Isolation and characterization of channel-forming proteins in the outer membrane of *E. coli* and *Borrelia* species

Dissertation

zur Erlangung des naturwissenschaftlichen Doktorgrades

der Fakultät für Biologie

an der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

Katrin Denker

Hansestadt Lübeck

Würzburg, 2005

Eingereicht am:

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. _____

1. Gutachter: Prof. Dr. R. Benz

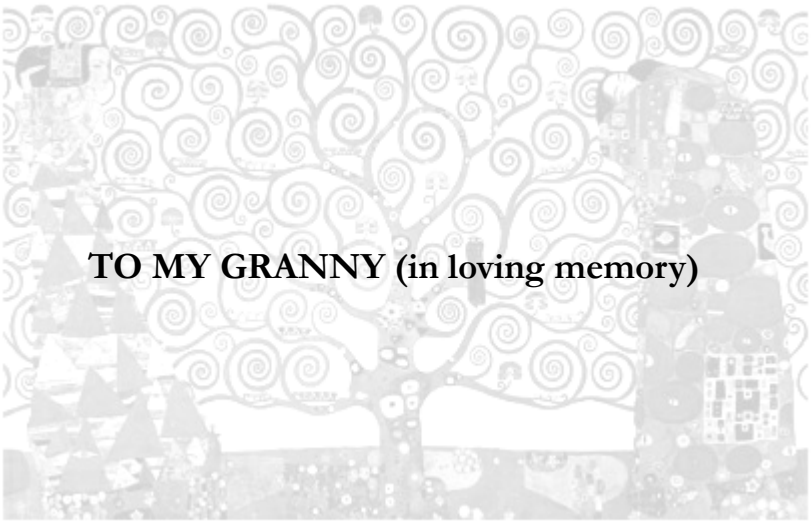
2. Gutachter: Prof. Dr. S. Bergström

Tag des Promotionskolloquiums: _____

Doktorurkunde ausgehändigt am: _____

Diese Dissertation wurde von mir selbständig und
nur mit den angegebenen Quellen und Hilfsmitteln angefertigt.

.....



TO MY GRANNY (in loving memory)

PUBLICATIONS

REGULAR PAPERS

- **Denker K., Orlik F., Schiffler B., and Benz R.** Site-directed mutagenesis of the greasy slide aromatic residues within the LamB (maltoporin) channel of *Escherichia coli*: effect on ion and maltopentaose transport. J Mol Biol. 2005 Sep 23;352(3):534-50.
  Reprinted with permission from Elsevier.

- **Denker K., Thein M., Larsson C., Mentele R., Lottspeich F., Bergström S., and Benz R.** Discovery of a channel-forming protein in the cell wall of the relapsing fever pathogen *Borrelia duttonii*. (submitted).

- **Denker K., Andersen C., Pinne M., Bunikis I., Bergström S., Sickmann A., and Benz R.** Identification of a 300 pS channel-forming protein in the *p66* knock out mutant of *Borrelia burgdorferi* senso stricto strain HB19. (submitted).

CONTENTS

CHAPTER 1

1.1 INTRODUCTION

Traditionally, the eubacteria are divided in the three subgroups (Mycoplasma, gram-negative and gram-positive bacteria) based on differences of their cell envelope. All three have the cytoplasmic membrane in common. The cytoplasmic membrane contains many transporter systems and determines what goes in and out of the organism (Madigan et al., 2003). All cells must take in and retain all the various substances needed for their metabolism.

One subgroup of the eubacteria is the cell wall free Mycoplasma and has just a cytoplasmic membrane. The major difference regarding the cell wall between the other two subgroups, gram-negative and gram-positive bacteria, is the restricted diffusion of hydrophilic solutes across the cell envelope. This is the result of a second permeability barrier besides the cytoplasmic membrane, the outer membrane of gram-negative bacteria (Nikaido and Vaara, 1985) (see Figure 1.1).

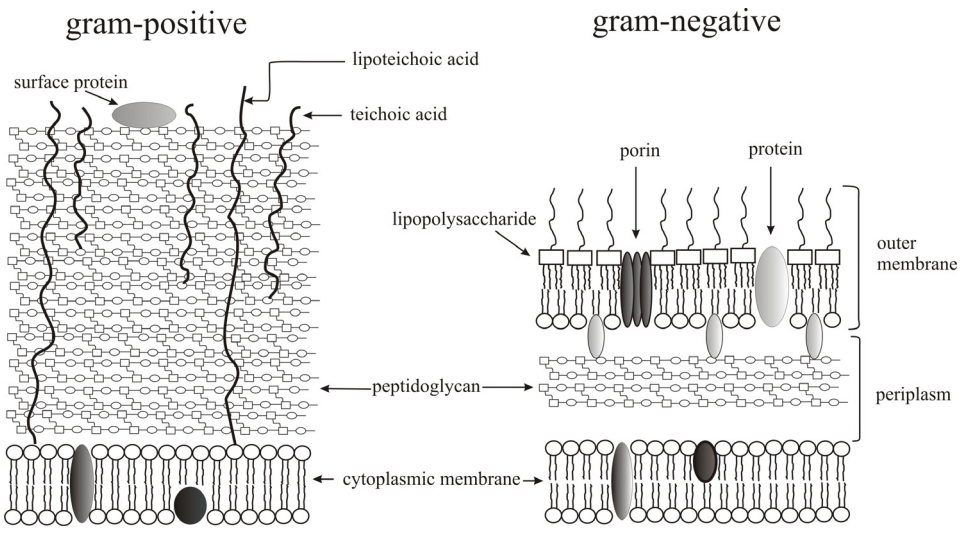


Figure 1.1 schematic illustration of gram-positive and gram-negative cell walls, based on figures in (Madigan et al., 2003)

A main component of the cell wall is the peptidogylcan. However, it occurs in different amounts in these two subgroups and indicates a second major structural difference between the two subgroups. The gram staining procedure makes the different properties of gram-negative and gram-positive cell walls visible.

Gram-positive bacteria have a thick mesh-like cell wall made of up to 40 layers of peptidoglycan, which is capable of retaining the crystal violet–iodine complex (violet dye) when it is dehydrated in the gram staining procedure. Gram-negative bacteria have a thinner cell wall than gram-positive bacteria. In addition to the cytoplasmic (inner) membrane, they have the outer membrane which contains lipids, lipopolysaccharides (LPS), and proteins and is separated from the cell wall by the periplasmic space, where just several layer of peptidoglygan are located. The thin layer of peptidoglycan is unable to retain the crystal violet–iodine complex and therefore gram-negative bacteria are not stained. Usually a counter stain with fuchsine dye is utilized to make gram-negative bacteria visible.

Two Gram-negative bacteria of different appearance: *E. coli* versus *Borrelia*

Within the group of gram-negative bacteria structural differences in the outer membrane and differences in other morphologic details have developed through evolution.

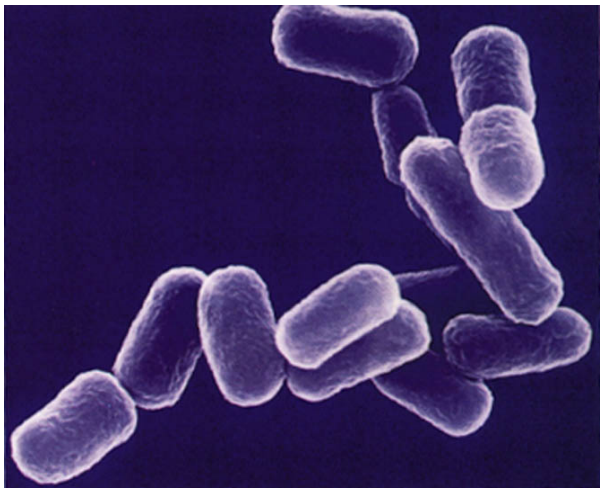


Figure 1.2 electron micrograph depicts a group of *E. coli* bacteria, taken from http://spacebio.net/modules/mb_resource/Ecoli.jpeg

E. coli as one of the youngest bacteria of the evolution belongs to the Proteobacteria-4γ (Gupta, 2000) and is a member of the enterobacteria family. In nature *E. coli* is found in the lower intestines of warm-blooded animals. It is characterized as a non spore forming, rod shaped, facultative anaerobic bacteria and it is an indicator for fecal contaminations (Schlegel, 1992). Because of simply cultivation properties and harmlessness for healthy humans *E. coli* is used as

a model organism for bacteria in general. The genomes of several *E. coli* strains are sequenced. The *E. coli* genome on a circular chromosome has a size of app. 4.6 Mbp (basepairs) and has a G-C content of around 50% (Blattner et al., 1997).

In some cases the generally harmless bacteria *E. coli* can become infectious, when they gain additional genetic information encoding a variety of virulence factors such as adhesins, capsular genes, siderophores or toxins. Uropathogenic *E. coli* strains (UPEC) can cause "honeymoon cystitis" when the bacteria enter the urinary tract. Furthermore some *E. coli* strains can produce very harmful toxins. In summary, it can be said that *E. coli* is a very important bacteria for the genetic and medical research.

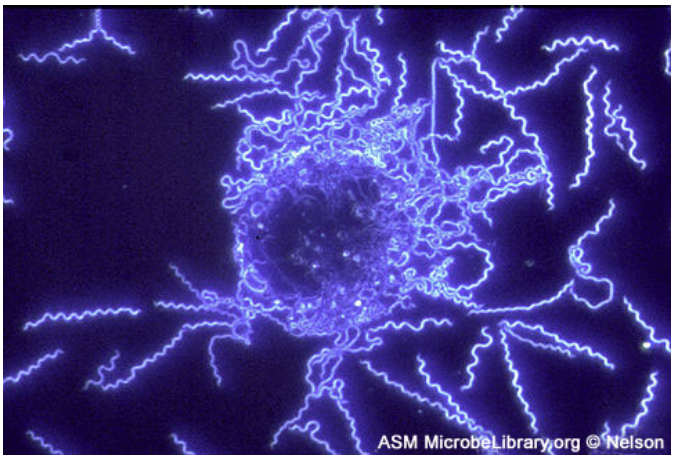


Figure 1.3 micrographs of *Borrelia burgdorferi* sento lato, taken from http://www.microbelibrary.org/images/jnelson/Images/borrelia.jpg)

The genus *Borrelia* belongs to the eubacterial phyla of the spirochetes, which is genetically very far related to the Proteobacteria (Gupta, 2000; Woese, 2000). Spirochetes have a characteristic shap (see Figure 1.3). They are helically shaped and very flexible. All species of the genus *Borrelia* are microaerophilic and transmitted to vertebrates by hematophagous arthropods (Barbour and Hayes, 1986). In the case of *B. burgdorferi Ixodidae* ticks are used as vectors for transmission of *Borrelia* to their reservoir, which are vertebrates (mostly small rodents and deer). The infection of humans happens accidentally and for *B. burgdorferi* it means a dead end. Infection of humans is called Lyme borreliosis or Lyme disease and can be treated with antibiotics (Nadelman et al., 2001; Steere et al., 2004). Diagnosis is not easy, if the typical symopton of early Lyme disease, an erythema migrans (red circular skin lesion) is absent, because other symptoms can be unspecific fever or flu like symptoms. In late states of infection the treatment with antibiotics can be problematic, because *Borrelia* disseminate to other organs, for example joints, which are not well supplied with blood (Steere et al., 2004). For the genus *Borrelia B. burgdorferi* is a model organism, because the genome of *B. burgdorferi* B31 is

sequenced and genetic manipulations have been established in it. The *B. burgdorferi* genome differs completely from any known bacterial genomes. It consists of a linear chromosome of 0.9 Mbp and at least 17 linear and circular plasmids, which have together an additional size of 0.5 Mbp (Casjens et al., 2000; Fraser et al., 1997). In contrast to *E. coli*, the G-C content in *B. burgdorferi* lies between 27-32%, which is low for a Gram-negative organism. One special feature of *B. burgdorferi* is the loss of some of its plasmids during cultivation concomitant with loss of virulence (Purser and Norris, 2000).

Another infectious *Borrelia* family causes relapsing fever. In contrast to *B. burgdorferi*, relapsing fever *B. recurrentis* is transmitted by lice and is spread over the whole world (Dodge, 1973; Dodge, 1973). *B. duttonii,* the relapsing fever agent in Africa, is transmitted by soft body ticks (*Ornithodorus moubata*) and it has been reported that humans are their only reservoir (Barclay and Coulter, 1990; Dutton JE, 1905). *B. hermsii* is the best studied relapsing fever agent causing relapsing fever in the US and it is transmitted by the soft body ticks *Ornithodorus hermsii* (Porcella et al., 2005).

Gram-negative cell wall (*E. coli* versus *Borrelia*)

The outer cell envelope of *E. coli* and *B. burgdorferi* are quite similar, however there are also characteristic differences (see Figure 1.4).

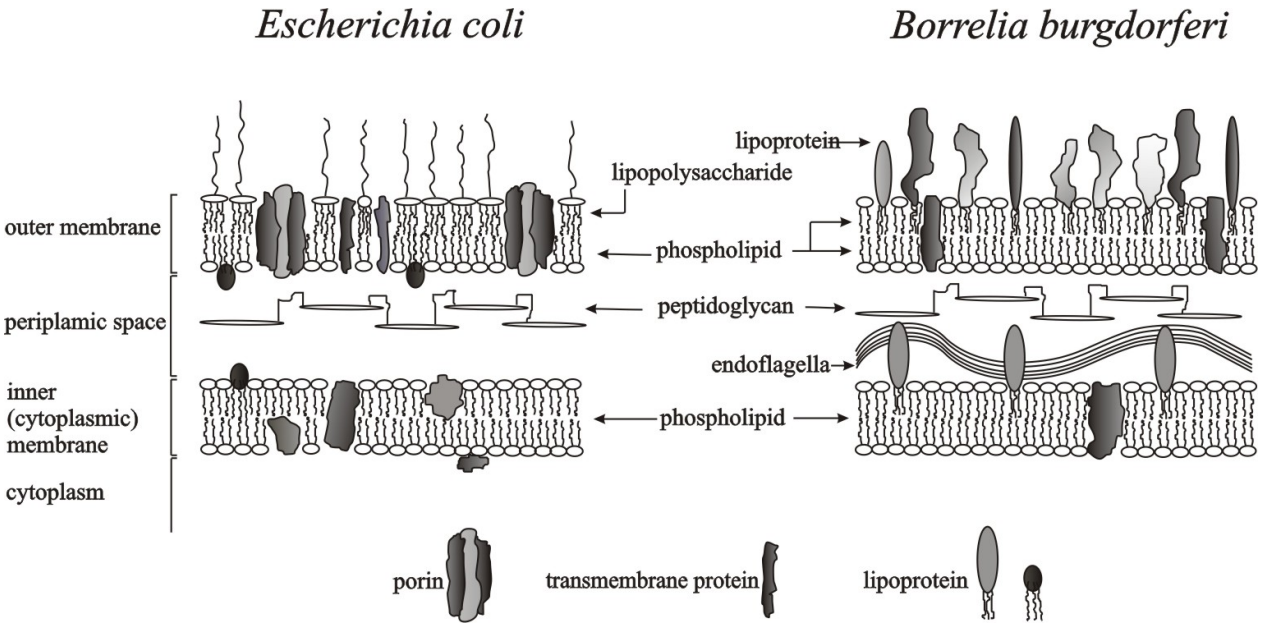


Figure 1.4 schematic illustration of the cell walls of *E. coli* and *B. burgdorferi* sento lato, based on (Tokuda and Matsuyama, 2004) and (Bergström et al., 2002)

In *E. coli* the outer leaflet of the outer membrane, which is the contact area to the environment is composed of lipopolysaccharides (LPS). LPS are completely missing in *Borrelia* (Takayama et al., 1987). *B. burgdorferi* in contrast has a huge amount of lipoproteins anchored in the outer leaflet.

Another tpyical feature of spirochaetes are endoflagella. The characteristic helical shape as well motility of *Borrelia* is established by flagella, which is located in the periplasmic space. The numbers of flagella in one cell varies in the genus *Borrelia*. For *B. burgdorferi* 7-11 flagella can be present (Barbour and Hayes, 1986).

Integral outer membrane proteins with the function as porins have also been reported for *Borrelia* (Ostberg et al., 2002; Shang et al., 1998; Skare et al., 1996; Skare et al., 1997). However, a structure of any channel-forming protein (porin) has so far not been determined.

Outer membrane proteins of *E. coli*

Outer membrane proteins of *E. coli* are well studied and the model organism *E. coli* can provide one prototype for each of the six functionally and structurally different families. While α-helical bundles have only been found in the cytoplasmic membranes, for *E. coli* it has been shown that β-barrels are restricted to the outer membrane and all six groups are structurally formed as β-barrels (Koebnik et al., 2000).

The first group of the outer membrane proteins are the small β-barrel membrane anchors composed of eight β-strands. A classical representative is OmpA, which serves as an anchor between outer membrane and peptidoglycan (Koebnik, 1999; Ried et al., 1994). Whereas OmpX belongs because of its structure to this group, however its function is the neutralization of the host defense mechanisms (Heffernan et al., 1994; Vogt and Schulz, 1999).

The second group are the membrane integral enzymes built by 12 transmembrane β-strands, like the outer membrane phospholipase A (OMPLA) (Snijder et al., 1999).

The barrel interior of OmpA, OmpX, and OMPLA is too small to allow the passage of ions or substrates. Thus, they do not act as pore-forming proteins.

Thirdly, the group of TonB-dependent receptors has the biggest β-barrel known so far. A representative of this group is FhuA, which is built out of 22 β-strands. These huge barrels of this group are necessary for the uptake of iron-siderophore complexes (FhuA) and large substrates like vitamin B12 (BtuB) (Chimento et al., 2003; Ferguson et al., 1998; Killmann et al., 2002; Killmann et al., 1995).

Recently the structure of the FadL family has been determined. The Fadl family is a transporter family for hydrophobic compounds, like long chain fatty acids. The transport across the membrane is based on diffusion and coupled with conformation changes in the 14 β-stranded barrel (van den Berg, 2005).

There are two more families of channel-forming proteins. The channel tunnels and porins are described in more detail in the following chapter "Channel-forming proteins of *E. coli*".

Outer membrane proteins of *Borrelia*

Outer membrane proteins of *Borrelia* are represented by a high quantity of lipoproteins. This chapter provides an overview of the ones that have been most important for this work. For a complete picture of *Borrelia* outer membrane proteins the review of Cullen et. al. (Cullen et al., 2004) is recommended. Lipoproteins of *Borrelia* are somehow comparable to the lipopolysaccharides (LPS) of *E. coli.* They are located on the outer leaflet of the outer membrane like the LPS in *E. coli*. In contrast to *Borrelia*, *E. coli* lipoproteins are anchored to the periplasmic surface of the inner leaflet of the outer membrane (Tokuda and Matsuyama, 2004). The lipoproteins in *B. burgdorferi* are named outer surface proteins (Osp's) and have several representatives. The crystal structure of OspA and its function as plasminogen receptor and adhesin in the tick midgut is known (Li et al., 1997) (Fuchs et al., 1994) (Pal et al., 2000). The expression of OspA is upregulated in ticks and it has been demonstrated that antibodies against OspA can block transmission of *Borrelia* from tick to mammalian host (de Silva et al., 1996). Based on this knowledge the first vaccine against Lyme disease was developed. For the lipoprotein OspB a similar functions as OspA has been reported (Cullen et al., 2004).

