this protein complex remains still unclear [68]. The P13 α-helical secondary structure is also not typical for bacterial porins. Only a few examples of α-helical porins have been described, all of them in Gram-positive bacteria and none of them with monomers spanning the membrane three times as supposed for P13 (Fig. 4) [77, 78]. These features make P13 to a possible new kind of pore forming protein not described before for any other bacteria.

The P13 C-terminal peptide has high pore-forming activity in artificial membranes. The P13 C-terminal peptide has been tested with the black lipid bilayer assay to check its pore-forming capacity. This small 28 amino acid peptide displayed a pore forming capacity with pores that vary in conductance from the pS range to bigger than 20 nS (Bárcena-Uribarri et al., unpublished results). A potential toxicity of this C-terminus for mammalian cells has still to be tested.

2.2.1 The paralogue family 48: The BBA01 protein

Eight P13 paralogues have been found within the genome of *Borrelia burgdorferi*, constituting the paralogue family [48]. P13 is a protein encoded in the main chromosome of *Borrelia.* All the paralogues are encoded in linear plasmids, some of them carrying two copies. Two of these paralogues are pseudogenes and do not produce functional proteins. A third paralogue displays an authentic frameshift producing a different protein and the other five produce conserved hypothetical proteins. BBA01 is from all of them the closest paralogue to P13 with a 54.1 % similarity on the amino acid level [79].

BBA01 displays porin activity and it is potentially interchangeable with P13. The recombinant expression and purification of BBA01 in *E. coli* revealed a porin activity similar to the one described for P13 [79]. An up-regulation of BBA01 in a P13 mutant raised the hypothesis of a possible function compensation of P13 [79].

2.3 BBA74: Oms28 a controversial porin

BBA74, also known as Oms28, was first described as a porin in the outer membrane of *Borrelia burgdorferi*. In 1995 Skare et al. described two pore-forming activities in the outer membrane vesicles coming from *Borrelia burgdorferi* [80]. The conductance of these two porins was 0.6 and 12.6 nS. A posterior study where the different proteins were isolated in different fractions by FPLC and SDS-Page elution attributed the 0.6 nS activity to a 28 kDa protein (Fig. 5) [81]. This protein was designated as outer membrane-spanning protein of 28 kDa, Oms28.

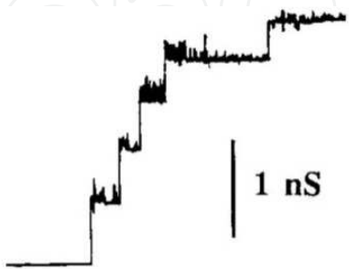

Fig. 5. Pore forming activity of 0.6 nS coming from outer membrane vesicles preparations of *Borrelia burgdorferi*. After isolation of outer membrane vesicles and further purification a clear 0.6 nS activity could be observed. This pore forming activity was attributed to BBA74. Taken from ref. [81] by permission.

The pore forming activity of Oms28 can be regained after separation in SDS-PAGE showing some resistant of this protein to the detergent SDS. Interestingly, certain oligomeric forms were observed after reducing the concentration of the detergent, removal of β-mercaptoethanol from the sample buffer and avoiding boiling prior electrophoresis [81].

Recombinant expression of BBA74 showed a similar pore-forming activity to the native protein. Recombinant production of BBA074 in *E. coli* and its posterior separation by SDS-PAGE and elution from the gel displayed a 1.1 nS pore forming activity. Native BBA074 has a 0.6 nS pore-forming activity. Possible explanations for the difference in conductance of the recombinant protein are an alteration in the tertiary structure folding or the preferential insertion as dimers [81].

BBA74 is transported to the periplasm. Computer analysis of the BBA74 protein sequence revealed a 24 amino acid signal sequence with a peptidase I cleavage site [81]. As described for outer membrane proteins BBA74 has a signal sequence to be transported from the cytoplasm to the periplasm. The initial protein of 28 kDa yields a mature protein of 25.3 kDa after the transport through the inner membrane and the cleavage of the signal sequence [81].