In contrast to OspA, OspC is upregulated when *B. burgdorferi* invades a human, but it can not be used as a new candidate for a vaccine because it is not highly conserved between different *Borrelia* strains (Wang et al., 1999). Furthermore, OspC is related to the variable surface proteins of *B. hermsi*, a representative of the relapsing fever *Borrelia* (Cadavid et al., 1997).

Variable surface proteins of relapsing fever *Borrelia* are subdivided into two groups. The variable large proteins (Vlp's) and the variable small proteins (Vsp's) define the serotypes of *B. hermsii* (Hinnebusch et al., 1998). The expression of the variable surface proteins is correlated to the pattern of the disease. At least three mechanisms of antigenic variation are known for the variable surface proteins (Kehl et al., 1986).

As mentioned before channel-forming proteins in the outer membrane, such as porins, have also been reported for *Borrelia*. The chapter "Channel-forming proteins in *Borrelia*" gives a detailed

description.

Channel-forming proteins of *E. coli*

Channel-forming proteins are necessary for the uptake of several substrates, like phosphate, sugars and nucleotides, but also for the efflux of antibiotics, dyes, or heavy metals and secretions of proteins like toxins or proteases.

For efflux processes channel tunnels are important as the outer membrane component of an export machinery spanning the whole cell envelope. They interact with inner membrane complexes comprising ABC transporter for secretion or proton antiporter for efflux (Andersen, 2003). The channel tunnel of *E. coli* TolC is a homotrimer with a 140 Å long cannon-shape structure. Each of the three monomers contributes four β-strands to form a single 40 Å long β-barrel, which is anchored in the outer membrane and is a single-channel forming domain (Koronakis et al., 2000). The β-barrel is prolonged by a 100 Å long tunnel domain formed exclusively by α-helices (see Figure 5). TolC homologues are ubiquitous among gram-negative bacteria, and already nearly a hundred have been identified (Koronakis, 2003).

The outer membrane protein family of the porins is necessary for the uptake of substrates and they are also usually trimers, but of totally different architecture as TolC. Porins form one barrel of 16 or 18 β-strands per monomer and therefore the trimeric assembly contains three separate channels (Koebnik et al., 2000). They can be subdivided into general (nonspecific) porins (OmpF) and substrate specific porins (LamB). OmpF is a classical diffusion pore and allows the diffusion of molecules smaller than 600 Da through a β-barrel build by 16 β-strands spanning the outer membrane (Garavito, 1994). An example for a specific pore is the well studied LamB, which is the outer membrane component of the *mal* regulon (Benz et al., 1986; Schirmer et al., 1995). The channel forms like OmpF a homotrimer and it is specific for maltose and maltodextrins. The specificity is caused by a special arrangement of amino acids within the channel. The loop 3 with the residue Y118 is inwardly folded and constricts the diameter of the channel. Furthermore inside the channel is a belt of six aromatic amino acids (“greasy slide”) lined up with polar amino acids (“polar tracks”). These aromatic residues form a pathway from the vestibule to the periplasmic outlet of the porin (see Figure 4.1B). Sugar binding obstructs the channel constriction leading to a blockage of the ion flow in black lipid bilayer experiments. By moving through the channel the sugar “glides” along the aromatic residues and hydrogen bonds between the sugar and the “polar tracks” are formatted and disrupted (Meyer and Schulz, 1997).

Recently the crystal structure of the nucleoside specific porin of *E. coli* Tsx was published (Ye

and van der Berg, 2004). The structure of Tsx is an exception within the channel forming protein family, because it forms monomers with 14 β-strands instead of trimers.

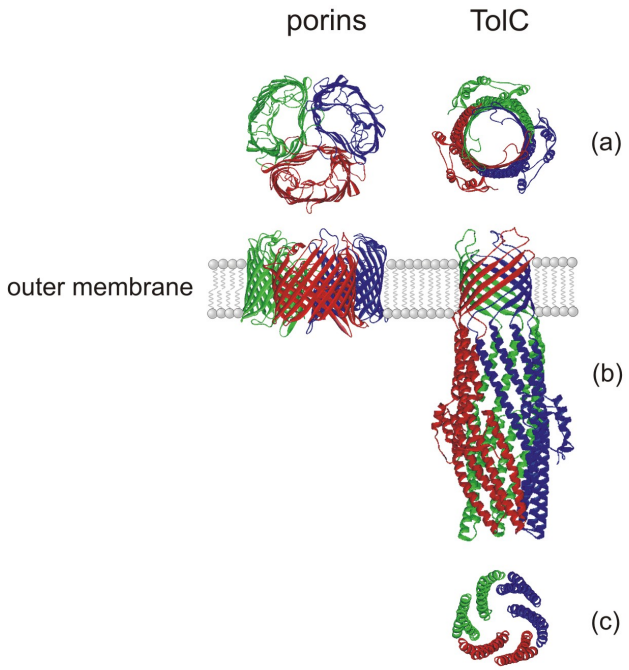


Figure 1.5 Porins and TolC: (a) view down the outer membrane channels, (b) side view, at right angles to the plane of the outer membrane, and (c) cross-section of TolC near the tunnel entrance; modified figure of (Andersen et al., 2001); with permission from Elsevier

Channel-forming proteins of *Borrelia*

By now, three pore-forming proteins of *B. burgdorferi* are described in literature. P66 is a 66 kDa protein, which has an integrin $\alpha_v\beta_3$ binding property and a channel forming ability with a huge single-channel conductance of 11.5 nS in 1 M KCl (Coburn et al., 1999; Skare et al., 1997).

Oms28 is a pore forming protein with a small single-channel conductance of 0.6 nS in 1 M KCl. Details about the function of this protein are not known so far (Skare et al., 1996).

P13, a 13 kDa integral outer membrane protein of *B. burgdorferi*, was identified as a porin using the black lipid bilayer assay (Ostberg et al., 2002). P13 has an unusually small molecular mass compared with other known porins having usually molecular masses of more than 30 kDa. The P13 protein is processed at the amino (N)-terminus and carboxyl (C)-terminus (Noppa et al., 2001). As for the other pore-forming proteins of *Borrelia*, the structure of P13 is not determined, but it would be of great interest to investigate how such a small protein arranges to build a transmembrane spanning barrel with a conductance of 3.5 nS.

Single-channel conductance measurements of the outer membrane fraction shows that *B.*

burgdorferi contains many other pore-forming proteins besides P66, Oms28, and P13. Various sizes of single channel conductance could be detected. Some were of very small size which leads to the conclusion that *B. burgdorferi* has like *E. coli* general diffusion porins and specific porins.

A channel tunnel such as TolC most likely is present in *B. burgdorferi*. A BLAST search revealed that a homologue (BB0142) is present in *B. burgdorferi* genome.

The relapsing fever agent *B. hermsii* was also studied for channel forming proteins. A P66 homologue was found and single-channel conductance fluctuations of various magnitudes were observed in the outer membrane preparation (Shang et al., 1998).

Aims of the work

The aim of the work was to functionally characterize outer membrane channels of the relapsing fever pathogen *B. duttonii* and the Lyme disease agent *B. burgdorferi*.

I have characterized an 80 pS channel of *B. duttonii.*

Furthermore I have identified the *B. burgdorferi* TolC homologue (BB0142) from a *p66* knock-out strain HB19/K02. The channel has single-channel conductance of 300 pS and shows different properties to *E. coli* TolC.

In addition, point mutations in the maltose binding protein LamB of *E. coli* could demonstrate the importance of the "greasy slide" for the sugar specificity of this channel.

CHAPTER 2

Discovery of a channel-forming protein in the cell wall of the relapsing fever pathogen *Borrelia duttonii*

2.1 SUMMARY

Outer membrane preparations of three *Borrelia* relapsing fever species have been studied for pore forming activity in the black lipid bilayer assay. Histograms of single-channel conductions fluctuations were obtained from single-channel experiments with outer membrane preparations of *B. hermsii, B. recurrentis* and *B. duttonii*. All strains had a different conductance fluctuation pattern with a broad range of single-channel conductance values varying from 0.5 ns – 11 nS. All three strains have a high pore forming activity at around 0.5 nS in common. Furthermore for one of the species, *B. duttonii*, a channel-forming protein with a single-channel conductance of 80 pS in 1M KCl could be isolated from the outer membrane preparation. Characterization of the 80 pS channel showed that it is slightly anionic selective and voltage independent.

2.2 INTRODUCTION

Old world tick-borne relapsing fever (RF), caused by the spirochetal pathogen *Borrelia duttonii*, is a common endemic disease in Central, East and South Africa. *B. duttonii* is transmitted to humans by the bite of an infected soft body tick *Ornithodorus moubata* (Barclay and Coulter, 1990). This was first time described in literature 1904 by Dutton (Dutton JE, 1905). Most infected people are farmers who live in traditional huts constructed of mud with flat, earth covered roofs. They sleep on the floor on animal skin and frequently share their accommodation with small livestock (Cutler et al., 1999). The bite by a tick occurs normally unrecognized, because they feed quickly within 30 minutes during night (Schwan et al., 2003); (Walton, 1962).

The pathogen is transmitted when infectious salivary and coxal secretions contaminate the feeding site (Barclay and Coulter, 1990). Man is the only reservoir for *B. duttonii* and the disease is characterized by high fever with chills, headache, myalgia and coughing. Each episode of high fever is followed by a period of well-being but several other episodes of high fever and other effects like abdominal pain, vomiting, diarrhea and photophobia may follow. The symptoms and recurrent fever of the disease have been linked to antigenic variation of and antibody production against different surface antigens presented by *B. duttonii* in the blood of the patient. The mechanism of antigenic variation has been thoroughly investigated in the closely related RF species *B. hermsii* and the recurring pattern of fever coincides with massive spirochetemia in the blood and is a consequence of the antigenic variation of the most abundant surface protein, variable major protein (Vmp), used by the bacterium to evade host antibody-mediated immune response (Barbour, 1990; Burman et al., 1990; Meier et al., 1985). The host develops immune response against these lipoproteins recently divided into two different families, Vsp (variable small proteins) and Vlp (variable large proteins) (Hinnebusch et al., 1998; Parola and Raoult, 2001). Also for the louse borne species *B. recurrentis*, which is very close related to *B. duttonii*, the Vmp is an important factor for infection. The Vmp is identified as the TNF-inducing factor (Scragg et al., 2000). A unique property of *B. duttonii*, is the ability to form erythrocyte aggregates which block precapillary blood vessels causing haemorrhage and inducing cell death and hypoxia (Burman et al., 1998; Shamaei-Tousi et al., 1999).

The extracellular pathogen *B. duttonii* is classified as a gram-negative microaerophilic mobile spirochete. The cell envelope consists of an outer membrane, periplasmic space and cytoplasmic membrane, similar to the cell envelope architecture of other gram-negative bacteria. However, the class Spirochaetes differs from other gram-negative bacteria because of the localization of the flagella in the periplasmic space (endo flagella). Furthermore, the outer membrane contains lipoproteins instead of lipopolysaccharides (LPS) (Takayama et al., 1987).

The cell wall of gram-negative bacteria acts as a molecular filter for hydrophilic solutes. These molecular sieving properties are the result of channel forming proteins in their outer membranes, called porins. Porins are integral proteins which form large water filled pores through the outer membrane (Benz et al., 1978). They are involved in the uptake of substances from their environment and can be subdivided into two classes. The porins of the first class can be described as general diffusion pores, such as OmpF of *E.coli* K12 (Benz et al., 1985). These porins sort mainly according the molecular mass of the solutes. The second class contains pores with a binding site inside the channel. These specific porins are responsible for the rapid uptake of classes of solutes such as carbohydrates (Benz et al., 1986; Ferenci et al., 1980), nucleosides

(Maier et al., 1988) or phosphate (Benz and Hancock, 1987).

Within the class Spirochaetes the species *B. burgdorferi* lacks genes involved in the synthesis of certain amino acids, fatty acids, co factors and nucleotides (Fraser et al., 1997). Consequently, *B. burgdorferi* needs complex media containing all these nutrients for effective growth. It has been demonstrated that these bacteria grow best in Barbour-Stoenner-Kelly media (BSK) supplemented with rabbit serum (Barbour et al., 1984). This suggests that *B. burgdorferi* and other *Borrelia* species may need special or specific porins (transport proteins) for uptake of these nutrients.

Only a few integral membrane proteins have previously been identified and characterized for *Borrelia* spp. Three porins of *B. burgdorferi*, the Lyme disease agent, are identified. These are P66 (Skare et al., 1997), P13 (Ostberg et al., 2002) and Oms28 (Skare et al., 1996). A homolog of P66 was identified in the outer membrane of the causative agent of new world relapsing fever *B. hermsii* (Shang et al., 1998).

Pore forming outer membrane proteins of gram-negative bacteria form ß-barrel cylinders. A prominent example is maltoporin of *E. coli* that is formed by a trimer of three identical polypeptide subunits (Schirmer et al., 1995). Porins of gram-negative bacteria are normally processed at the N-terminal end. P13 of *B. burgdorferi* represents in this respect an exception because it is processed at the N-terminal and the C-terminal end. Secondary structure prediction showed possible α-helices as transmembrane spanning domain of P13 (Noppa et al., 2001; Pinne et al., 2004).

To date, little is known about outer membrane proteins of *B. duttonii* and their involvement in pathogenesis of relapsing fever and life cycle besides the Vsp's and Vlp's. In this study we compared the channel forming activity of the outer membrane protein fraction of *B. duttonii*, *B. hermsii* (tick borne) and *B. recurrentis* (louse borne). Clear differences were detectable in the appearance of single channel conductance. Here we also describe the purification and characterization of a channel forming protein of *B. duttonii.* The biophysical characterization was performed using the black lipid bilayer assay. The channel forming protein has a single-channel conductance of 80 pS and is slightly anion selective. Parts of the amino acid sequence of a putative channel forming candidate were identified by mass spectrometric analysis. Search within the known protein sequences of other relapsing fever agents such as *B. hermsii* and *B. turicatae* demonstrated that this protein is a possible homologue of the lipoprotein of the Vsp family but it is presumably not a channel former.

2.2 MATERIAL AND METHODS

2.2.1 Bacterial strain and growth conditions

The relapsing fever *Borrelia* strains used in this study were *Borrelia duttonii* 1120 and *B. recurrentis* A1, a kind gift of Guy Baranton, Institut Pasteur, Paris, France and *Borrelia hermsii* ATCC 35209, a kind gift of Alan Barbour, University of California Irvine, USA. Bacteria were grown at 37°C in Barbour-Stoenner-Kelly-II (BSKII) medium (Barbour, 1984) or BSK-H (Sigma, St. Louis, Mo.) supplemented with 10% rabbit serum and 1.4% gelatine.

2.2.2 Separation of outer membrane proteins and purification of the 80 pS channel

Outer membrane fractions (so called B-fraction) of *B. duttonii*, *B. recurrentis* and *B. hermsii* were prepared as described elsewhere (Magnarelli et al., 1989). Purification of the native 80 pS channel was performed with a dry hydroxyapatite (HTP) Bio-gel (Biorad) column. 100 µl of B-fraction of *B. duttonii* (app. 100 ng proteins) was diluted with 400 µl 2% Genapol X-080 (Fluka) and preheated for 10 min at 50 °C. Subsequently the mixture was applied to a dry HTP column made from 0.3 g hydroxyapatite in an Econa-Column (Biorad) with the dimensions 0.5 cm diameter and 5 cm length. The column was washed with six column volumes of a buffer containing 2% Genapol (Roth), 10 mM Tris-HCl (pH 8.0). The bound 80 pS channel protein was released from the column by increasing the ionic strength of the elution buffer. Three column volumes of a buffer containing 2% Genapol (Roth), 1 M KCl and 10 mM Tris-HCl (pH 8.0) (Merck) were passed through the column. Fractions of 1.0 ml were collected. Similarly, the 80 pS channel could also be obtained by using immobilised metal ion affinity chromatography (IMAC). About 100 ng of *B. duttonii* outer membrane B-fraction was dissolved in 400 µl 1% Genapol, 0.02 M Na_2HPO_4, 0.75 M NaCl (pH 7) (Merck) and subsequently applied to an Econa-Column (Biorad) with the dimensions 0.5 cm diameter and 5 cm length filled with 5 ml Cu^{2+} loaded Chelating Sepharose Fast Flow (Amersham Bioscience). The column was washed with 1% Genapol, 0.02 M Na_2HPO_4, 0.75 M NaCl (pH 7) until the zero line was reached again. The bound 80 pS channel protein was eluted with a solution containing 10 mM imidazol (Roth), 1% Genapol, 0.02 M Na_2HPO_4, 0.75 M NaCl (pH 7). Fractions of 1.0 ml were collected.

2.2.3 Protein electrophoresis

Proteins samples were precipitated by the protocol of Wessel and Flügge (Wessel and Flugge, 1984) and loaded with 4x sample buffer on SDS polyacrylamide gels (SDS-PAGE). Proteins were separated on 12 % SDS-PAGE or 4-15 % gradient SDS acryl gels (Biorad) under native or

denatured conditions (boiled for 5 min in 4x SDS sample buffer before loading the gel) by using a Biorad electrophoresis system according to the Laemmli method (Laemmli, 1970). The gels were stained either with Coomassie R250 (Sigma) or silver stain (Blum et al., 1987).

2.2.4 Sequencing of the 27 kDa protein peptides and 27 kDa antibodies

For peptide sequencing SDS-PAGE was performed as described above using 12% gels. Protein bands were cut out of the gel and digested with trypsin, endoproteinnases Lys-G and Asp-N (Roche Diagnostics) according to Eckerskorn and Lottspeich (Eckerskorn and Lottspeich, 1993). The resulting peptides were separated by reversed-phase HPLC (Sykam) with Luna C-18 column, 150 x 1 mm and subjected to N-terminal sequence and MALDI-TOF mass spectrometry. Microsequencing was performed using a gas-phase sequencer Procise 492 cLC (Applied Biosystems GmbH) according to the instructions of the manufacturer. Mass spectrometry was performed using a Bruker reflex III MALDI-TOF mass spectrometer equipped with a 337 nm nitrogen laser (Bruker-Franzen).

The 27 kDa protein was purified by preparative SDS-PAGE (Jordy et al., 1996). Polyclonal antibodies against the purified 27 kDa protein were raised in rabbits and obtained by Agrisera (Sweden).

2.2.5 Lipid bilayer experiments

The methodology for the black lipid bilayer experiments was described earlier (Benz et al., 1978). The membrane cell consisted of a Teflon chamber with two aqueous compartments connected by small circular holes (surface area 0.5 mm²). Membranes were formed by painting onto the hole a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids) in *n*-decane. The aqueous salt solutions (Merck) were used unbuffered and had a pH around 6. The temperature was kept at 20°C throughout. Small amounts of the different protein fractions were added to the aqueous phase on one or both sides of black lipid bilayer membranes.

The membrane current was measured with a pair of Ag/AgCl electrodes switched in series with a voltage source and an electrometer (Keithley 617). For single-channel recordings the electrometer was replaced by a highly sensitive current amplifier (Keithley 427). The amplified signal was recorded with a strip chart recorder.

The zero-current membrane potentials were measured as described previously (Benz et al., 1979). The membranes were formed in a 100 mM salt solution containing a predetermined

protein concentration so that the membrane conductance increased about 100-1000 fold within 10-20 min after membrane formation. At this time the instrumentation was switched to the measurements of the zero-current potential and the salt concentration on one side of the membrane was raised by adding small amounts of concentrated salt solutions. The zero-current membrane potential reached its final value 2-5min after addition of the concentrated salt solution.

Titration experiments were performed with multi-channel experiments. The protein sample containing the 80 pS channel was added to black diphytanoyl phosphatidylcholine/n-decane membranes at concentration of about approximate 50 ng/ml. 30 min after the addition of the proteins, the rate increase in conductance had slowed down considerably. At this time small amounts of concentrated solutions of different substrates (see Table 3) were added to the aqueous phase to both sides of the membrane, with constant stirring to allow equilibration. The conductance data of the titration experiments were analyzed using the following equations used earlier for the carbohydrate-induced block of LamB from *Escherichia coli* (Benz et al., 1986; Benz et al., 1987). The conductance, G(c), of a membrane containing many channels in the presence of a substrate with the stability constant, K (half saturation constant K_S) and the carbohydrate concentration, c, is given by the maximum conductance (without substrate), G_{max} times the probability that the binding site is free:

$$G(c) = \frac{G_{max}}{(1 + K \cdot c)} \qquad (1)$$

Equation 1 may also be written as:

$$\frac{(G_{max} - G(c))}{G_{max}} = \frac{K \cdot c}{(K \cdot c + 1)} \qquad (2)$$

which means that the conductance is a function of the substrate concentration and can be analyzed using Lineweaver-Burke plots.

The voltage dependence of the porin channel was checked as described elsewhere (Riess and Benz, 2000) by using membrane potentials as high as –150 to + 150 mV.

Single-channel measurements of incubated protein sample with 27 kDa protein antibodies (in a ratio 2:1 for 1h at 20 °C) were performed to check is the 27 kDa protein is the channel former in the black lipid bilayer.

2.3 RESULTS

2.3.1 Channel-forming activities in the outer membrane fractions of different *Borrelia* strains

The outer membrane fractions obtained from the three RF *Borrelia* species were dissolved in 1% Genapol and investigated for channel-forming activity in the black lipid bilayer assay. All outer membrane preparations contained porins. Figure 2.1 shows histograms of conductance fluctuations obtained from single-channel experiments with outer membrane preparations of the three *Borrelia* strains. The histograms show a broad range of single-channel conductance values caused presumably by different channel-forming proteins reconstituted into the lipid bilayer membranes. The three *Borrelia* strains showed a different conductance fluctuation patterns. The B-fraction of *B. duttonii* (A) formed channels with single-channel conductance centered on 0.25 nS, 2.5 nS, 5.5 nS and 11 nS. For *B. hermsii* (B) highest single-channel conductance was found to be around 4 nS, which is different to that reported previously by Shang et al. (Shang et al., 1998). The histogram obtained for total outer membrane of *B. duttonii* and *B. recurrentis* (panel C) showed also high single-channel conductance above 4 nS. Both contained large channels with conductance around 9.5 nS and 11 nS. Smaller channels could not be observed for outer membrane preparations of the three strains because channels in the range below 200 pS are masked by the appearance of the high conductance channels.

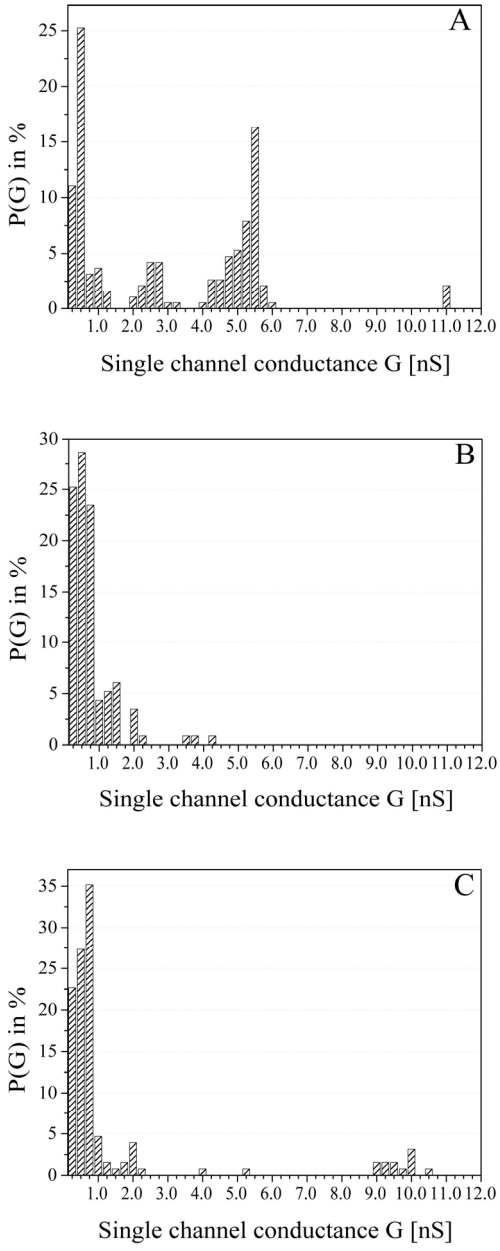


Figure 2.1 Histogram of the probability P(G) for the occurrence of given conductivity steps observed with membranes formed of 1% diphytanoyl phosphatidylcholine/n-decane in the presence of outer membrane fractions from *B. duttonii* (A), *B. hermsii* (B) and *B. recurrentis* (C). P(G) is the probability that a given conductance step G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance step by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; T = 20°C. The total numbers of conductance steps were 195 for *B. duttonii* (A), 116 for *B. hermsii* (B) and 135 for *B. recurrentis* (C).

2.3.2 Single channel analysis of the 80 pS channel

When the proteins of the B-fraction of *B. duttonii* was separated using a dry HTP column or immobilized metal ion affinity chromatography (IMAC) the high ionic strength or the competitive ligand eluates contained a channel-forming protein that formed stable channels with a single-channel conductance of 80 pS in 1 M KCl (see Figure 2.2A). Control experiments with Genapol alone showed that the addition of the detergent did not result in channel formation. It is noteworthy that the 80 pS channels could not be observed in the lipid bilayer assay when the B-fractions of *B. hermsii* and *B. recurrentis* were separated in the same way (data not shown). Figure 2.2B shows a histogram of 121 conductance steps of the 80 pS channel of *B. duttonii* in 1

M KCl at a membrane potential of 20 mV. The average single-channel conductance was about 80 pS, therefore the channel forming protein was named 80 pS channel for further reference.

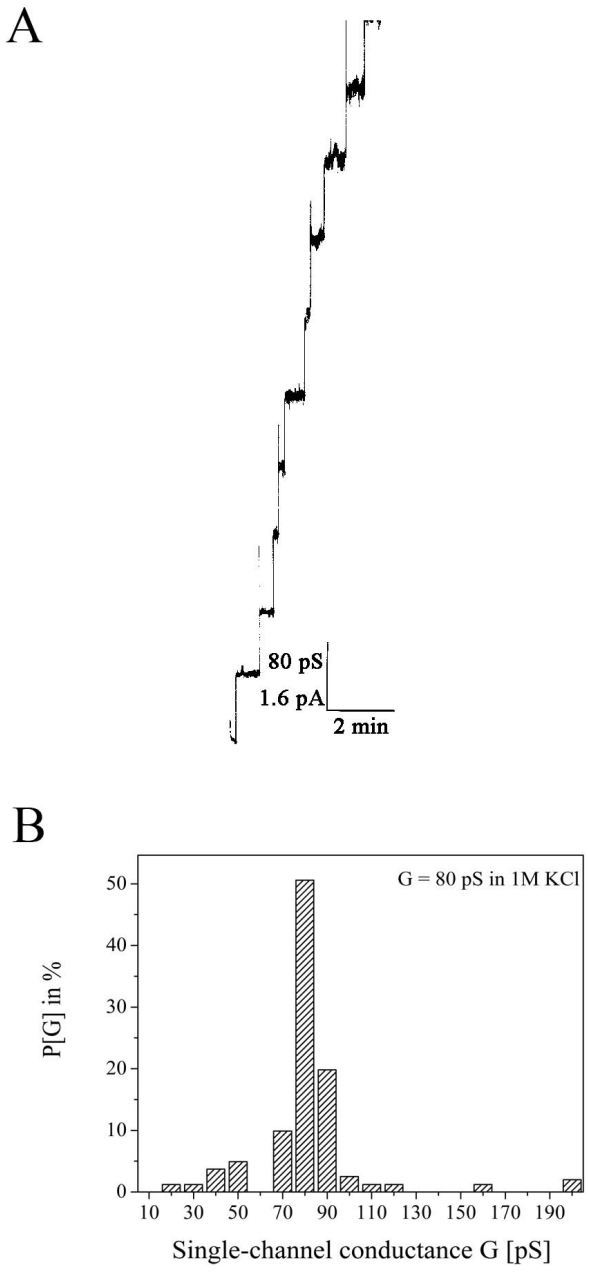


Figure 2.2
(A) Single channel recording of a 1% diphytanoyl phosphatidylcholine/*n*-decane membrane in the presence the HTP protein fraction of *B. duttonii*, which forms 80 pS channels. 50 ng/ml protein was added to the aqueous phase bathing a black lipid bilayer membrane.
(B) Histogram of the conductance steps observed in the presence of the protein sample of *B. duttonii* forming the 80 pS channel. The aqueous phase contained 1 M KCl. The applied voltage was 20 mV; T = 20°C. The average single-channel conductance was about 80 pS for 121 steps.

The conductance of the 80 pS channel was studied as a function of different electrolytes and concentrations. The results are summarized in Table 2.1. The single-channel conductance was a linear function of the KCl-concentration between 0.1 and 3 M demonstrating that the channel did not contain a binding site for ions inside the channel. The channel conductance did not vary much when the salts contained different cations. The replacement of K^+ by Li^+ resulted in a decrease from 80 pS to 50 pS (1.6-fold). However, replacement of chloride by the less mobile

acetate anion had a more substantial effect on the single channel conductance. It decreased from 80 pS to 35 pS by a factor of 2.7. This result suggested that the 80 pS channel is more specific for anions than for cations.

Table 2.1 Average single channel conductance, *G*, of the 80 pS channel as a function of concentration *c* of different salt solutions

Salt	Concentration	Average single channel conductance
	M	pS
LiCl	1	50
KCl	0.1	15
	0.3	30
	1	80
	3	250
CH_3COOK, pH 7	1	35

The membranes were formed from 1% diphytanoyl phoshatidylcholine dissolved in *n*-decane. The pH of the aqueous salt solutions was approximatively 6. The single-channel conductance is given as the mean of at least 100 single steps. The applied voltage was 20 mV and the temperature was 20°C.

2.3.3 The 80 pS channel is anion selective

Zero-current membrane potential experiments were carried out in the presence of salt gradients to study the selectivity of the 80 pS channel. After the insertion of 100 - 1000 channels into the PC membranes, the KCl concentration was raised on one side from 100 to 500 mM by addition of 3 M KCl, while stirring to allow equilibration. Table 2.2 shows the results of these measurements using KCl, LiCl and potassium acetate. In all three cases we observed negative potentials at the more diluted side of the membrane (100 mM) indicating preferential movements of anions through the channel. The zero-current membrane potentials for the three salts had values between –23 mV (LiCl), –14.4 mV (KCl) and –8.4 mV (potassium acetate). Analysis of these potentials using the Goldman-Hodgkin-Katz equation (Benz et al., 1979) suggested that cations also have a certain permeability through the channel because of the radios of permeability P_{cation} to P_{anion} were 0.24 (LiCl), 0.41 (KCl) and 0.58 (potassium acetate). These results demonstrate that the 80 pS channel is weakly anion selective, which agrees with the

single-channel experiments.

Table 2.2 Zero-current membrane potential, V_m, of diphytanoylphoshatidylcholine/*n*-decane membranes in the present of the HTP protein fraction measured for a tenfold gradient of different salts**.**

Salt	V_m	Permeability ratio P_{cation}/P_{anion}
	mV	
KCl	- 14.4	0.41
LiCl	- 23	0.24
KCH_3COO, pH 7	- 8.4	0.58

V_m is defined as the difference between the potential at the diluted side (100 mM) and the potential at the concentrated side (500 mM). The pH of the aqueous salt solution was 6 unless otherwise indicated. T = 20°C. P_{cation}/P_{anion} was calculated with the Goldman-Hodgkin-Katz equation (Benz et al., 1978; Benz et al., 1985) from at least three experiments.

2.3.4 The 80 pS channel does not contain a binding site for substrates

Because of the small conductance and anion selectivity of the 80 pS channel it seems possible that the channel is specific for anionic and other substrates. To check such a possibility titration experiments were performed to determine specific binding of anionc and other substrates to the channel. In particular, we tested substrates that are reported to bind to small specific porins such as LamB (Benz et al., 1987) and Tsx (Maier et al., 1988). Table 2.3 contains a list of substrates that are available in the host or vector of *B. duttonii* and may be transported across its outer membrane. So far we did not find a pronounced conductance decrease for these compounds within the concentration range, which could be used here. Assuming that we would have noticed 10% decrease of conductance G_{max} following addition of the compounds we calculated the lower limit of the half saturation constant; K_S, from eqn. (2) (see Table 2.3). This means that the half saturation constant for binding of these molecules, if it existed, is in most cases above 100 mM, which is outside the physiologically relevant concentration range. Taken together it is clear that the 80 pS channel does not show a significant specificity for the tested substrates.

Table 2.3 Lower limit of the half saturation constant K_S for the binding of different substrates to the *B. duttonii* 80 pS channel as derived from titration experiments.

Substrate	K_S [M]
Maltopentaose	>0.1
Glutamate, pH7	>0.4
KH_2PO_4	>0.8
Adenosintriphosphate	>0.04
Citrate, pH6	>0.8

The membranes were formed from 1% diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl and small amounts of the HTP protein fraction containing the 80 pS channel. The lower limit of the half saturation constants, K_S, was calculated using equation (2) and assuming that a 10% decrease of conductance G_{max} followed the addition of the highest concentration of the compounds.

2.3.5 Voltage-dependence of the 80 pS channel

Certain outer membrane porins show voltage-dependent closure despite the fact that no voltage-dependent closure was observed so far in *in vivo* experiments, which make it likely that the voltage dependence is a reconstitution artifact (Lakey and Pattus, 1989; Sen et al., 1988, Benz, 1994). To check if also the 80 pS outer membrane channel of *B. duttonii* is voltage-dependent we performed experiments with many 80 pS channels. The results demonstrate that this channel did not show any voltage-induced closure even at voltages as high as ± 150 mV (see Fig. 2.3).

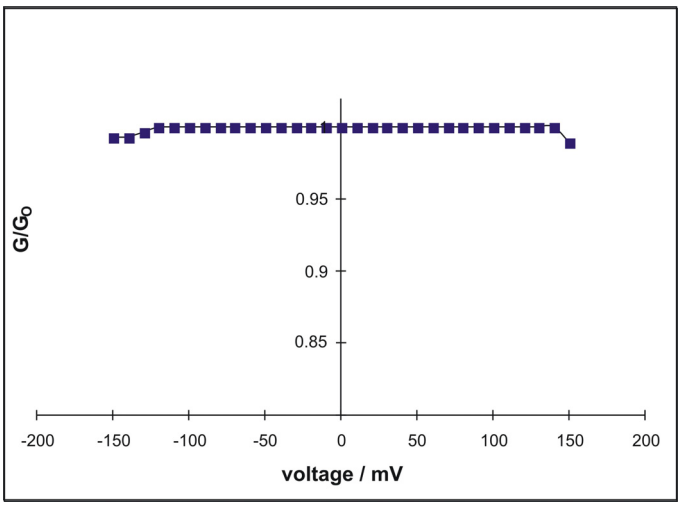


Figure 2.3 Ratio of the conductance G at a given membrane potential (V_m) divided by the conductance G_o at 10 mV as a function of the applied voltage. The closed squares show measurements in which 50 ng/ml protein was added to the both sides of the membranes. The aqueous phase contained 1 M KCl. The membranes were formed from 1% diphytanoyl phosphatidylcholine/*n*-decane. T = 20 °C. Means of three experiments are shown.

2.3.6 Attempts to identify of the protein responsible for the 80 pS channel

As described above the 80 pS channel was found in a protein fraction eluted with high ionic strength or competitive ligand buffer from HTP or IMAC column chromatography. After SDS-PAGE and Coomassie staining of the gels, a 27 kDa protein was visible in both active fractions (see Fig. 2.4). To identify the protein responsible for the formation of the 80 pS channel the 27 kDa protein band was cut out of the gels. The protein was digested by trypsin, endoproteinases Lys-G or Asp-N treatment followed by HTP chromatography purification and mass spectrometry analysis of the digested fragments. The partial amino acid fragments were used for BLAST search. The analysis identified the 27 kDa band as a lipoprotein of the variable small protein (Vsp) family, which shows a high similarity to the Vsp33 of the *B. duttonii* strain Ly (Tabuchi et al., 2002) (see Fig. 2.5). After analysis of the protein in terms of secondary structure predictions and comparison with other channel forming lipoproteins it seems to be rather unlikely that the lipoprotein is a channel-forming component of the outer membrane. Also single-channel experiments with an active protein fraction incubated with the 27 kDa antibodies resulted in a constant activity of the 80 pS channel. Antibodies make proteins water soluble so they can not enter into the membrane. This is also a further proof that the 27 kDa lipoprotein is not the responsible channel former.