The association of BBA74 with the outer membrane resulted in some discrepancies in different studies. Skare et al. [81] showed the BBA74 association with the outer membrane by treating *Borrelia* cells with harsh salt solutions. BBA74 was retained in the membrane pellet as expected for integral membrane proteins [81]. Controversially, in cells subjected to Triton X-114 phase partitioning BBA74 partitioned exclusively into the aqueous phase whereas typical transmembrane proteins stay in the detergent phase [81, 82]. These results have to be considered with some caution because Pinne and Haake [83] demonstrated in a independent study that Triton X-114 can be problematic for the localization of outer membrane spanning proteins in spirochetes and these investigations need to be complemented with other methods to address this question accurately [83].

BBA74 lacks typical porin features. To complement the information about the possible localization of BBA74 additional experiments were performed. Its secondary structure was study by CD spectroscopy revealing a vast majority of α-helical folding [82]. The surface exposure of BBA74 was investigated by proteinase K digestion and immunofluorescence microscopy. No digestion of BBA74 or fluorescence could be observed in wild type strains of *B. burgdorferi* indicating that this protein has no accessible surface-exposed regions [82]. Usually bacterial porins partition in the detergent fraction when treated with Triton X-114, they have a β-barrel tertiary structure and they have some accessible surface exposed loops. Because BBA74 lacks this properties it has been hypothesized that it could be a protein only associated to the inner leaflet of the outer membrane.

Extracellular secretion of BBA74 has been described. Radiolabeled amino acids were used to study the secretion of proteins in *B. burgdorferi*. Free protein medium was supplemented with radiolabeled methionine and cysteine. After some growing time the cells and the medium were separated by centrifugation. In the medium some proteins were found, among them BBA74 (Oms28) and Bgp [84]. This fact supports the hypothesis of a possible BBA74 extracellular secretion.

However, it is known that *Borrelia* releases outer membrane vesicles and blebs containing BBA74, OspA and OspB under stressful conditions. To rule out the possibility of this being the cause for the detection of BBA74 in the media, immunoblots against these three proteins were carried out. While BBA74 was found in the cells and in the free-protein medium, OspA-B were only found in the cell pellet [84].

BBA74 was mainly secreted during the mid- to late-logarithmic phase. In the same study dealing with a possible secretion of BBA74 its secretion pattern was examined. Recollection of samples during the growing process revealed that BBA74 expression was at its highest level during the logarithmic phase while during the stationary-phase the amount of BBA74 was considerably smaller [84].

The *bba74* gene is transcribed exclusively during larval and nymphal blood meals. The sigma factor 70 is responsible for its transcription while RpoS independent and dependent mechanisms stops the transcription in response to arthropod and mammalian host-specific signals [85].

BBA74 is not expressed during murine infection and the loss of the gene does not seem to affect the infectivity or the transit between the tick and the mouse [85].

2.4 DipA / Oms38: A specific porin for dicarboxylates

In contrast to the general diffusion porin P66 with its huge single-channel conductance, DipA is a substrate-specific porin and exhibits a very small single-channel conductance. DipA is responsible for the rapid influx of compounds belonging to the dicarboxylates (Thein et al. unpublished).

DipA was first identified in knock-out mutants of *B. burgdorferi*, *B. burgdorferi* Δp66 and *B. burgdorferi* Δp13/Δp66 [54]. During black lipid bilayer experiments with isolated outer membranes of those mutants, high channel-forming activities in the conductance range between 10 and 100 pS were detected, which could not be related to one of the previously

described OM pores P13, Oms28, P66 and BesC. This finding indicated the presence of a porin with a small single-channel conductance in the outer membrane. Interestingly, after subjecting the outer membrane fraction of *B. burgdorferi* to hydroxyapatite chromatography, a pore-forming protein could be purified with an apparent molecular mass of 36 kDa (Thein et al. unpublished).