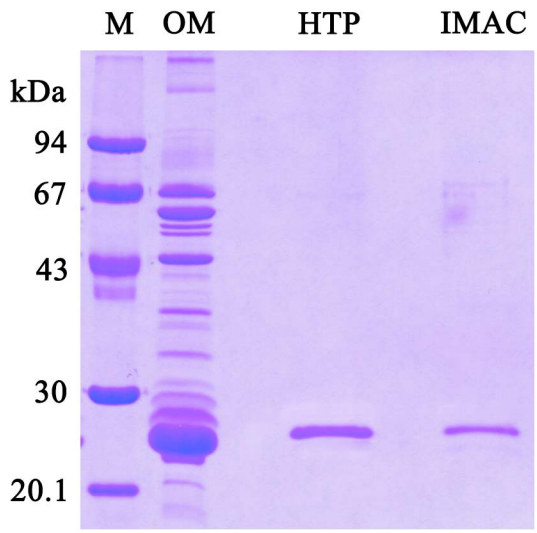


Figure 2.6 Protein separation by 12% SDS-PAGE of different protein preparations of *B. duttonii*. Lane OM shows total outer membrane proteins. Lane HTP shows the high ionic strength eluate of the HTP column and lane IMAC shows the competitive ligand eluate of the IMAC column. In each lane, 5 µg proteins were separated and visualized by Coomassie staining. Molecular mass standard in kilodaltons is indicated at the left of the gel. The HTP and the IMAC proteins resulted in formation of the 80 pS channel in lipid bilayer experiments.

```
                    10        20        30        40        50        60
                     |         |         |         |         |         |
peptide                                          KGDGSVIDLGV-SKKIEDAVAFAES
Vsp33Bd      -------MVLGILMVVMGCCNSGGGIKEGDEGKAKKGDGSVIDLKVIGEKIKSAVEFAGS
Vsp2Bhe      MKKNTLSAILMTLFLFISCNNGGPELKKGE---VTKSDGTVLDLSKISANIKNAVTFAAS
VspABtu      MKRITLSALLMTLFLLMSCNNSGTSPKDGQ---AAKSDGTVIDLATISKNIKDTVAFAKS
OspCBbu      MKKNTLSAILMTLFLFISCNNSGGDSASTN---PDES-AKGPNLTVISKKITDSNAFLLA

                   70        80        90       100       110       120
                    |         |         |         |         |         |
peptide      VKEVKTLVKSVD                           DNGSLLAGVHSVISAVNTKL
Vsp33Bd      VKEVHALVRSVGEFAKAIGKKVTQNTGVIAADAGGNNNGGLIAGAYSLISELNTKVEVLG
Vsp2Bhe      VQEVETLVKSIDELAKAIGQKV--NADGLTAEA--NKNDSLVAGVYQLISDVQGKLTKLE
VspABtu      VKDVHTLVKSIDELAKAIGQKIQQNSDQFANDG--AHNGSLISGAFQVILTVETKLKSLE
OspCBbu      VKEVEALLSSIDELSKAIGKKIK-NDGTLDNEA--NRNESLIAGAYEISKLITQKLSVLN

                  130       140       150       160       170       180
                    |         |         |         |         |         |
peptide            DELKVKIVAVAKEGKAFLDK                             TKDKGAT
Vsp33Bd      KKDGNSSELKSKFDDLNKKCKAFLDKVKGD-AELCKKDVTDENAQKALDVNNATKDKGAS
Vsp2Bhe      IGASKFAGLKEKVVAAKKGSDDFLTKVKAQHNNLGQS----AEAPKAIKKGNADSTKGAE
VspABtu      DTVGLSDTLKTKVTSSKIASRAFLDKVKSKHTELGKEGASDADAKAAILVSNGTKDKGVD
OspCBbu      -----SEELKEKIKEAKDCSQKFTTKLKDSHAELGIQSVQDDNAKKAILKTHGTKDKGAK

                  190       200       210       220
                    |         |         |         |
peptide      ELAKLNGTIDELLKASNKILSDVLAELVVKPTT
Vsp33Bd      ELDALNTSIDGLLIVIKKLVEDAVNELTTSS----------
Vsp2Bhe      ELGKLNTAIDELLTAAKDAVEAAIADVTAAPAKPATPVKP-
VspABtu      ELVKLNTEIDALLTAAEAAVTAAI-NALSTPAKSDAPAQSN
OspCBbu      ELEELFKSLESLSKAAQAALTNSVKELTN-PVVAESPKKP-
```

Figure 2.5 Amino acid comparison of the full-length Vsp33 protein of *B. duttonii* Ly, Vsp protein of *B. hermsii*, VspA protein of *B. turicatae* and OspC protein of *B. burgdorferi* using Pole Bioinformatique Lyonnaise Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr). Identical amino acids are given in red. Partial peptide sequences of the 27 kDa protein (see Figure 4) were obtained by mass spectrometry.

The silver stained gels of the active fraction contained also other bands that could be responsible for channel formation (see Fig. 2.6). In particular, the gels contained a 37 kDa band. However, the concentration of the 37 kDa protein did not allow staining by Coomassie. Therefore it was not possible to identify this protein by mass spectrometry. Further attempts to concentrate the 37 kDa protein in the active fraction were not successful, which means that the channel-forming component could not be identified.

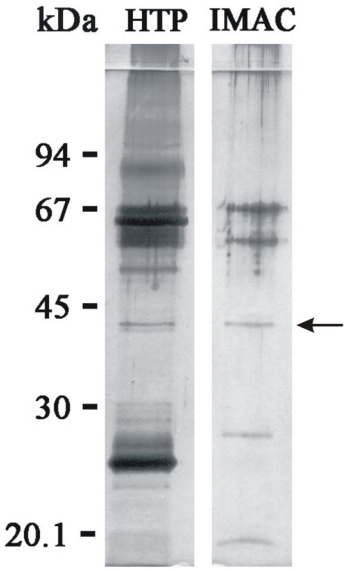


Figure 2.6
12 % SDS-PAGE of the proteins samples from *B. duttonii* as obtained by chromatography across HTP and IMAC columns. The protein sample obtained from the IMAC column was precipitated by the method of Wessel and Flügge (Wessel and Flugge, 1984), which resulted in the loss of the 27 kDa lipoprotein presumably because it was soluble in the chloroform-methanol mixture. The arrow marks the band that both protein samples have in common. The proteins in the gel were visualized by silver staining. Molecular mass standards in kDa are indicated at the left side of the gel.

2.4 DISCUSSION

In this study we investigated outer membrane preparation of three relapsing fever species, *B. duttonii, B. hermsii* and *B. recurrentis* for channel forming activity in the outer membrane using the lipid bilayer assay. All outer membranes preparations contained channel-forming proteins. From the histograms of the conductance fluctuation of the three different B-fractions (see Fig.2.1) it is obvious that the channel forming activity of all three strains are caused by different proteins and all showed a strain specific cluster of channel-forming activity. This means that relapsing fever *Borrelia* have several pore forming proteins in their cell wall similar as has been reported for other gram-negative bacteria (Benz, 2001; Nikaido, 2003). Gram-negative bacteria, such as *E. coli*, have pore forming proteins in their outer membrane, which have a different single-channel conductance. Outer membrane channels in enteric bacteria with a single-channel conductance higher than 1 nS in 1M KCl represent often general diffusion pores (Benz et al., 1985). Small channels with a much lower single channel conductance are often responsible for

uptake of specific substrates such as carbohydrates or nucleosides (Benz et al., 1986; Maier et al., 1988). This should also be possible for *Borrelia* when the different habitat and the metabolic needs of these bacteria are considered. Thus it seems conceivable that they also developed individual uptake mechanisms for specific substrates. The histogram of channel-forming proteins from the outer membranes of *B. hermsii* differs from earlier published data. This may be explained either by genetic differences in the investigated strains or by differences between the methods used to prepare outer membrane proteins (Shang et al., 1998).

In the second part of the study we investigated the biophysical properties of an 80 pS channel of *B. duttonii* in the lipid bilayer assay. The 80 pS channel of *B. duttonii* was discovered in a fraction obtained by high ionic strength elution from the HTP column and by competitive ligand elution from an IMAC column of the outer membrane preparation. We could demonstrate that the 80 pS channel forms a water filled channel with a conductance in the range of the specific outer membrane porins such as LamB (150 pS in 1 M KCl) (Benz et al., 1993) and Tsx (10 pS in 1 M KCl) (Maier et al., 1988). Its single-channel conductance was a linear function of the aqueous salt concentration. Interestingly, the 80 pS channel of *B. duttonii* showed no voltage dependence and was anion selective with a cation/anion permeability ratio of 0.41 in KCl solution. This indicates a specificity of the channel for anionic substrates. Voltage independence is frequently found for specific outer membrane channels, because oppositely charged residues are distributed in a more balanced way within the channel in contrast to general diffusion pores (Koebnik et al., 2000). However, our titration experiments demonstrated that the 80 pS channel does not contain a binding site for a variety of substrates such as maltopentaose, glutamate, KH_2PO_4, adenosintriphosphate or citrate (Table 3). This was concluded from the experimental observation that the membrane conductance was not influenced by the addition of these substrates. When we take a previously proposed formalism for the binding of these substrates to a specific channel into consideration it is possible to calculate an upper limit for the stability constant of substrate binding to the 80 pS channel. For this we assume that the 80 pS channel is a single-file channel (Benz et al., 1986). This means that the 80 pS channel is open when no substrate is bound, and closed when it is occupied. The conductance G(c) of a 80 pS channel containing membrane in the presence of a substrate (concentration c), which binds with the stability constant, K, is given in equation (2) in the Material and Method part, where G_{max} is the membrane conductance before the start of the substrate addition to the aqueous phase. When we assume that we could observe at least a 10 % decrease of the conductance during the addition of the substrate we can calculate an upper limit for the stability constant for substrate binding by using Eqn. (2) and the maximum substrate concentration given above. For maltopentaose,

glutamate, KH_2PO_4, adenosintriphosphate and citrate we calculated stability constants that should be below 10, 2.5, 1.25, 25 and 1.25 1/M, respectively. The corresponding half saturation constants (lower limits) should be higher than 0.1, 0.4, 0.8, 0.04 and 0.8 M, respectively.

These possible half saturation constants are in a range that is not of physiological relevance for effective substrate scavenging because the concentration of these substrates is in the low millimolar range in the hosts of *Borrelia*. This means that the substrate concentration probably never reaches those of the half-saturation constants if these are about 0.04 M and higher. This result suggests that the 80 pS channel does not contain a binding site for these substrates inside the channel. Taken together it is clear that the 80 pS channel is unspecific with respect to the tested substrates. Nevertheless, there exist indications that the channel could be specific for a not identified substrate.

We tried to identify the protein responsible for channel forming activity of the 80 pS channel. During our search we identified a 27 kDa protein of *B. duttonii* as a possible candidate (see figures 4 and 5). Secondary structure analysis of this protein led to the conclusion that it is presumably not a channel former because its secondary structure lacks the typical amphipatic transmembrane β-strands of gram-negative bacterial porins (data not shown). By BLAST search and amino acid sequence alignment it was shown that the 27 kDa protein belongs presumably to the Vsp family. It has a high amino acid identity to Vsp33 of *B. duttonii* Ly strain (Tabuchi et al., 2002). In addition, Vsp-proteins share a high degree of amino acid and DNA sequence identity with OspC of *B. burgdorferi* (Carter et al., 1994). The structure of OspC of *B. burgdorferi* is known and shows no transmembrane domain that could form a channel. Furthermore, inactivation of the channel forming activity by antibodies against the 27 kDa lipoprotein was not possible. Our attempts to relate the channel-forming activity porin activity of the 80 pS channel to bands excised and eluted from preparative SDS-PAGE failed also because of the general susceptibility of *Borrelia* porins towards SDS-treatment (data not shown). Comparison of silver stained SDS-PAGE of the active fractions obtained from the two purification procedures (hydroxyapatite and IMAC columns) finally led to the conclusion that a 37 kDa protein could be the channel former, responsible for the 80 pS activity (Fig. 6). This seems to be possible because outer membrane porins of gram-negative bacteria have molecular masses between 30 and 50 kDa, which is sufficient to form β-barrel structures of either 16 or 18 β-strands (Koebnik et al., 2000).

The identification of this candidate as a channel forming protein was difficult because the genome of *B. duttonii* is not known. The analysis of the primary structure of the 37 kDa protein could have solved this problem. However, it was not possible to obtain this protein in sufficient quantity (at least a visible protein band on a gel stained by Coomassie) to perform N-terminal sequencing (Eckerskorn and Lottspeich, 1993). Small protein amounts which are visible by silver staining can be used for identification of proteins using peptide mass fingerprints (Sickmann et al., 2002). However, this procedure needs the knowledge of the entire chromosome of *B. duttonii*. This means that further attempts are necessary to identify the protein responsible for the formation of the 80 pS channel.

This study demonstrates that the relapsing fever strains, *B. duttonii*, *B. hermsii* and *B. recurrentis* contain channel forming proteins in their outer membrane. Further investigations are needed to understand the role of porins such as the 80 pS channel in the physiology and virulence of these relapsing fever *Borrelia*.

CHAPTER 3

Identification of a 300 pS pore forming protein in the *p66* knock out mutant of *Borrelia burgdorferi* sensu stricto strain HB19

3.1 SUMMARY

Outer membrane preparations of *B. burgdorferi* HB19 and *p66* knock-out strain HB19/K02 were characterized in the black lipid bilayer assay. Comparing the histograms of single-channel conductions fluctuations of both strains showed no single-channel activity at 11.5 nS for the *p66* knock-out strain. This verifies earlier studies that P66 is a pore-forming protein in *B. burgdorferi*. Furthermore, one fraction obtained by anion exchange chromatography of the *p66* knock out outer membrane preparation showed a uniform channel forming activity with a single channel conductance of 300 pS. The biophysically characterization showed that the 300 pS channel is ionselective and not voltage dependent. By mass spectrometry using peptide mass finger prints, BB0142 could be identified as the sole channel forming candidate in the active fraction. A BLAST search and a conserved domain search showed that BB0142 is a putative TolC homologue in *B. burgdorferi*. For the first time a putative drug efflux system of *B. burgdorferi* could be identified and the outer membrane component BB0142 biophysically characterized.

3.2 INTRODUCTION

Lyme borreliosis is a medical term of an infection caused by the pathogen *Borrelia burgdorferi* sensu lato transmitted by the bite of an infected hard body tick of the genus *Ixodes*. *B.*

burgdorferi, was primarily described as a causative agent for Lyme borreliosis in 1975 by Burgdorfer in Lyme, Connecticut USA (Burgdorfer et al., 1982). Beside this, two other species, *B. afzelii* (Canica et al., 1993) and *B. garinii* (Baranton et al., 1992), are also known to cause a similar disease in Eurasia (Hubalek and Halouzka, 1997); (Baranton et al., 1998; Li et al., 1998). Today it is clear that Lyme borreliosis is a bacterial infection limited to the northern hemisphere. The disease is closely correlated to the distribution of the *Ixodes* hard bodied ticks (Eisen et al., 2002; Hudson et al., 1998).

Infection of humans by *B. burgdorferi* results in a wide variety of clinical manifestations (Steere et al., 2004). The genus *Borrelia* represents highly mobile spirochetes. Following an initial local infection of the skin (erythema migrans) the bacteria spread into other tissues, where they trigger inflammation in distant dermis, nervous tissue, joints, and the heart (Steere et al., 2004). The bacteria can survive for a long time in humans by evading the immune system. Chronic infections result in some cases in arthritis, dermatitis or neuroborreliosis. Diagnosis of *Borrelia* infections is sometimes difficult and problematic, in particular because of ambiguous symptoms in late infection state. To date antibiotic treatment with tetracycline or β-lactam is successful in most cases (Steere et al., 2004), especially when diagnosed at the early stage. However, it is also known that *B. burgdorferi* has a natural resistance towards several antibiotics. This resistance may rely on different mechanism known from other bacteria, such as enzymes, differences of the antibiotic target, and efflux pumps. On the other hand, the cause of antibiotic resistance of *Borrelia* is still an open question.

Until now only little is known about the physiology and metabolic of *B. burgdorferi* in different hosts. However, the recently published genome of *B. burgdorferi* sensu stricto provides an interesting insight into its metabolism (Fraser et al., 1997). Gene products necessary for the synthesis of several essential metabolic substrates are lacking. Nevertheless, *B. burgdorferi* can adapt in its tick vector (*Ixodes*) and escape from the immune system of its mammalian host, such as humans, small rodents, and deer.

In comparison to enteric gram-negative bacteria, the density of transmembrane proteins is low. Nevertheless, *B. burgdoferi* has within the group of spirochetes the highest concentration of transmembrane proteins (Radolf et al., 1994; Walker et al., 1991). This means presumably that *Borrelia* has an effective uptake mechanism for several amino acids, fatty acids, co-factors and nucleotides, which it cannot synthesize (Fraser et al., 1997). The proteins responsible for solute transport across the outer membrane of gram-negative bacteria are porins. Further knowledge on the structure and function of these porins would be useful for understanding of the pathogenesis

during Lyme borreliosis.

Porins can be subdivided into two classes. General diffusion pores such as OmpF of *E. coli* are needed for the uptake of smaller solutes because these porins sort according to the molecular mass (Benz et al., 1985). Specific porins like the maltoporin of *E. coli*, containing binding sites (Benz et al., 1986), are necessary parts of uptake systems for classes of solutes. Furthermore, porins often serve as receptors for bacteriophages (Wang et al., 2000).

Three putative porins are known for *B. burgdorferi*, Oms28 (Skare et al., 1996), P13 (Noppa et al., 2001; Ostberg et al., 2002) and P66 (Skare et al., 1997).

In this study we investigated the outer membrane fraction of a *p66* knock out mutant strain of *B. burgdorferi* HB19/K02 (Coburn and Cugini, 2003) for its channel forming properties. Channel activity with 11.5 nS single channel conductance as reported for P66 could not be detected in outer membrane fractions of the mutant. However, it contained a channel with a single-channel conductance of 300 pS in 1 M KCl, which could only be detected because the giant P66 pore was absent in the lipid bilayer assay. We determined the biophysical properties of the 300 pS channel and identified the protein by mass spectrometry. It is very likely that the 300 pS channel is caused by a TolC homolog, which could mean that *B. burgdorferi* possesses also one or several export systems for harmful drugs, which could be part of its antibiotic resistance (Andersen, 2004)

3.3 MATERIAL AND METHODS

3.3.1 Bacterial strain and culture conditions

The *Borrelia burgdorferi* strains used in this study were *Borrelia burgdorferi* HB19 and the P66 knock out strain *B. burgdorferi* HB19/K02, a kind gift of Jennifer Coburn, Tufts- New England Medical Center, Boston, USA. The bacteria were cultured in Barbour-Stoenner-Kelly (BSK-II) (Barbour and Hayes, 1986) or BSK-H (Sigma, St. Louis, Mo.) supplemented with 6 % rabbit serum at 32°C. In the case of *B. burgdorferi* HB19/K02, the growth media contained also 200 µg/ml kanamycin.

3.3.2 Preparation of outer membrane fraction and purification of BB0142

Outer membrane fractions (fraction B) of *B. burgdorferi* HB19 and HB19/K02 were prepared as described previously (Magnarelli et al., 1989). Purification of native BB0142 was performed

with an anion exchange MonoQ column in combination with fast protein liquid chromatography (FPLC) (Amersham Biosciences). About 200 µg of *B. burgdorferi* HB19/K02 fraction B were dissolved in 800 µl 2% lauryl-dimethyl-amine-oxide (LDAO, Sigma) and were applied to the column. The column was first washed with 7.5 ml 0.4% (LDAO) buffered with 10 mM Tris-HCl (pH 8.0). Bound proteins were eluted with a linear NaCl gradient (0 to 1 M) containing 0.4% LDAO buffered with 10 mM Tris-HCl (pH 8.0). Fractions showing a peak in the FPLC protocol (see Fig. 2.3) were further analyzed by SDS-PAGE and the black lipid bilayer assay.

3.3.3 Protein precipitation, electrophoresis and immunoblotting

Two hundred µl of each FPLC fraction 33, 35, and 36 was added to 500 µl ice cold ethanol (analytical grade, Merck) and stored for 18 h at –20 °C followed by centrifugation for 45 minutes at 20,000 g and 4°C. The supernatant was discarded and the pellet was dried under vacuum centrifugation (Speedvac, Eppendorf). Protein samples dissolved at 30°C and 100°C (5 min) in sample buffer were separated by SDS polyarcrylamide gel electrophoresis (SDS-PAGE) using the method of Lämmli (Laemmli, 1970). SDS-polyacrylamid gel were stained either with Coomassie blue R-250 (Sigma) or silver stain (Blum et al., 1987). The monomeric band of BB0142 (51 kDa) was purified by preparative SDS-PAGE (Jordy et al., 1996). Polyclonal antibodies against BB0142 were raised in rabbits and obtained by Agrisera (Sweden). The BB0142 antiserum was used for Western blots of the protein bands transferred on a polyvinylidene difluoride membrane (PDVF, Roth) using standard procedures (Towbin et al., 1979). For detection of bind BB0142 antibodies secondary antibodies raised against rabbit antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) were used. The blot was developed with the ECL Western Blotting Detection kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

3.3.4 Mass spectrometry (MS) of BB0142

The FPLC fractions showing channel-forming activity were subjected to SDS-PAGE followed by silver staining (see Fig. 3.3). The different bands were analyzed by nano LC-mass spectrometry as described elsewhere (Sickmann et al., 2003). Data interpretation of the obtained MS/MS datasets was performed by the mascot algorithm (Perkins et al., 1999).

3.3.5 Planar lipid bilayer assay experiments

The methods used for the black lipid bilayer experiments have been described previously (Benz

et al., 1978). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by small circular holes (surface area about 0.5 mm²). Membranes were formed by painting onto the hole a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids) in *n*-decane. All salts (analytical grade) were obtained from Merck, Germany). The aqueous salt solutions were used without buffering and had a pH around 6. The temperature was kept at 20°C throughout. The channel forming protein solutions were highly diluted in 1% Genapol X-080 (Fluka) and added to the aqueous phase after the membrane turned black.

The membrane current was measured with a pair of calomel electrodes switched in series with a voltage source and an electrometer (Keithley 617). For single-channel recordings the electrometer was replaced by a highly sensitive current amplifier (Keithley 427). The amplified signal was recorded with a strip chart recorder. The zero-current membrane potentials were measured as described previously (Benz et al., 1979). The membranes were formed in a 100 mM salt solution containing a predetermined protein concentration so that the membrane conductance increased about 100-1000 fold within 10-20 min after membrane formation. At this time the instrumentation was switched to the measurements of the zero-current potential and the salt concentration on one side of the membrane was raised by adding small amounts of concentrated salt solutions. The zero-current membrane potential reached its final value after 2-5min.

The voltage dependence of the porin channel was checked as described elsewhere (Riess and Benz, 2000) by using membrane potentials as high as –150 to + 150 mV.

3.4 RESULTS

3.4.1 Porin activity in the outer membrane fraction of *p66* knock-out mutant

In a first approach we checked the outer membrane preparation (fraction B) of *B. burgdorferi* strain HB19 (wildtype) and *p66* knock-out strain HB19/K02 for channel forming activity in the lipid bilayer assay. For these outer membrane fractions of *B. burgdorferi* strains HB19 (wildtype) and HB19/K02 were diluted 1:1000 in 1% Genapol and added to the 1 M KCl solution bathing a lipid bilayer membrane. Figure 3.1A and B show the histograms of conductance fluctuations obtained in single-channel experiments with both B-fractions. The histograms illustrate a broad range of single-channel events caused by different channel-forming proteins reconstituted into the membranes. For the wildtype B-fraction a high channel-forming activity with a conductance around 11-12 nS was detected, whereas in the B-fraction of the *p66* knock out mutant strain HB19/K02 only a small channel-forming activity was observed with a

conductance of about 11 nS. This result indicates that P66 is responsible for channel formation with a single-channel conductance of about 11.5 nS. It is noteworthy that control experiments with Genapol alone at the same concentration as with the B-fraction demonstrated that the detergent alone did not lead to any appreciable increase of the specific membrane conductance.

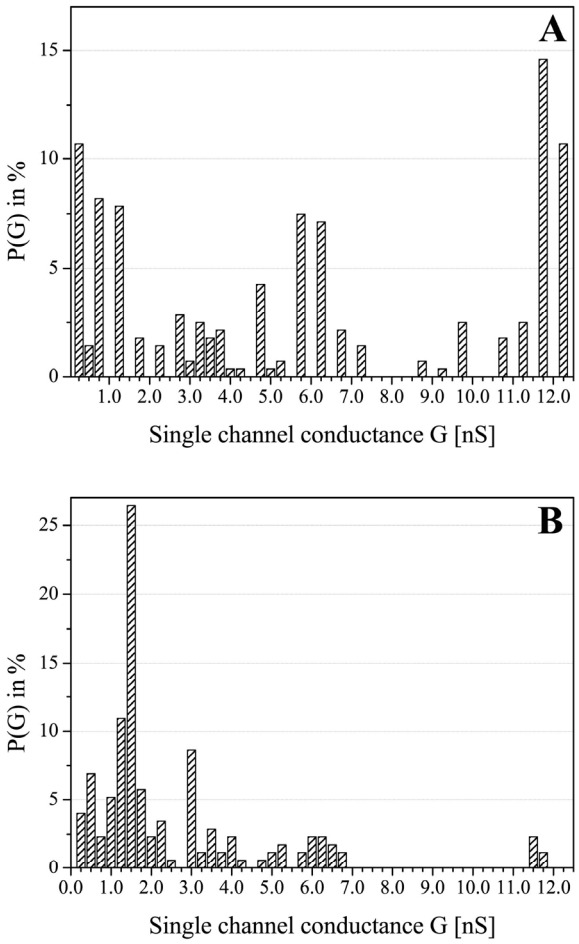


Figure 3.1 Histograms of channels formed by outer membrane proteins of *B. burgdorferi* HB19 (wildtype) (A) and the *p66* knock out strain *B. burgdorferi* HB19/K02 (B) in diphytanoyl phosphatidylcholine/n-decane membranes. The B-fractions of both strains were dissolved in 1% Genapol. The aqueous phase contained 1 M KCl. The applied voltage was 20 mV; T = 20°C. The total numbers of events were 281 for *B. burgdorferi* HB19 wild type (A) and 174 for *B. burgdorferi* HB19/K02 (B).

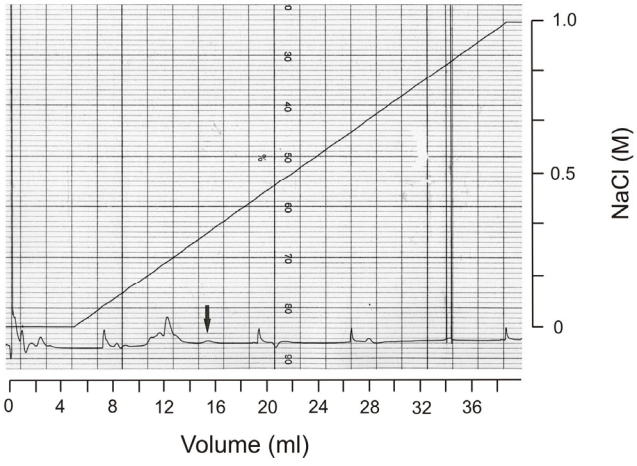


Figure 3.2 Protocol of the purification procedure of BB0142 using FPLC across a MonoQ column. Detergent-solubilized B-fraction of *B. burgdorferi* HB19/K02 was loaded on a MonoQ column and bound proteins were eluted with a linear NaCl gradient (0 to 1 M) containing 0.4% LDAO buffered with 10 mM Tris-HCl (pH 8.0). The arrow indicates the fraction containing BB0142. The solid line shows the increasing NaCl concentration. The abscissa indicates the volume of the fractions taken from the column.

3.4.2 Identification of proteins involved in channel forming activity

Outer membrane proteins of the *p66* knock out mutant *B. burgdorferi* HB19/K02 were separated by chromatography across a MonoQ anion exchange column. Proteins were eluted with elution buffer supplemented with increasing concentration of NaCl and checked in the lipid bilayer assay for channel forming activity. The fractions eluted at 250 mM NaCl showed a uniform channel-forming activity with a single-channel conductance of about 300 pS (see below). The protocol of the elution taken at 280 nm showed a small protein peak when the elution buffer contained 250 mM NaCl (see Fig. 3.2). To check the protein content of the corresponding fractions 200 µl of the active fraction were precipitated and separated by SDS-PAGE. The proteins in the silver stained bands were analyzed by mass spectrometry and identified using peptide mass fingerprints (see Fig. 3.3). Several proteins of known and unknown function were identified (data not shown). The protein of great interest was BB0142, which is a potential homologue of the outer membrane protein TolC of *E. coli*. To verify that the corresponding fractions contained BB0142 we performed a Western blot with polyclonal antibodies against BB0142 (see Fig. 3.4). The BB0142 monomer was detected in the membrane-active FPLC fraction and in the B-fraction of *B. burgdorferi* strain HB19/K02. When the membrane-active protein sample was solubilized at 30°C a high molecular mass band was observed with an apparent molecular mass of approx. 115 kDa on 12% SDS-PAGE that was not visible when the samples were boiled (see Fig. 3.5). This band reflects presumably the trimer of BB0142 similar to the formation of trimers by TolC on SDS-PAGE (Benz et al., 1993; Andersen et al., 2000). However, the protein concentration of the oligomeric band was not high enough to be identified by mass spectrometry or Western blot (data not shown).

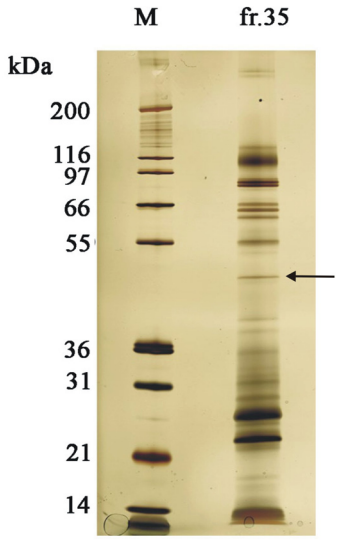


Figure 3.3 Fraction 35 of the MonoQ FPLC that was active in the lipid bilayer assay was precipitated and separated by 10% SDS-PAGE followed by silver staining. The arrow indicates the band where BB0142 was found by mass spectrometry. Molecular mass standards in kilodaltons (kDa) are indicated to the left.

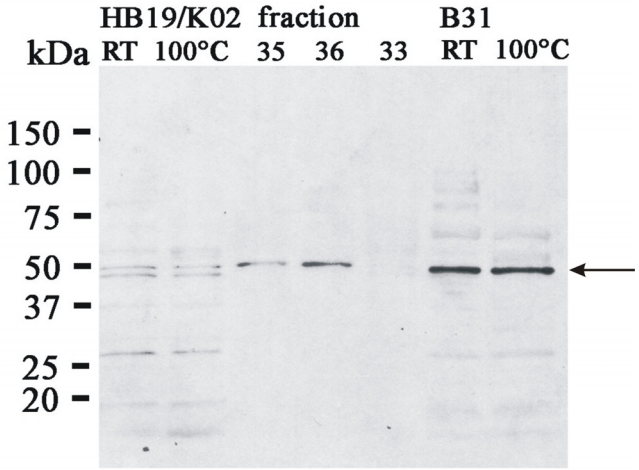


Figure 3.4 Western blot of the B-fraction of *B. burgdorferi* HB19/K02 (first two lines from left), of the fractions 35, 36 and 33 of the MonoQ FPLC (middle three lines) and of *B. burgdorferi* B31 lp (right two lanes). The proteins were dissolved in sample buffer at room temperature (RT) or 100°C (100), separated by 12 % SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The proteins were probed with polyclonal rabbit serum (RS) against BB0142. Molecular mass standards in kilodaltons (kDa) are indicated to the left.

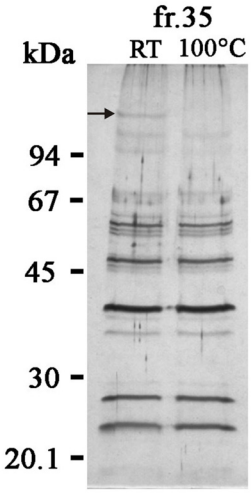


Figure 3.5 12% SDS-PAGE of fraction 35 of the separation of the B-fraction of *B. burgdorferi* HB19/K02 using MonoQ FPLC. The protein sample was loaded on the SDS polyarcylamid gel after dilution with sample buffer at room temperature (RT) and 100°C. The gel was stained with silver. The arrow marks the possible trimer of BB0142. Molecular mass standards in kilodaltons (kDa) are indicated to the left.

3.4.3 Analysis of the channels formed by BB0142

Single-channel experiments with FPLC fractions containing BB0142 resulted in a stepwise increase of the membrane conductance. Figure 3.6A shows a single-channel recording of a lipid bilayer membrane in the presence of a very low concentration of BB0142 at a membrane voltage of 20 mV. Panel B shows a histogram of 122 conductance steps observed in single-channel experiments with BB0142 containing FPLC fractions. This protein forms obviously channels with an average single-channel conductance of 300 pS in 1 M KCl. Channel-formation by the 300 pS channel could be completely block by incubation with polyclonal antibodies against BB0142. After addition of the polyclonal antibodies to the protein fraction in a ratio 1:2 and incubation for 1h at room temperature, the mixture showed no channel-forming activity in the lipid bilayer assay. Interestingly, the 300 pS pore did not show any voltage dependence even at voltages as high as ± 150 mV (data not shown). This demonstrates that the 300 pS channel did not exhibit voltage dependent closure.

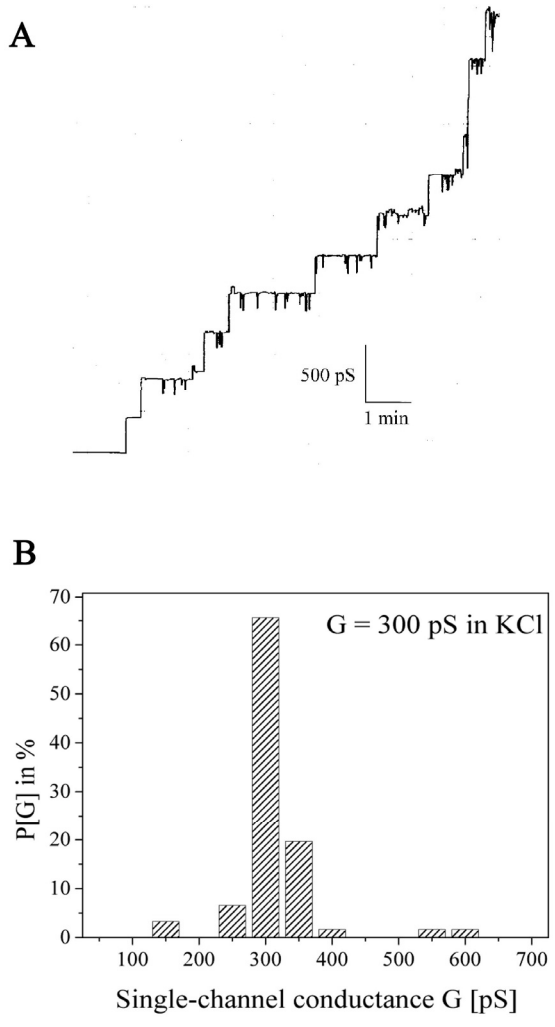


Figure 3.6 (A) Single-channel recording of a diphytanoyl phosphatidylcholine/*n*-decane membrane observed in the presence of 100 ng/ml of fraction 35 of the MonoQ-FPLC of the B-fraction of *B. burgdorferi* HB19 K02. The aqueous phase contained 1 M KCl; V_m = 20 mV; T = 20°C.
(B) Histogram of the probability P(G) for the occurrence of a given conductivity unit observed with membranes formed of 1% diphytanoyl phosphatidylcholine/n-decane in the presence of fraction 35 of the MonoQ-FPLC of the B-fraction of *B. burgdorferi* HB19 K02. P(G) is the probability that a given conductance increment G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The average single-channel conductance for 122 single-channel events was 300 pS

The channels formed by BB0142 were permeable for a variety of different ions. The conductance of the 300 pS channel was found to be dependent on the type of electrolyte and its concentration (see Table 3.1). The KCl concentration varied from 0.1 to 3 M and the conductance behaved as a linear function of the electrolyte concentration. This means that the channel does not contain a binding site for potassium ions or chloride. Replacement of chloride against the less mobile acetate had a strong effect on the single-channel conductance, which decreased by 50% from 300 pS for 1 M KCl to 150 pS for 1 M potassium acetate. The exchange of the cation from K^+ to Li^+ had in contrast to this a relatively small effect: the single-channel conductance decreased only from 300 pS (1 M KCl) to 250 pS (1 M LiCl). This result demonstrates that the 300 pS channel shows some preference for anions over cations.

Table 3.1 Average single channel conductance, *G*, of the channels formed by BB0142 as a function of different salt solutions of concentration *c*

Salt	Concentration	Average single channel conductance
	M	pS
LiCl	1	250
KCl	0.1	30
	0.3	90
	1	300
	3	900
CH_3COOK, pH 7	1	150

The membranes were formed from 1% diphytanoylphoshatidylcholine dissolved in *n*-decane. The pH of the aqueous salt solutions was 6 if not indicated otherwise. The single-channel conductance is given as the mean of at least 100 single events. The applied voltage was 20 mV and the temperature was 20°C.

3.4.4 The channels formed by BB0142 are not selective

Zero-current membrane potential experiments were performed for the analysis of the ion selectivity. Table 3.2 shows the results of the measurements in the presence of 5-fold salt

gradients for KCl, LiCl and potassium acetate. After insertion of 100 to 1000 channels into the PC membrane, the salt concentration on one side of the membrane was raised from 100 to 500 mM by addition of 3 M salt solution. The aqueous phase was stirred for equilibration and 10 min after every increase of the salt gradient the zero-current potential across the membrane was measured. For potassium acetate the potential was found to be positive on the more diluted side of the membrane, whereas the potential was found to be negative for LiCl on the more diluted side of the membrane. The zero current membrane potential was close to zero for KCl, which means that the ion permeability through the BB0142 follows the aqueous mobility of the ions. The analysis of the zero-current membrane potential using the Goldman Hodgkin Katz equation (Benz et al., 1979) confirm that anions and cations have similar permeabilities through the channel because the ratios P_{cation}/P_{anion} were 0.6 (LiCl), 0.9 (KCl) and 2.4 (potassium acetate).