Mass-spectrometric analysis of the protein revealed its sequence and confirmed it to be a homologue of the Oms38 porin of relapsing fever *Borreliae* [86]. The deduced amino acid sequence contains an N-terminal cleavage site, which is typical for proteins localized in the outer membrane. The localization in the outer membrane was confirmed by electron-micrographs of immunogold-labeled *B. burgdorferi* cells decorated with antibodies against the identified 36 kDa protein. Computational analysis of the deduced amino acid sequence predicted putative ß-strands, which suggested that the secondary structure of DipA may contain many ß-sheets similar as is known for the ß-barrel cylinders of well-studied bacterial porins [87, 88]. All these findings indicated the identification of another porin in *B. burgdorferi*, and the protein was consequently named DipA, which stands for "dicarboxylate-specific porin A", due to its function as a dicarboxylate-specific porin (see below) (Thein et al. unpublished).

DipA was extensively characterized in the black lipid bilayer assay (Thein et al. unpublished): it forms pores in the artificial membrane which exhibit a small single-channel conductance of 50 pS in 1 M KCl. DipA pores were also investigated for voltage-dependent behavior. In the range from -120 V to +120 V the voltage does not show any influence on the conductance demonstrating that DipA is not voltage-dependent up to these potentials (Thein et al. unpublished).

DipA is a porin selective for anions. This was shown by multi-channel experiments under zero-current potential conditions. The permeability ratios of cations over anions through DipA were calculated from the zero-current potentials using the Goldman-Hodgkin-Katz equation [58]. They revealed together with the zero-current membrane potential that DipA is preferentially anion selective, because the ratios of the permeability coefficients P_{cation}/P_{anion} were 0.57 (in KCl), 0.47 (in LiCl). The P_{cation}/P_{anion} in KCH_3COO was 1.65, which means that also cations have certain permeability through the DipA pore (Thein et al. unpublished).

Strikingly, the DipA single-channel conductance of 50 pS is much smaller than the one of typical general diffusion pores [76]. This small single-channel conductance and the fact that growth of *Borrelia* is highly dependent on the uptake of nutrients [89, 90] suggests that DipA is a channel specific for essential nutrients and contains a binding site for them in a similar way as the carbohydrate-specific *E. coli* channel LamB [91, 92]. This hypothesis was tested by titration experiments using different classes of substrates as described previously for titration of LamB with carbohydrates [45, 92]. Interestingly, many classes of substrates that are necessary for bacterial growth including carbohydrates, such as glucose, fructose, sucrose, maltose and lactose, nucleosides, such as adenosine, and other anionic molecules, like acetate, carbonate, phosphate and adenosine triphosphate, do not show any interaction with DipA. Interestingly, DipA can be partly blocked by dicarboxylates (Thein et al. unpublished).

DipA-mediated channel-conductance can be partly blocked by addition of dicarboxylates. After DipA channel reconstitution into lipid bilayer membranes and having an approximately stationary membrane conductance, concentrated solutions of different dicarboxylates were added to the aqueous phase at both sides of the membrane. As a consequence, the DipA-

mediated membrane conductance was dose-dependently blocked. For example, the the DipA conductance decreases by 23% after addition of malate, 29% after addition of 2-oxoglutarate and 25% in after addition of phthalate at substrate concentrations of 27 mM, 9 mM and 4 mM, respectively (Fig. 6). Strikingly, DipA can be blocked by a variety of dicarboxylates and other related organic anions with high biological relevance. The tested compounds include oxaloacetate, succinate, malate, fumarate, maleate, 2-oxoglutarate, phthalate, citrate, aspartate, glutamate, and pyruvate. All anions listed previously block the ion current through DipA with a maximum block of channel conductance ranging from 20% for pyruvate to 31% for oxaloacetate (Thein et al. unpublished).