Table 3.2 Zero-current membrane potential, V_m, of diphytanoylphoshatidylcholine/*n*-decane membranes in the present of BB0142 measured for a tenfold gradient of different salts.

Salt	V_m	Permeability ratio P_{cation}/P_{anion}
	mV	
KCl	- 0.1	0.9
LiCl	- 8.7	0.6
KCH_3COO, pH 7	10	2.4

V_m is defined as the difference between the potential at the diluted side (100 mM) and the potential at the concentrated side (500 mM). The pH of the aqueous salt solution was 6 unless otherwise indicated. t = 20°C. P_{cation}/P_{anion} was calculated with the Goldman-Hodgkin-Katz equation (Benz et al., 1978; Benz et al., 1985) from at least three individual experiments

3.4.5 Alignment of BB0142 and *E. coli* TolC

To demonstrate that BB0142 is a homologue to *E. coli* TolC we performed an amino acid sequence alignment using Pole Bioinformatique Lyonnaise Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr) (see Fig. 3.7). The alignment suggests that the amino acid sequence identity between BB0142 and TolC is only 10.92%. However, additional annotations of key structural features of TolC suggest that the two proteins share important structural determinants. For example BB0142 contains prolines at position P44 and P251 that are highly conserved within the TolC protein family. These prolines residues are strictly required to accommodate the

abrupt turn that links the β-barrel to the α-helical motifs. Similarly, the glycines residues at positions 151 and 370 are also conserved and are situated in the turns near the closed end of the TolC channel. However there exist also differences in the amino acid sequence between both proteins. The BB0142 protein, which has 405 residues, is 71 amino acids shorter than the *E. coli* TolC. This depends on a gap of eight residues in the loop 2 in BB0142. In addition, the equatorial domain is missing because BB0142 ends directly after helix 8. It is noteworthy that variations in the loop regions and the deletion of the equatorial domain have been reported also for other TolC homologues (Andersen et al., 2000). Moreover differences in the amino acid sequence are that none of the reported links for intra and intermolecular connections between the monomers are conserved. Also the aspartate ring at the TolC tunnel entrance from the periplasm is not conserved (Andersen et al., 2002; Koronakis et al., 2000).

Furthermore an NCBI conserved domain search has been performed and produced a significant alignment (*E* value = 10^{-21}) of BB0142 with the outer membrane efflux protein (OEP) family, including the *E. coli* outer membrane protein TolC (Marchler-Bauer and Bryant, 2004). This is an important hint for BB0412 being a TolC homologue.

One further observation on the genome level is that the gene *bb0142* is located in an operon encoding an efflux system. The gene *bb0142* encodes the outer membrane compartment of the efflux system which interacts with the acriflavine resistance protein (acrB) BB0140 and a membrane fusion protein (mtrC) BB0141 (Fraser et al., 1997).

Figure 3.7

```
                    10        20        30        40        50        60
                     |         |         |         |         |         |
                      H1                  H2                  S1        L1
BB0142      EIIQISPKQAVNMALENSLDSENALYKENIKKLYKNNAWNVFVPNVNLSSTLSRNPSALS
TolC        ----ENLMQVYQQARLSNPELRKSAADRDAAFEKINEARSPLLPQLGLGADYTYSNGYRD
Consensus       enlkQay#qArennl#lrnaaadr#aakekiN#ArnpllP#lnLgadlsrnngard
Prim.cons.  --------Q----A--------------------N-A-----P---L------------

                    70        80        90       100       110       120
                     |         |         |         |         |         |
                    S2                            H3
BB0142      ELERDYWGFGFGVGINLSL-SPSVLKRMQLVMLEYESAKIERESAVRNIKLNVLKSYNQL
TolC        ANGINSNATSASLQLTQSIFDMSKWRALTLQEKAAGIQDVTYQTDQQTLILNTATAYFNV
Consensus   aner#snafgaglqinqSi dmSklra$qLqelaaeiad!er#saqrniiLNtakaYn#l
Prim.cons.  -----------------S-----------L--------------------LN----Y---

                   130       140       150       160       170       180
                     |         |         |         |         |         |
                                        T1                  H4
BB0142      IALKSTLKVFESQIQNSKLKFEQARIAYNNGLISEIDFLDAQLKYKKSQPDLDGHIINFE
TolC        LNAIDVLSYTQAQKEAIYRQLDQTTQRFNVGLVAITDVQNARAQYDTVLANEVTARNNLD
Consensus   iaaidtLkyf#aQi#aikrql#Qarqa%NnGL!aeiDfq#AraqYdksqa#edgarnNl#
Prim.cons.  ------L-----Q---------Q-----N-GL----D---A---Y------------N--
```

```
                    190       200       210       220       230       240
                     |         |         |         |         |         |
                           S3                  H5                  H6
BB0142       KSKEIFKLLIGLDHDQDFEIIGELPDETIDFSLFNEALNFNESLEIKDLNMRLKMTEQLI
TolC         NAVEQLRQITG-NYYPELAALNVENFKTDKPQPVNALLKEAEKRNLSLLQARLSQDLARE
Consensus    nakEqlrqiiG #hdq#laaineendeTddfqlfNaaLneaEkr#ikdL#aRLkqdeare
Prim.cons.   ---E------G---------------T------N------E------L--RL-------

                    250       260       270       280       290       300
                     |         |         |         |         |         |
                          S4          L2              S5
BB0142       DSLWL-DTYLPSLSLSFSYSPYKSFHENSK--------GFSTGFLASFSLNYGLTEIFPF
TolC         QIRQAQDGHLPTLDLTASTGISDTSYSGSKTRGAAGTQYDDSNMGQNKVGLSFSLPIYQG
Consensus    #irqa DghLPsLdLsaStgisdsfhenSK        gddsnmganksgnsglleI%qg
Prim.cons.   ------D--LP-L-L-S-----------SK-------------------------I---

                    310       320       330       340       350       360
                     |         |         |         |         |         |
                                         H7
BB0142       SKSFTKIQDNNYQLKILQNNVEGKIRNLKSSIVQKRKDIRRYKAILDASKINVELANKNY
TolC         GMVNSQVKQAQYNFVGASEQLESAHRSVVQTVRSSFNNINASISSINAYKQAVVSAQSSL
Consensus    gksnsq!q#a#Y#lkgaq##lEgahRnlkqs!rqkrn#Irasiaii#AsKqaVelA#knl
Prim.cons.   -----------Y---------E----------------I--------A-K-V---A----

                    370       380       390       400       410       420
                     |         |         |         |         |         |
                       T2                H8                            H9
BB0142       QMAFNAFNSGVMDLSKLNDIELVYKQSDLKFIEDKLNYANSILEYKNLINSLD-------
TolC         DAMEAGYSVGTRTIVDVLDATTTLYNAKQELANARYNYLINQLNIKSALGTLNEQDLLAL
Consensus    #aaeaa%nsGtrdisdlnDaeltlk#adqela#arlNYannqL#iKnainsL#
Prim.cons.   ---------G--------D-----------------NY----L--K-----L-

                    430       440       450       460       470
                     |         |         |         |         |
                S6
BB0142       ------------------------------------------------------------
TolC         NNALSKPVSTNPENVAPQTPEQNAIADGYAPDSPAPVVQQTSARTTTSNGHNPFRN
```

Alignment data :

Alignment length : 476
Residues conserved for 90 % or more (upper-case letters) : 52 is 10.92 %
Residues conserved for 50 % and less than 90 % (lower-case letters) : 306 is 64.29 %
Residues conserved less than 50 % (white space) : 78 is 16.39 %
IV conserved positions (!) : 4 is 0.84 %
LM conserved positions ($) : 1 is 0.21 %
FY conserved positions (%) : 3 is 0.63 %
NDQEBZ conserved positions (#): 32 is 6.23 %
Sequence 0001 : BB0142 (403 residues).
Sequence 0002 : TolC (471 residues).

Modified alignment of *B. burgdorferi* BB0142 and *E.coli* TolC (without signal sequence) using Pole Bioinformatique Lyonnaise Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr). Identical amino acids are represented in red. Amino-acid sequence of TolC is annotated with the elements of secondary structure (**H** = α-helix, **T** = turn, **S** = β-sheet) (Koronakis et al., 2000).

3.5 DISCUSSION

Pore forming activity of *Borrelia* outer membrane proteins has been described in previous publications (Ostberg et al., 2002; Shang et al., 1998; Skare et al., 1996; Skare et al., 1997). The previously indentified channel-forming proteins are P66, Oms28 and P13. The channel-forming activity of the latter one has been demonstrated by comparison of the current fluctuations of outer membrane protein preparations of *B. burgdorferi* wildtype and a *p13* knock out strain, respectively (Ostberg et al., 2002). In the first part of our study we performed similar experiments with a *p66* knock out *B. burgdorferi* strain. This strain was used by Coburn and Cugini (2003) to demonstrate the function of P66 as integrin $\alpha_v\beta_3$ adhesion protein (Coburn and Cugini, 2003). We observed for outer membrane protein preparations of the knock out strain a clear reduction of the 11.5 nS activity that is typical for reconstitution experiments with P66 (Skare et al., 1997). This suggests that the outer membrane protein P66 is a bifunctional protein. It is an integrin $\alpha_v\beta_3$ adhesion protein but it acts also as a channel forming protein with a single channel conductance of 11.5 nS in 1 M KCl. P66 is the largest outer membrane channel present in *B. burgdorferi*. Electrophysiological analysis of its properties suggest that it is a general diffusion pore such as the 1.7 nS OmpF of *E. coli* and in contrast to the smaller outer membrane channels such as LamB and Tsx, which are solute-specific (Benz et al., 1985; Benz et al., 1986; Maier et al., 1988; Maier et al., 2001). P66 has a very high single-channel conductance for a general diffusion pore as compared to the channels of the outer membrane of enteric bacteria. However it is not the largest outer membrane pore reported in literature. *Thermus thermophilus* outer membrane contains a giant pore with a single-channel conductance of 20 nS in 1 M KCl (Maier et al., 2001).

Absence of the giant P66 outer membrane channel represents a considerable advantage for purification of other *B. burgdorferi* channel forming proteins. Separation of outer membrane proteins of the *p66* knock out strain by anionic exchange chromatography resulted in a fraction, which showed a uniform channel forming activity in the black lipid bilayer assay with a single-channel conductance of 300 pS in 1 M KCl. By mass spectrometry using peptide mass fingerprints (Sickmann et al., 2002) we identified the protein BB0142 as the sole protein with a putative pore forming function. A BLAST search and a NCBI conserved domain search identified BB0142 as a homologue of *E. coli* TolC. TolC is the outer membrane component of type I secretion systems and multidrug efflux pumps (Koronakis, 2003). By itself, TolC forms channels with a single-channel conductance of 80 pS in 1 M KCl, which is in the similar range as the observed channel in the isolated FPLC fraction (Andersen, 2004; Benz et al., 1993;

Koronakis, 2003; Koronakis et al., 2000). TolC is a homotrimer with a 140 Å long cannon-shape structure. Each of the three monomers contributes four β-strands to form a single 40 Å long β-barrel, the so-called channel domain, which is anchored in the outer membrane (Koronakis et al., 2000). The β-barrel is prolonged by a 100 Å long tunnel domain formed exclusively by α-helices. The alignment of BB0142 with TolC (see Fig. 7) depicts conserved residues, which are important for the correct folding of the channel-tunnel family. Among these are prolines and glycines, which are important for the transitions between the β-sheets of the channel and the α-helices of the tunnel (Andersen et al., 2001). An immunoblot analysis with BB0142 antibodies showed that BB0142 is present in the FPLC fraction, which is active in the black lipid bilayer. When the FPLC fraction was incubated with BB0142 antibodies and added to the black lipid bilayer assay the channel forming activity was completely abolished, confirming that BB0142 is responsible for the channel forming activity. A further purification of the putative oligomer protein band of BB0142 by preparative SDS-PAGE failed (data not shown). We assume that this occurs because of the sensitivity of BB0142 to SDS, as it is reported for other channel forming protein of *B. burgdorferi* (Ostberg et al., 2002).

For TolC of *E. coli* it is known that residues lining the periplasmic entrance are critical for the electrophysiological behavior. The aspartate ring consisting of six aspartates residues at the TolC tunnel entrance from the periplasm is responsible for its high cation selectivity (Andersen et al., 2002). In BB0142 this aspartate ring is replaced by positive lysine residues (K366) and negative aspartate residues (D369) resulting in an unselective BB0142 channel, which is in contrast to the selective TolC channel. The lysine residue (K366) and the aspartate residue (D369) could compensate each other and might form a salt bridge. This means that no net charge exists in the tunnel entrance. This consideration is supported by titration experiments with multivalent cations, which do not block the BB0142 channel in contrast to the *E. coli* TolC (data not shown) (Andersen et al., 2002). Another difference between BB0142 and TolC is that the BB0142 channel has a nearly four times higher conductance in 1M KCl than TolC (80 pS in 1M KCl) (Benz et al., 1993). TolC of *E. coli* contains a circular network of inter- and intramolecular connections between the three monomers that is presumably responsible for the stability of the closed state of the channel-tunnel (Andersen et al., 2002). None of the residues forming these inter- and intramolecular connections are conserved in BB0142. Thus it is possible that other residues build a circular network of inter and intramolecular connections, keeping the tunnel entrance closed otherwise the single-channel conductance should be much higher. Structural modeling or crystal structure determinations of BB0142 could answer the questions what kind of network is responsible for the closed state of the channel.

Looking at the location of the *bb0142* gene within the chromosome of *B. burgdorferi* it is evident that it is a part of an operon coding for a multidrug efflux pump. This means that BB0142 is interacting with the putative inner membrane complex formed by the AcrB homologue BB0140, which serves as a inner membrane transporter, and the MtrC homologue BB0141, a periplasmic accessory protein (Pos et al., 2004; Veal et al., 1998). These three components are necessary to form a transport system spanning the two membranes for pumping out noxious compounds from the cytoplasm (Andersen, 2003; Sulavik et al., 2001). So far, the finding of a multidrug efflux system in *B. burgdorferi* makes sense, because *B. burgdorferi* is carrying a natural resistance for several antibiotics (Hunfeld et al., 2001). Resistance can originate from multidrug efflux pumps, as it is demonstrated for several other gram-negative bacteria (Eswaran et al., 2004; Grkovic et al., 2002; Mata et al., 2000; Sulavik et al., 2001). Expression of BB0142 occurs also under cultivation conditions and this shows that the only drug efflux pump found in the *B. burgdorferi* B31 genome is active. For further studies it would be interesting to investigate, if the protein is expressed in all phases of the *Borrelia* life cycle or if expression is special for certain conditions. Another future interest would be to identify the substrates of the multidrug efflux pump. This could be done by investigation of a *bb0142* knock out mutant.

Taken together we have here in demonstrated that P66 is a channel forming protein besides its function as integrin $\alpha_v\beta_3$ adhesion protein because the typical 11.5 nS channels were absent in the B-fraction of the *B. burgdorferi* knock out strain. In addition we identified and characterized BB0142 as a TolC homologue of *B. burgdorferi* in the black lipid bilayer assay. In future studies it would be of interest to investigate the role of the TolC homologue BB0142 in virulent and in drug resistant *Borrelia* strains as well as performing complementation studies in other bacterial species.

CHAPTER 4

Site-directed mutagenesis of the greasy slide aromatic residues within the LamB (maltoporin) channel of *Escherichia coli*: Effect on ion and maltopentaose transport.

4.1 SUMMARY

The 3D-structure of the maltooligosaccharide-specific LamB-channel of *Escherichia coli* (also called maltoporin) is known from X-ray crystallography. The 3D-structure suggest that a number of aromatic residues (Y6, Y41, W74, F229, W358 and W420) within the channel lumen is involved in carbohydrate and ion transport. All aromatic residues were replaced by alanine (A) scanning mutagenesis. Furthermore, LamB mutants were created in which one, two, three, four, and five aromatic residues were replaced to study their effects on ion and maltopentaose transport through LamB. The purified mutant proteins were reconstituted into lipid bilayer membranes and the single-channel conductance of the mutants was studied in conductance experiments. The results suggest that all aromatic residues provide some steric hindrance for ion transport through LamB. Highest impact is provided by Y6 and Y41 that are localized opposite to Y118, which form the central constriction of the LamB channel. Stability constants for binding of maltopentaose to the mutant channels were measured using titration experiments with the carbohydrate. The mutation of one or several aromatic amino acid led to a substantial decrease of the stability constant of binding. The highest effect was observed when all aromatic amino acids were replaced by alanine because no binding of maltopentaose could be detected in such a case. However, binding was again possible when Y118 was replaced by tryptophane (W). The carbohydrate-induced block of the channel function could also be used for the study of current noise through the different mutant LamB-

channels. The analysis of the power density spectra of some of the mutants allowed the evaluation of the on- and off-rate constants (k_1 and k_{-1}) of carbohydrate binding to the binding-site inside the channels. The results suggest that both on- and off-rate constants were affected by the mutations. For most mutants k_1 decreased and k_{-1} increased. The possible influence of the aromatic residues of the greasy slide on carbohydrate and ion transport through LamB is discussed.

4.2 INTRODUCTION

The cell envelope of gram-negative bacteria consists of different layers. The inner or cytoplasmic membrane contains the respiration chain, proteins for the transport of nutrients and proteins involved in the synthesis of phospholipids, peptidoglycan and lipopolysaccharides (Beveridge, 1981; Nikaido and Vaara, 1985). The periplasmic space between the membranes is an aqueous compartment isoosmolar to the cytoplasm(Benz, 1994). It contains the peptidoglycan and a large number of different proteins. The outer membrane is composed of protein, lipid and lipopolysaccharide (Nikaido and Vaara, 1985). It contains only a few major proteins. At least one of constitutive outer membrane proteins called porin forms a general diffusion pore with a defined exclusion limit for hydrophilic solutes within the outer membrane (Benz and Bauer, 1988; Danelon and Winterhalter, 2004; Hancock et al., 1986; Nikaido and Vaara, 1985). In addition to the constitutive porins the outer membrane may contain porins, which are induced under special growth conditions (Brass et al., 1985; Hancock, 1981; Szmelcman et al., 1976; Tommassen and Lugtenberg, 1980). They often form solute-specific channels and contain binding sites for neutral substrates such as carbohydrates (Benz et al., 1986; Ferenci et al., 1980), nucleosides(Maier et al., 1988) and charged solutes such as phosphate (Benz and Hancock, 1987; Hancock, 1981). Many of these specific porins are part of uptake and degradation systems, such as the mal-system of *Escherichia coli* (Schwartz, 1987) or the single copy plasmid pUR400 of enteric bacteria (Schmid et al., 1988). These systems confer to the bacteria the possibility of growth on maltose and maltooligosaccharides and on sucrose as carbon source (Schmid et al., 1982; Szmelcman et al., 1976).