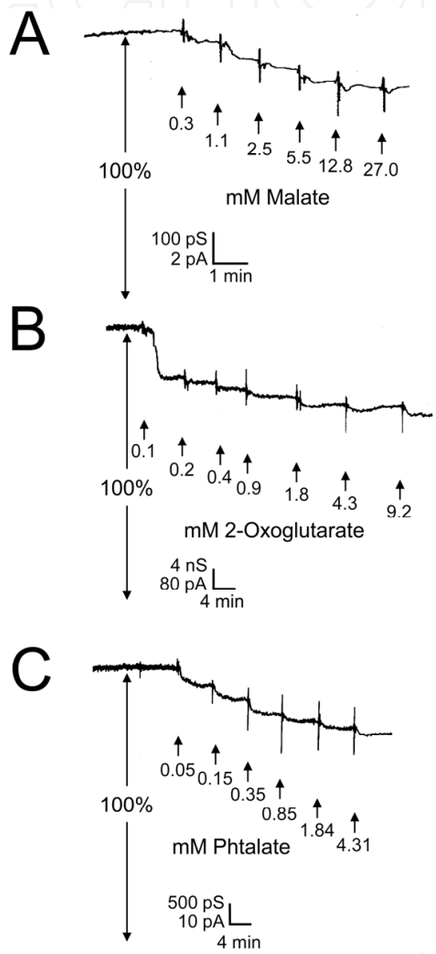

Fig. 6. Titrations of DipA-mediated conductance with different dicarboxylates. Membranes saturated with DipA were titrated with increasing concentrations of A) Malate, B) 2-Oxoglutarate, C) Phtalate. (Thein et al., unpublished). The binding of these molecules to the channel interior slows the translocation of KCl because of partial blocking of ion flux through the channel.

From these titration experiments, the binding affinities of the tested dicarboxylates to DipA were analyzed in a similar way as used for the characterization of carbohydrate-binding channels of Gram-negative bacteria [45, 92]. Binding of dicarboxylates to DipA yield high stability constants for oxaloacetate (K = 19,900 ± 5,100 l/mol), succinate (K = 6,100 ± 2,200

l/mol), malate (K = 1,300 ± 520 l/mol), maleate (K = 28,300 ± 950l/mol) and 2-oxoglutarate (K = 3,500 ± 140 l/mol). This means that binding of the tested compounds to the DipA channel show a significant specificity. The detailed study of the DipA specificity revealed that the stability constants depended strongly on the specific structure of the organic anion showing a maximum for C4-dicarboxylates oxaloacetate and maleate. The binding specificities to certain substrates are distinctly depending on the number of carboxylic acid groups and on side groups of the anions like oxo-, hydroxyl- or amino- groups (Thein et al. unpublished).

In analogy to other bacterial specific porins, it is likely that the DipA binding site with its high affinity for dicarboxylic anions increases the permeability of the channel for these metabolites as has been demonstrated previously: The presence of a binding site leads to an accelerated transport of carbohydrate through LamB and of phosphate transport through OprP, especially at very low substrate concentrations [40, 45]. Thus, the permeability of a substrate-specific porin can surpass that of a general diffusion pore by orders of magnitude in spite of its smaller cross-section [45].

Dicarboxylates, such as malate, succinate, oxaloacetate and 2-oxoglutarate, are major intermediates of the tricarboxylic acid cycle and are also used for synthesis of amino acids. For example, oxaloacetate and 2-oxoglutarate are important substrates for the biosynthesis of asparagine, aspartic acid and glutamic acid, respectively, which are essential proteinogenic amino acids. In addition, C4 dicarboxylates other than succinate cannot be metabolized due to the lack of a functional tricarboxylic acid cycle in anaerobic energy metabolism of most bacteria [93]. Taking these points into consideration, a potential dependence of the growth of *Borrelia* on this group of chemicals is likely. This hypothesis is additionally supported by the fact that the serum-supplemented mammalian tissue-culture medium for in vitro cultivation of *Borreliae* is supplemented by pyruvate and the tricarboxylic citrate. Amongst others, these compounds have been shown to specifically bind to DipA. Consequently DipA plays an important role in the uptake of dicarboxylates and related compounds across the outer membrane.