The proteins coded on the maltose operon contain also a specific outer membrane porin that is

specific for maltose and maltooligosaccharides. LamB of *Escherichia coli* and other enteric bacteria is essential for the uptake of maltooligosaccharides across the outer membrane (Szmelcman et al., 1976), but it has a much lower efficiency for the uptake of sucrose(Andersen et al., 1998; Schmid et al., 1982). LamB has been crystallized and its 3D-structure is known from X-ray analysis (Schirmer et al., 1995). The individual channel within a LamB-trimer is formed by 18 antiparallel β-strands, which form a cylinder with a diameter of about 2.5 nm. The diameter of the channel is reduced by the external loop 3 folding into the channel lumen to form a central constriction with a size of about 0.5 x 0.3 nm (see Figure 4.1). Carbohydrate transport through the channel is mediated partially by van der Waals interaction of the maltooligosaccharides with six aromatic residues lining up the channel interior (the greasy slide, Y6, Y41, W74, W358, W420 and F227) and by hydrogen bonds between the hydroxy groups of the carbohydrates and amino acid residues within the constriction zone such as R8 (Dutzler et al., 1996; Jordy et al., 1996; Schirmer et al., 1995). The aromatic amino acids are lined up from the external surface in the series W74 (provided by a neighboring monomer), Y41, Y6, W420, W358 and F227 close to the periplasmic space (see Figure 1). Localized opposite to these amino acids of the greasy slide is tyrosine 118 (Y118) (see Figure 1). This amino acid has a major impact of ion and carbohydrate transport through LamB (Orlik et al., 2002; Orlik et al., 2002). Furthermore, alanine-scanning mutagenesis of single aromatic residues of the greasy slide has shown that they influence carbohydrate binding *in vitro* and maltose uptake *in vivo* (Van Gelder et al., 2002).

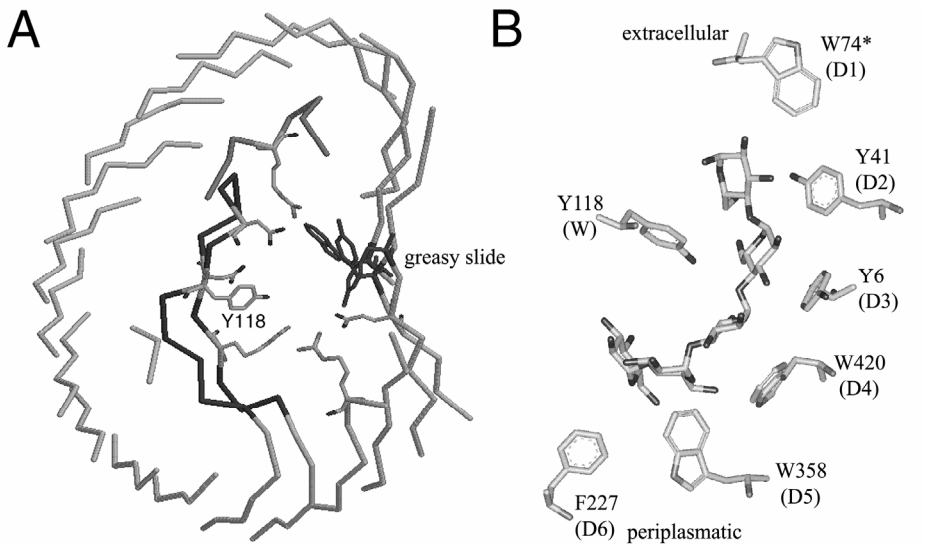


Figure 4.1 Cross-section of the *E. coli* LamB monomer (maltoporin) (A, left side panel) and arrangement of the greasy slide together with a bound maltopentaose (B, right side panel). The left side panel shows loop 3 (dark gray) and tyrosine 118 (denoted with its number from the mature N-terminal end) that are relevant for passage of carbohydrates and ions through the

central constriction. The amino acids of the greasy slide are localized opposite to Y118 and are given in dark gray. The strands of the β-barrel cylinders of the porins are given in light gray. The coordinates of maltoporin were taken from the crystallographic data of Schirmer et al. (1995) (Schirmer et al., 1995). The right side panel shows the arrangement of the aromatic residues of the greasy slide (denoted with their numbers from the N-terminal end and of the deletions) with respect to a bound maltopentaose molecule. Note that the amino acids W74 (D1; denoted with an asterix) is provided from a neighboring LamB monomer. The coordinates of bound maltopentaose were taken from the crystallographic data of Dutzler et al. (1995)(Dutzler et al., 1996).

Carbohydrate binding to the LamB channel has been studied in detail by assuming a symmetrical one-site two-barrier model for sugar transport and titrating the ion current through LamB with increasing concentrations of different carbohydrates (Benz et al., 1987). Carbohydrate binding affinity increases with the chain length of the maltooligosaccharides. The kinetics of carbohydrate transport can be derived from carbohydrate-induced current noise of the LamB-channel (Andersen et al., 1995; Jordy et al., 1996; Nekolla et al., 1994). In this study we give a quantitative description of the effect of alanine scanning mutagenesis of all aromatic residues of the greasy slide on ion transport and carbohydrate binding through the LamB-channel. Besides single mutations, two, three, four, five and all six amino acids of the greasy slide were replaced by alanine. Some of the mutants included also mutagenesis of Y118W together with mutations of the greasy slide residues. All mutants were purified to homogeneity and ion transport as well as maltopentaose binding was studied in detail using the lipid bilayer assay. In addition, for some of the mutants it was possible to evaluate maltopentaose-binding kinetics by the measurement of the current noise. The results suggest that all amino acids of the greasy slide contribute to certain extent to ion and carbohydrate transport properties of LamB. Highest impact on channel function have the aromatic amino acids Y41, Y6 and W420 localized opposite Y118 and Y118 itself. When all six residues of the greasy slide are mutated to alanine, the mutation Y118W is sufficient to confer to LamB maltopentaose transport *in vivo* and maltopentaose binding *in vitro*.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Diphytanoyl phosphatidylcholine (DiphPC) was obtained from Avanti Polar Lipids (Alabaster, AL). All salts were analytical grade (Merck, Darmstadt, Germany). Ultrapure water was obtained by passing deionized water through a Milli-Q equipment (Millipore, Bedford, MA).

The QuikChange™ site-directed mutagenesis kit and the *One Shot®* Top10F' competent cells were purchased from Stratagene (Amsterdam, The Netherlands). Maltopentaose was obtained from Seikagaku (Japan) distributed by Medac (Germany).

4.3.2 Plasmids and DNA manipulations

The generation of LamB mutant Y118W was described previously (Orlik et al., 2002). All other LamB mutants were constructed according to standard genetic manipulations using the QuikChange™ site directed mutagenesis kit. Table 1 contains a list of the mutants and the primers used for the mutation. Plasmid pAM117 encoding wild-type LamB (Heine et al., 1988) was used as a template for *in vitro* site directed mutagenesis. For each mutant two synthetic oligonucleotide primers were designed (purchased from Carl Roth, Karlsruhe, Germany) each complementary to opposite strands of the plasmid and containing the desired mutation (see Table 1). The oligonucleotide primers were extended during temperature cycling by using *Pfu-turbo* DNA polymerase. Incorporation of the primers generates a mutated plasmid containing staggered nicks. After temperature cycling the product was treated with *DpnI*. The *DpnI* endonuclease (target sequence: 5'-Gm^{6}ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template. This led to the selection of the mutation-containing synthesized DNA. The nicked vector DNA was then transformed into *One Shot®* Top10F' competent cells, where the nicks in the mutated plasmid were repaired. For the generation of double mutations the plasmid coding for a single mutation was used as a template for further *in vitro* site directed mutagenesis. Similarly, using the already existing plasmids coding for mutant porins as templates triple and higher mutations were created. The mutants containing the mutation Y118W were likewise created. All mutated plasmids were controlled by DNA sequencing.

Table 4.1 Primers for the greasy slide mutants of LamB

Mu-tant	Muta-tion	Primer
D1	W74A	5´-GCA CAA CAG AAT GAC GCG GAA GCT ACC GAT CCG-3
		5´-CGG ATC GGT AGC TTC CGC GTC ATT CTG TTG TGC-3´
D2	Y41A	5´-GT CTT GGC AAC GAA TGT GAA ACT GCT GCT GAA TTA AAA TTG GG-3´
		5´-CC CAA TTT TAA TTC AGC AGC AGT TTC ACA TTC GTT GCC AAG AC-3´
D3	Y6A	5´-GTT GAT TTC CAC GGC GCT GCA CGT TCC GGT ATT G-3´
		5´-C AAT ACC GGA ACG TGC AGC GCC GTG GAA ATC AAC-3´
D4	W420A	5´-GGT GCC CAG ATG GAA ATC GCG TGG TAA TAG CAA AAC-3´
		5´-GTT TTG CTA TTA CCA CGC GAT TTC CAT CTG GGC ACC-3´
D5	W358A	5´-GC GAC AGC ATC GCG TCA CGC CCG GCT ATT C-3´
		5´-G AAT AGC CGG GCG TGA CGC GAT GCT GTC GC-3´
D6	F227A	5´-CAG AGT GTC CTG AAG GGC GCT AAC AAG TTT GTT GTT C-3´
		5´-G AAC AAC AAA CTT GTT AGC GCC CTT CAG GAC ACT CTG-3´

4.3.3 Growth of bacteria and purification of LamB-mutants

The plasmids that contained the genes of the LamB-mutants were transformed via electroporation into competent cells of the strain *E. coli* KS26 or Omp8 (which both lack most of the outer membrane porins ($LamB^-$, $OmpF^-$, $OmpC^-$ and $TolC^-$) (Schulein et al., 1995). The strains harboring the plasmids were grown in DYT-Medium at 37 °C. Although the LamB gene in this plasmid has no controllable promoter, the expression of the LamB gene is sufficient and is not lethal for the cells (Heine et al., 1988). The cells containing the LamB mutants were harvested at an OD of 1.0 and passed three times through a French pressure cell at 900 psi. Unbroken cells were removed by centrifugation. The cell envelopes were pelleted in an ultracentrifuge (Beckman Omega XL90) at 48.000 rpm for 60 min. Further isolation procedure has been described in detail elsewhere (Jordy et al., 1996). Briefly, the LamB mutants were isolated by (i) extraction of cell envelopes with sodium dodecyl sulfate (SDS) at 30 °C and (ii) release of LamB mutants from the protein-peptidoglycan complex by treatment with 0.4 M NaCl-solution. The supernatant of a subsequent centrifugation step was applied to a starch column (starch coupled to Sepharose 6B as described by Ferenci *et al.*(1980)[11]). The column was washed first with a buffer containing 0.1 M $NaHCO_3$, 1 % Triton X-100, pH 8.6 (buffer 1) and then with the same buffer supplemented with 1 % SDS (buffer 2) to remove unspecifically bound proteins. The column was eluted with buffer 2 containing in addition

20% maltose to remove the bound mutant protein from the column. Some of the mutants did not bind sufficiently tight to the starch column to allow the use of this protocol for purification. In these cases purification of the LamB mutants was performed by preparative SDS-PAGE as has been described previously (Jordy et al., 1996). All mutant LamB proteins were pure and in their trimeric form as judged by SDS-PAGE using a solubilization temperature of 30 °C.

4.3.4 Growth experiments with the strains containing LamB or the LamB-mutants

The growth experiments with the LamB mutant strains were performed in M9 minimal medium containing 6.4 % Na_2HPO_4, 1.5 % KH_2PO_4, 0.25 % NaCl, 0.5 % NH_4Cl, (all weight/vol), 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$. The salt solution was autoclaved and then supplemented with sterile filtered 0.4% (4.8 mM) maltopentaose, 5mg/l thiamin and 50 mg/ ampicillin. The wild-type *lamB* gene and all the mutated alleles were expressed from pAM117 derived plasmids in KS26, a strain, which lacks most of the outer membrane porins (Orlik et al., 2002; Schulein et al., 1995). It is noteworthy that the LamB and the LamB mutant genes are not under the control of an inducible promoter and have an expression rate of about 40 % of the level of fully induced, wildtype, chromosomally encoded protein (Heine et al., 1988).

4.3.5 Lipid bilayer experiments

Single channel conductance measurements

Black lipid bilayer membranes were formed as described previously (Benz et al., 1978). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole with a surface area of about 0.4 mm^2. Membranes were formed by painting onto the hole a 1 % solution of diphytanoyl phosphatidylcholine in n-decane across the holes. The aqueous KCl solutions were used unbuffered and had a pH of 6. The LamB mutants were added from concentrated stock solution to the aqueous phase bathing a membrane in the black state. The temperature was kept at 20°C throughout. The membrane current was measured with a pair of Ag/AgCl electrodes switched in series with a voltage source and a current amplifier (a current to voltage converter home made using a Burr Brown operational amplifier). The amplified signal was monitored with an oscilloscope and recorded with a strip chart recorder or a PC. For all conditions used here at least 100 conductance steps were recorded and averaged to give the single-channel conductance value. Its standard

deviation was generally below 10 % of its mean value.

Titration experiments

Wildtype LamB can be blocked for ion transport when a carbohydrate is bound to the binding site inside the channel(Benz et al., 1986). We studied whether the mutant channels also bind carbohydrates. These measurements were performed with multi-channel experiments under absolutely stationary conditions (Andersen et al., 1995; Nekolla et al., 1994). The LamB mutants were added to black diphytanoyl phosphatidylcholine/n-decane membranes at concentration of about 500 ng/ml. After about 30 minutes the current was stationary. At that time small amounts of concentrated solutions of maltopentaose were added to the aqueous phase to both sides of the membrane, with stirring to allow equilibration. In these experiments we observed a strong dose-dependent decrease of the membrane conductance. The conductance data of the titration experiments were analyzed using the following equations used earlier for the carbohydrate-induced block of wildtype LamB (Benz et al., 1986; Benz et al., 1987). The conductance, *G(c)*, of a LamB mutant channel in the presence of maltopentaose with the stability constant, *K* (half saturation constant K_S) and the carbohydrate concentration, *c*, is given by the maximum conductance (without carbohydrate), G_{max} times the probability that the binding site is free:

$$G(c) = \frac{G_{max}}{(1 + K \cdot c)} \qquad (1)$$

Equation 1 may also be written as:

$$\frac{(G_{max} - G(c))}{G_{max}} = \frac{K \cdot c}{(K \cdot c + 1)} \qquad (2)$$

which means that the conductance as a function of the carbohydrate concentration can be analyzed using Lineweaver-Burke plots.

Noise analysis

The membrane current was measured by a pair of silver/silver chloride electrodes switched in series with a battery operated voltage source and a current amplifier (Keithley 427 with a four pole filter or a home-made operational amplifier with a three pole filter). Feedback resistors between 0.01 and 10 GΩ were used in the experiments. The amplified signal was monitored by a strip chart recorder and simultaneously fed through a low pass filter (4 Pole Butterworth Low-Pass Filter) into an AD-converting card of an IBM-compatible PC. The digitized data were analyzed with a homemade fast Fourier transformation program. The spectra were composed of 400 points and they were averaged either 128 or 256 times. The spectra were analyzed using commercial graphic programs. Assuming small perturbations of the number of closed channels due to microscopic variations of the number of bound maltopentaose molecules, the power density spectrum, $S(f)$ is given by a "Lorentzian" function (De Felice, 1981; Nekolla et al., 1994; Verveen and DeFelice, 1974):

$$S(f) = \frac{S_0}{1 + (f / f_c)^2} \qquad (3)$$

S_0 is the plateau value of the power density spectrum at small frequencies and f_c is the corner frequency given by

$$\frac{1}{\tau} = 2\pi \cdot f_c = k_1 \cdot c + k_{-1} \qquad (4)$$

A plot of 1/τ as a function of the ligand concentration c allows the derivation of the on- and off-rate constants, k_1 and k_{-1} of the chemical reaction. These rate constants describe the jump and the inverse movement, respectively, of maltopentaose from the aqueous phase to the binding-site inside the channel. The stability constant of the binding of maltopentaose to the binding-site is given by $K = k_1 / k_{-1}$ (i.e. the half saturation constant is $K_S = k_{-1} / k_1$)

4.4. RESULTS

4.4.1 Growth experiments with KS26 strains containing wildtype LamB and different greasy slide mutants

To study the functional integrity of the greasy slide mutants of LamB *in vivo* we performed growth experiments of some of the mutant strains in an synthetic M9 medium supplemented with 0.4% maltopentaose, which means that it contained maltopentaose as sole carbon source. For these investigations the plasmid pAM117 carrying the genes for wildtype LamB and the different LamB greasy slide mutants (Heine et al., 1988) were transferred into the KS26 strain that lacks most outer membrane porins (Schulein et al., 1995). An aliquot corresponding to 0.02 OD of an overnight culture, grown in LB media was added to 10 ml M9 minimal medium and the growth was followed over at least one day. The growth curves showed a substantial difference between the growth of the porin deficient strain KS26 and KS26 transfected with the plasmids encoding for wildtype LamB and the LamB mutants. KS26 showed absolutely no growth on maltopentaose, but the strains that expressed wildtype LamB and most of the LamB greasy slide mutants showed approximately the same growth rate. This result indicated that most of the LamB mutants were able to transport maltopentaose, i.e. the mutants were functional. However, we observed one exception. The six-fold greasy slide mutant (D1/2/3/4/5/6), which lacks all aromatic residues of the greasy slide showed no growth similar to KS26. Interestingly, the six-fold greasy slide mutant that contains the additional mutation Y118W shows again growth. This means presumably that maltopentaose transport sufficient for growth is restored when tyrosine 118 is mutated to tryptophane (see also Discussion). For most of the mutants the outer membrane was not rate limiting under the conditions employed here because 0.4% maltopentaose used in the growth experiments corresponds to a concentration of 4.8 mM maltopentaose, which is above the half saturation of most of the LamB mutants used in this study (see below).

4.4.2. Effect of the aromatic residues of the greasy slide on single-channel conductance

In a first set of experiments we investigated the effect of the replacement of single greasy slide amino acids by alanine. The data were compared to wildtype LamB (Benz et al., 1986) Y118W and Y118A, which had been studied previously (Orlik et al., 2002). All mutants formed well-defined channels in lipid bilayer membranes indicating no gross perturbation of the channel structure induced by the mutations. Examples for single channel recordings are

shown in Figure 4.2 for the mutants D1 (A), D1/2 (B), D1/2/3/6+Y118W (C) and D1/2/3/4/5/6 (D). The single-channel conductance of the mutants was analyzed in histograms (see Figure 3) showing a homogeneous distribution for all LamB mutants. Table 2 shows a summary of the single-channel conductance of the six single mutant channels in 1 M KCl solution. The data indicate that the single-channel conductance was in many cases influenced by the mutation of the aromatic residues.