3. BesC: A channel-tunnel part of an efflux pump in the genus *Borrelia*

Many bacteria live in hostile environments where other organism or the infected hosts produce antimicrobial substances. To avoid possible toxicity of these compounds many bacteria have developed transport systems for export of harmful substances out of the cells called multi-drug resistance efflux systems [94, 95]. There are different types of these systems involved in the efflux of different substances like toxins, endogenous metabolic waste products or antibiotics. One of the most important multi-drug resistance efflux systems are the so called resistance-nodulation division (RND) transporters. This family of transporters is present in many living organisms but plays a crucial role in the export of toxic substances in Gram-negative bacteria.

The RND transporters in Gram-negative bacteria are composed of three components [95, 96]. An energy dependent transporter spanning the cytoplasmic membrane, a channel-tunnel crossing the outer membrane and the periplasmic space and a fusion protein located in the periplasmic space that connects both transporter and channel-tunnel. The best studied example of this kind of efflux pump is the multi-drug resistance pump AcrA-AcrB-TolC in *Escherichia coli*. In this case TolC is the protein situated in the outer membrane of *E. coli*, AcrB

is thought to be a proton-driven translocase in the inner membrane and AcrA is the fusion protein connecting AcrB and TolC.

Only one study has been published about this type of systems in *Borrelia*. The *Borrelia burgdorferi* B31 genome sequencing allowed the identification of a TolC homologue called in *Borrelia* BesC (Fig. 7). The genes flanking this gene showed also high homology to AcrA and AcrB and they were called BesA and BesB respectively. The name of the genes Bes comes from *Borrelia* Efflux System. Analysis of the RNA coding for these genes showed that they were co-transcribed and transcriptional linked [97].

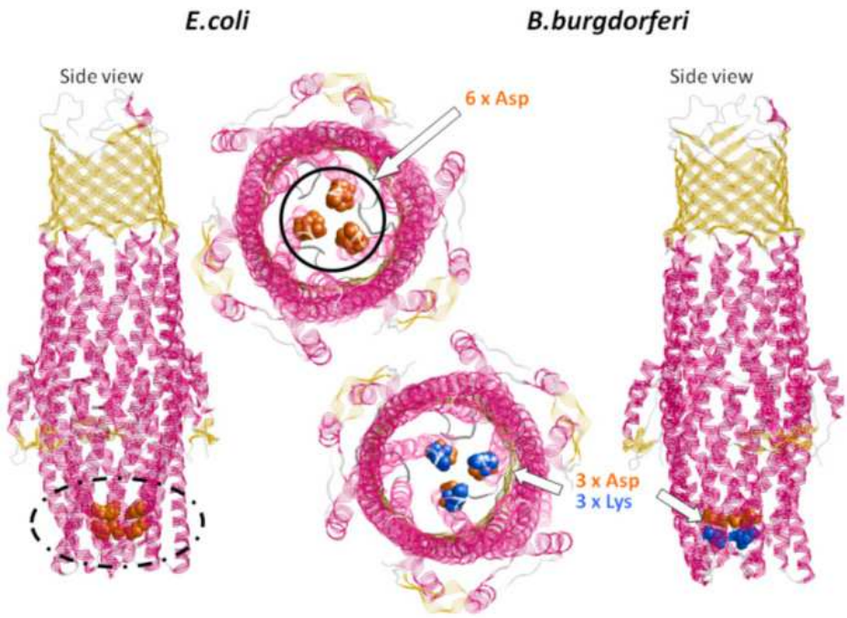

Fig. 7. Predicted 3D-structure of BesC based on TolC crystal structure [95]. Computer predictions for the structure of the TolC homologue BesC reveals a similar one as TolC. In contrast to TolC that has a positively charged periplasmic entrance, BesC has an entrance where positive and negative charges are compensated. Provided by Ignas Bunikis by courtesy.

BesC is essential for antibiotic resistance and necessary for mammal infection. A knock-out of the BesC gene resulted in a 2 to 64 fold decrease in the resistance to antibiotics compared to the wild type. Both the MIC (minimal inhibitory concentration) and MBC (minimal borreliacidal concentration) values were lowered by the lost of BesC [97]. Studies done in a mouse model showed that the BesC knock-out strain could not be recovered two weeks after mouse infection from heart, bladder, knee and ear biopsies. Whereas the knock-out strain was unable to survive in the mouse for that short period of time wild type and a complemented strain grew well in BSKII media after being collected from mice [97].

BesC forms pores in the outer membrane of *Borrelia*. Studies of BesC using the black lipid bilayer assay showed that BesC had an average single channel conductance of about 300 pS in 1M KCl at 20 mV transmembrane potential [97]. Channel-forming activity of pure BesC samples could be inactivated by their incubation with antibodies against BesC [97]. This provided further evidence that the channel-forming activity of the 300 pS channels were caused by BesC.

Biophysical studies of BesC suggested a slight anion selectivity and voltage independence. Zero-current membrane experiments were performed to study the ion selectivity of BesC. The increase of different salt concentrations in one of the membrane sides revealed no preference for translocation of anions or cations through the BesC channel [97]. Similarly, application of different positive and negative voltages up to 150 mV to a membrane saturated with BesC channels showed no reduction in the channel conductance revealing a very stable and voltage-independent channel [97].

The structure of BesC shows some differences to that of TolC [97]. Modeling of the BesC structure taking advantage of the known structure of TolC and TolC homologues revealed some variations especially at the N-terminal and C-terminal ends. A wider tunnel entrance in the BesC model could explain its 4-fold increased conductance in relation with TolC of *E. coli*. The missing ion selectivity of BesC could be explained by the substitution of one negatively charged aspartic acid residue by positively charged lysines at the periplasmic entrance of the three BesC monomers. The substitution of three negative charges at the entrance of the BesC trimer could counterbalance the residual three negative aspartates at the entrance, thus explaining the missing ion selectivity (Fig. 7).

All together there are five pore-forming proteins described in *Borrelia*. Four of them are porins and BesC is a channel-tunnel forming part of an efflux pump system. Pore forming proteins are usually characterized by different biophysical features such as its single channel conductance, ion selectivity, voltage dependence and possible specificities for substrates. The biophysical properties of P66, P13, BBA74, DipA and BesC are summarized in Table 1.

4. Conclusions

Spirochetes differ in many ways to other groups of bacteria. Similar to most bacteria, *Borrelia* obtain the nutrients from the surrounding media. The first step in the acquisition of essential substrates is their transport through the outer membrane. In all bacteria including *Borrelia* this transport is accomplished by protein channels located in the outer membrane named porins. However, the porins described so far in *Borrelia* seem to have special characteristics.

P66 is a special porin and remains an interesting research object, due to the fact that it shows a dual function as an adhesin and as a porin. Furthermore, it has an extremely high single-channel conductance and probably a peculiar molecular organization. Anyway, the exact molecular structure remains to be revealed by crystallization. Considering the high single-channel conductance of P66 and the organization as a molecular sieve with defined cut-off in the outer membrane of *Borrelia* cells, P66 seems to play a major role in the first import steps of general nutrients and other molecules.

P13 is probably one of the most intriguing proteins in *Borrelia*. Despite its small molecular weight and its secondary α-helical structure it forms channels in the outer membrane of *Borrelia*. The organization of P13 in protein complexes is required to form a channel given its small size. Apart from that, the presence of a periplasmic-cleaved C-terminal peptide which function is not completely understood is unique among porins. A very remarkable feature of P13 is the presence of up to eight paralogues in the genome of *Borrelia burgdorferi*. From those only BBA01 has been studied, showing similar pore forming characteristics as P13. The reason for the high number of copies for this gene is still not understood but it reinforces the idea of P13 being an essential outer membrane protein. The up regulation of BBA01 in a P13 knock-

out to probably compensate its lack and the impossibility to obtain a P13-BBA01 double mutant reinforces this theory.

The function of BBA74 (Oms28) is controversial. It was described first as a porin with a single channel conductance of 0.6 nS. Its fractionation in the aqueous solution after treatment with Triton X-114 instead of in the detergent fraction where transmembrane proteins normally are found motivated later on an additional study. It concluded that BBA74 lacks the typical porin properties and it is just associated with the internal leaflet of the outer membrane. An independent study showed an extracellular secretion of BBA74 together with other proteins. This secretion seems to happen during the logarithmic growth phase and it is independent from the production of blebs or vesicles. Further studies are required to unify the knowledge of this protein and why the investigations created quite contradictory results.

Protein	M.W. [kDa]	Conductance [nS] in 1 M KCl	Selectivity	Voltage-dependence	Function
P66	66	11.0	not selective	yes	adhesin, porin
P13	13	3.5	for cations	no	porin
BBA074 (Oms28)	25	0.6	*n.d.*	no	porin-like properties?
DipA	36	0.05	for anions	no	dicarboxylate-specificity
BesC	48	0.3	not selective	*n.d.*	part of efflux- system

Table 1. Biophysical properties of the pore-forming proteins described in the outer membrane of *Borrelia burgdorferi*. M.W. means molecular weight of the processed protein; *n.d.* means not determined; conductance means average single-channel conductance measured in 1 M KCl.

DipA does not form general diffusion pores, but it is a specific porin. Its permeability properties are determined by charges in the channel that act like a filter. Thus, DipA is the first identified *Borrelia* porin exhibiting a substrate specificity and therefore has presumably a well-defined function in the biology of this spirochete. Its small conductance and its presence next to channels up to about two hundred times bigger such as P66 are very remarkable. This fact can only be explained by some kind of specificity of these channels for some indispensable substrates for *Borrelia*.

BesC is a well conserved homologue of the extensively studied TolC of *E. coli*. BesC forms part of a bigger complex similar as TolC that spans both membranes in *Borrelia*. This whole complex is involved in the export of toxic substances and antibiotics and plays presumably an important role for the infection in mammals.

The *Borrelia* porin research could have important consequences for the development of new strategies in diagnosis and vaccination to improve the treatment of infections by these bacteria. The ability of *Borrelia* to change their surface antigens and escape the immune system has made its correct diagnosis and treatment a really hard task. Surface-exposed essential proteins of the bacteria are the perfect candidates to be used as diagnosis/treatment targets. Porins could be used as ideal targets because they are important proteins for the biology of bacteria and can be fundamental in an infection

procedure. The identification of the specific function, structure and expression profile of a porin is therefore relevant and a fascinating field to research on.

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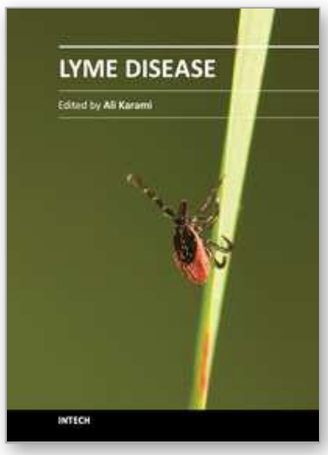

Lyme Disease
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Lyme disease, or Lyme borreliosis, is an emerging infectious disease caused by bacteria belonging to the genus borrelia. Borrelia burgdorferi, in the strict sense. This book deals mostly with the molecular biology of the Lyme disease agent orrelia burgdorferi. It has been written by experts in the relevant field and is tailored to the need of researchers, advanced students of biology, molecular biology, molecular genetics of microorganism. It will also be of use to infectious disease experts and people in other disciplines needing to know more about Lyme borreliosis. The book contains chapters on the molecular biology of the Lyme disease agent, zoonotic peculiarities of Bb, advancement in Bb antibody testing, the serology diagnostic schemes in Bb, discovering Lyme disease in ticks and dogs, adaptation to glucosamine starvation in Bb, and porins in the genus borrelia.

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