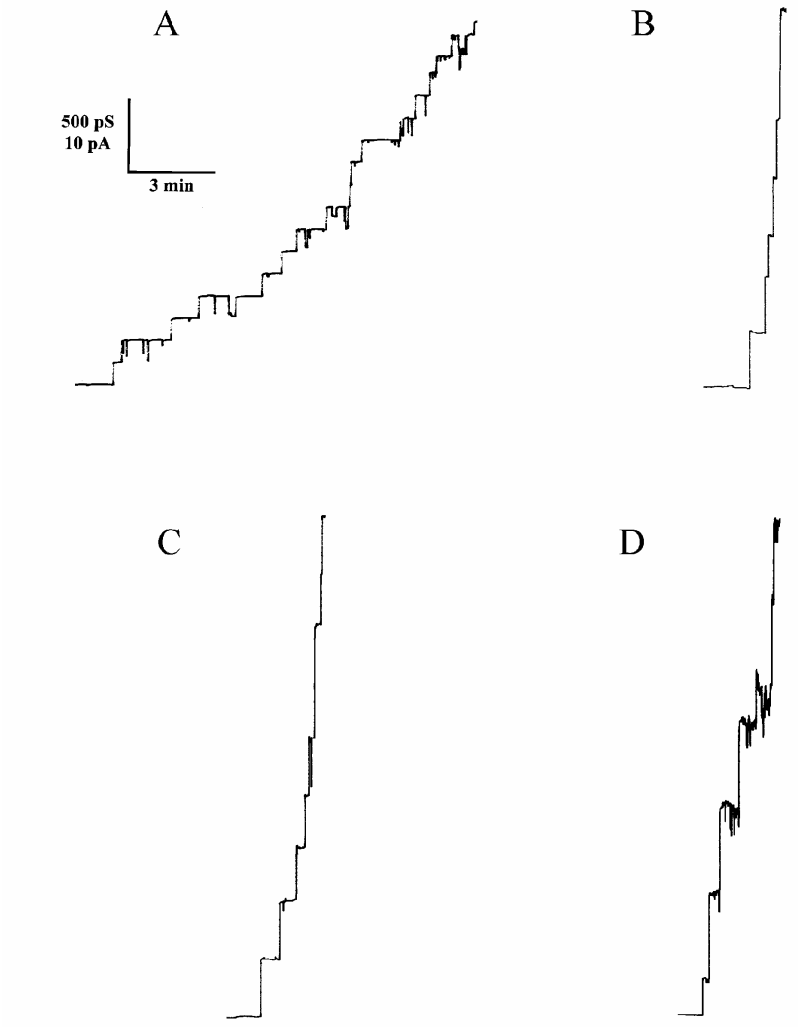


Figure 4.2 Single-channel recordings of diphytanoyl phosphatidylcholine/n-decane membranes in the presence of 4 different maltoporin greasy slide mutants D6 (A), D1/2 (B), D1/2/3/4+Y118W (C) and D1/2/3/4/5/6 (D). The aqueous phase contained 1 M KCl (pH 6) and 10 ng/ml LamB mutants. The applied membrane potential was 20 mV; T = 20°C. Note that current noise of the single-channel recording of wildtype LamB is similar to that of the mutants indicating that the mutation did not induce a major change of the channel structure.

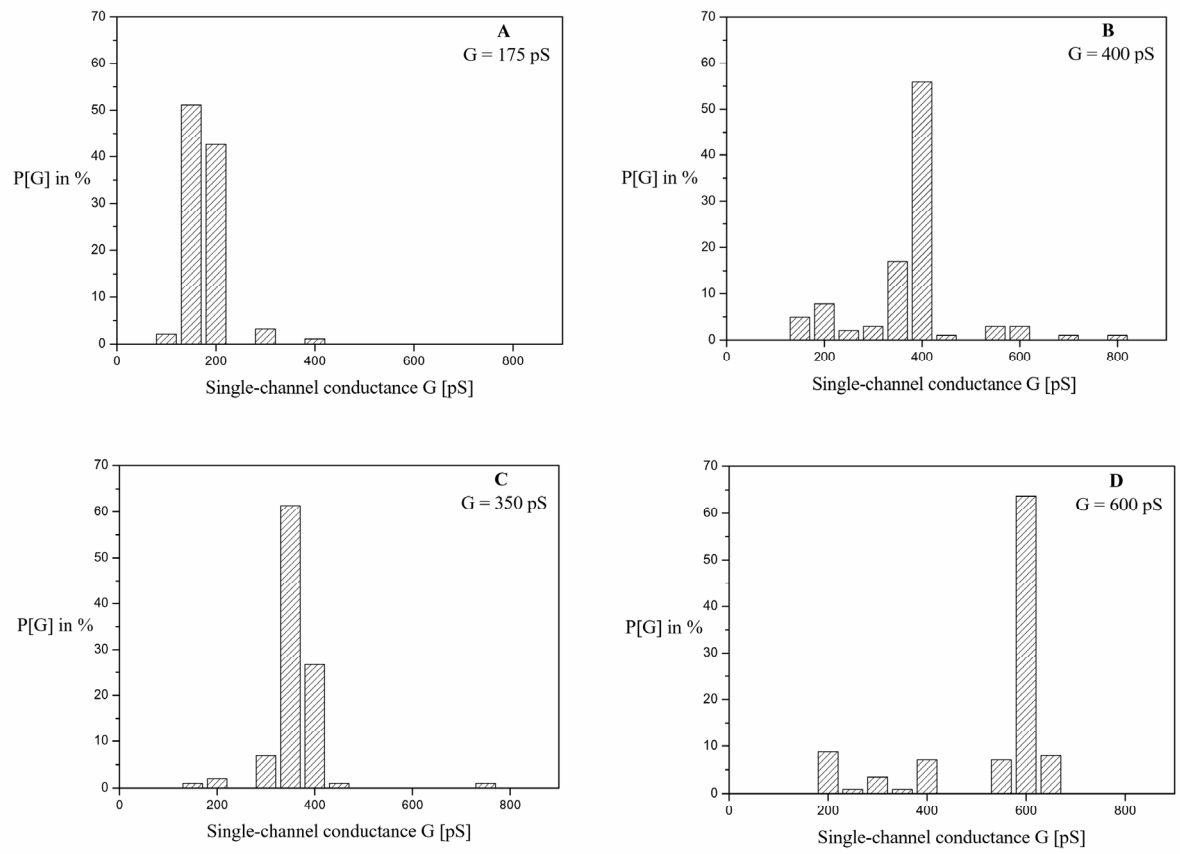


Figure 4.3 Histogram of the probability of the occurrence of certain conductivity units observed with membranes formed of diphytanoyl phosphatidylcholine/n-decane in the presence of 10 ng/ml of the LamB greasy slide mutants D6 (A), D1/2 (B), D1/2/3/4+Y118W (C) and D1/2/3/4/5/6 (D). The aqueous phase contained 1 M KCl. The applied membrane potential was 20 mV; T = 20°C. The average single-channel conductance was 175 pS for 94 single-channel events (D6), 375 pS for 102 events (D1/2), 350 pS for 101 events (D1/2/3/4+Y118W) and 600 pS for 113 events (D1/2/3/4/5/6).

From all single mutations, the single mutations D1 (W74A), D2 (Y41A) and D3 (Y6A) showed the highest effect on the single-channel conductance, which increased for these mutants (300, 350 and 275 pS, respectively) approximately a factor of two over LamB wildtype (155 pS). All the other three single mutations influenced the conductance of the LamB mutant channels only little. This result has presumably to do with the location of Y6, Y41 and W74 with respect to the central constriction of the LamB-channel, which is localized between Y118 on one side and Y6 and Y41 on the other side (see Figure 4.1). In particular Y118 plays a major role in ion transport because its mutation to alanine leads to the highest increase of the single-channel conductance for all mutants in which aromatic residues were changed by alanine scanning mutagenesis. In this case the increase was by a factor of about

six as compared to wildtype LamB (Orlik et al., 2002).

The effect of double mutations on single-channel conductance was in all three cases not much different compared to the single mutations (see Table 4.2). The combination D1/2 resulted in the highest conductance, which is well understood because the single mutations D1 and D2 showed already a high conductance. The two triple mutations (D1/2/3 and D4/5/6), in which the outer and the inner aromatic residues were deleted, respectively, follow this line. The effect of the deletion of the outer residues on ion permeability is higher than that of the inner ones. Similar results were obtained for the mutants where more than three aromatic residues were deleted (see Table 4.2). Ion permeability through the mutant LamB-channels was high when all three of the outer residues were mutated together with one or two of the inner aromatic amino acids (W420, W359 and F227; see Table 4.2). Highest single-channel conductance was observed, when all six aromatic residues were removed. This result indicates that all these aromatic amino acids represent some steric hindrance for ion transport though LamB.

In previous papers, it has been demonstrated that the replacement of Y118 by phenylalanine and tryptophane had the smallest influence on ion transport through LamB probably because the bulky tyrosine side chain was replaced by other bulky groups (Jordy et al., 1996; Orlik et al., 2002). The mutation Y118W had a substantial effect on carbohydrate binding (Orlik et al., 2002). Therefore some mutants were investigated where besides greasy slide mutations Y118 was replaced by tryptophane. Single-channel experiments with these mutants suggested that the mutation Y118W had a substantial influence on the conductance of the mutants. In all cases the single-channel conductance decreased when Y118W was introduced in addition to greasy slide mutations (see Table 4.2). This means that the bulky side chain of tryptophane controls ion flux through the central constriction in a similar way as phenylalanine or tyrosine (Orlik et al., 2002).

Table 4.2 Single-channel conductance of the LamB greasy slide mutants in 1 M KCl. Up to six aromatic amino acids of the greasy slide were replaced by alanine[a].

Mutant number	Mutation	Single channel conductance [pS]
	LamB wild-type	155*
	Y118A	850**
	Y118W	90**
	Single Mutation	
D1	W74A	300
D2	Y41A	350
D3	Y6A	275
D4	W358A	175
D5	W420A	175
D6	F227A	175
	Double Mutations	
D1/2	Y41A/W74A	400
D3/4	Y6A/W420A	300
D5/6	F227A/W358A	300
	Triple Mutations	
D1/2/3	W74A/Y41A/Y6A	500
D4/5/6	W420A/W358A/F227A	175
	More than three Mutations	
D1/2/3/4	W74A/Y41A/Y6A/W420A	500
D3/4/5/6	Y6A/W420A/W358A/F227A	250
D1/2/3/4/5	W74A/Y41A/Y6A/W420A/W358A	450
D2/3/4/5/6	Y41A/Y6A/W420A/W358A/F227A	500
D1/2/3/4/5/6	W74A/Y41A/Y6A/W420A/W358A/F227A	600
	Mutations together with Y118W	
D1/2+Y118W	Y41A/W74A+Y118W	225
D3/4+Y118W	Y6A/W420A+Y118W	300
D5/6+Y118W	F227A/W358A+Y118W	300
D1/2/3/4+Y118W	W74A/Y41A/Y6A/W420A+Y118W	350
D3/4/5/6+Y118W	Y6A/W420A/W358A/F227A+Y118W	275
D1/2/3/4/5/6+Y118W	W74A/Y41A/Y6A/W420A/W358A/F227A+Y118W	550

The membranes were formed of diphytanoyl phosphatidylcholine dissolved in n-decane. The 1 M

KCl solutions were unbuffered and had a pH of 6. The applied voltage was 20 mV, and the temperature was 20°C. The average single-channel conductance was calculated from at least 100 single events: The single-channel conductance of wildtype LamB* (Benz et al., 1986) and the Y118A** mutant (Orlik et al., 2002) are given for comparison.

4.4.3 Evaluation of the stability constants for maltopentaose binding to the different greasy slide mutants

The stability constants for maltopentaose binding to LamB-mutants were evaluated from titration experiments similar to those performed previously to study carbohydrate binding to LamB wildtype and ScrY (Benz et al., 1987; Schulein et al., 1995). Figure 4.4 shows an example for such experiments. LamB mutant Y6A (D3) was added while stirring from a concentrated stock solution to the aqueous phase (concentration about 500 ng/ml) bathing a black lipid bilayer membrane. The corresponding current increase was monitored on a strip chart recorder. After about 30 min the conductance was almost stationary. The titration experiment was started by the addition of concentrated solution of maltopentaose to both sides of the membrane. This led to a decrease of membrane conductance in a dose dependent way as is shown in Figure 4.4. At a maltopentaose concentration of 5.4 mM the current through the membrane almost decreased to zero, which means that the LamB mutant Y6A (D3) channels were almost totally blocked for the flux of ions caused by the binding of maltopentaose to the binding site. The stability constant K for the binding of maltotriose to the binding site inside the Y6A (D3) channel was evaluated using Lineweaver-Burke plots according to eqn. (2). An example for the fit of the data of Figure 4.3 is given in Figure 4.5. The stability constant for maltopentaose binding to the Y6A (D3) mutant was 973±16 1/M (half saturation constant 1.03 mM). This has to be compared with a stability constant of 14,000 1/M (half saturation constant about 70 μM) for maltopentaose binding to wildtype LamB (Benz et al., 1987).

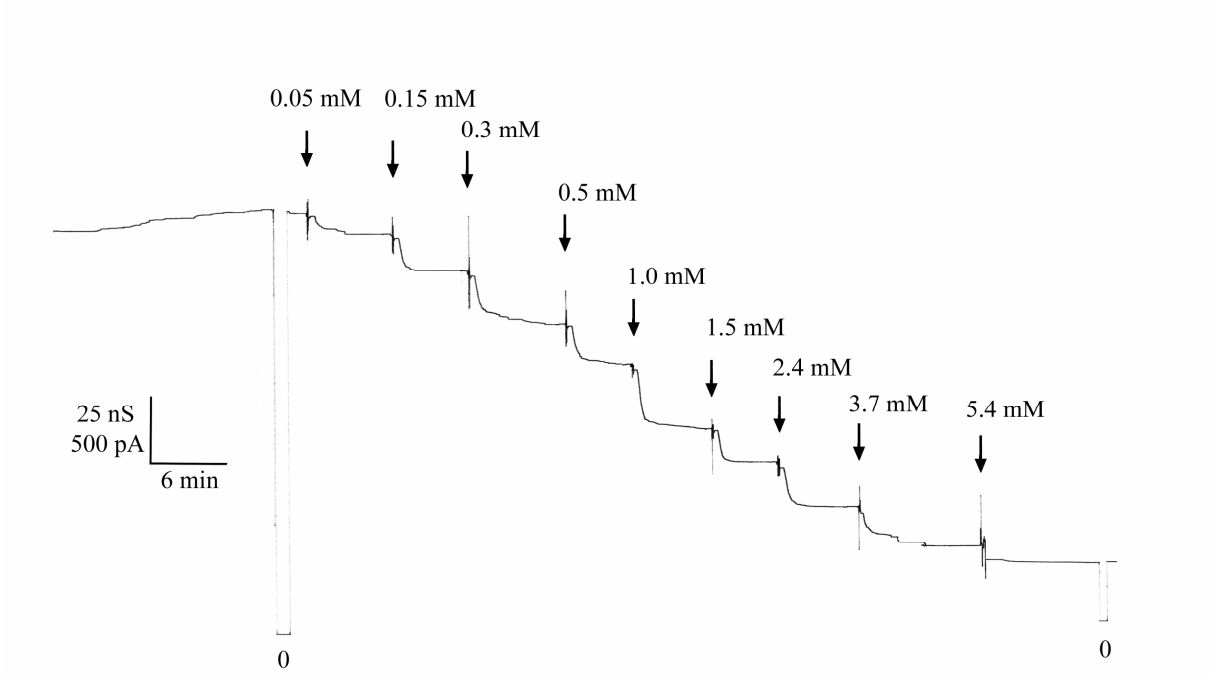


Figure 4.4 Titration of LamB-mutant Y6A (D3) induced membrane conductance with maltopentaose. The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 500 ng/ml protein, 1 M KCl, and maltopentaose at the concentrations shown at the top of the figure. The temperature was 20°C and the applied voltage was 20 mV.

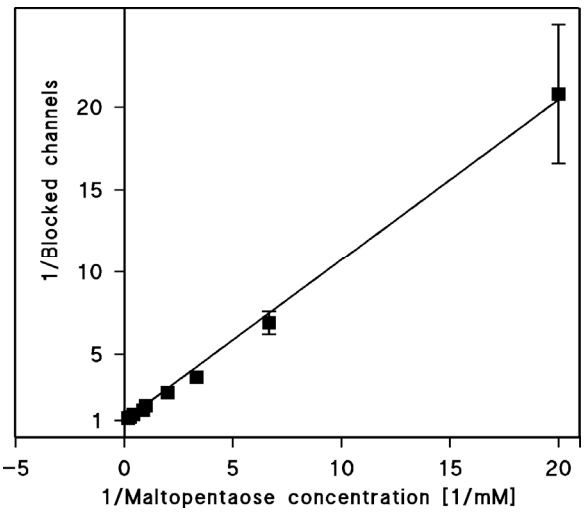


Figure 4.5 Lineweaver-Burke plot of the inhibition of the LamB Y6A (D3) mutant-induced membrane conductance by maltopentaose. The data taken from Figure 4.4 and of two similar experiments were analyzed using eqn. (2). The data points reflect the mean of the measurement ± SD. Note that the standard deviation was for most of the measurements so small that it lies within the data points. The straight line corresponds to a least squares fit of the data points. The fit corresponds to a stability constant K, for maltopentaose binding to LamB mutant Y6A (D3) of 973±16 l/mol (KS = 1.03 mM).

It is noteworthy that replacement of the individual aromatic amino acids of the greasy slide by alanine led to different effects on the stability constants for maltopentaose binding. The mutation of W74A (D1), Y41A (D2) and F227A (D6) had a small influence on the stability constant. The effect of D3 (Y6A), D4 (W358A) and D5 (W420A) on maltopentaose binding

was more substantial (see Table 4.3) and *K* decreased by factors between about 7 and 30 as compared to wildtype LamB (Benz et al., 1987). This result indicated that the influence of the inner amino acids of the greasy slide (which are localized opposite to the Y118 amino acids in the central constriction of the LamB channel) on carbohydrate binding is higher than that of the aromatic amino acids localized at the periphery of the channel. This result agrees nicely with a previous study where Y118 was exchanged by a variety of other amino acids (Orlik et al., 2002). The effect of the mutation of Y118 on carbohydrate binding was much higher as measured here for the single greasy slide mutation. Y118A led to a dramatic decrease of maltopentaose binding by a factor of about 20, whereas the mutation Y118W resulted in very strong increase of the stability constant by a factor of more than 200 (see Table 4.3) (Orlik et al., 2002).

Table 4.3 Parameters of maltopentaose induced transport noise and stability constants of maltopentaose binding to different LamB greasy slide mutant channels.

Mutant	$k_1/10^6$ $M^{-1}s^{-1}$	k_{-1}/s^{-1}	$K/10^3M^{-1}$	$K^*/10^3M^{-1}$	K_S^*/mM
LamB wild type*	5.3	420	13	14	0.07
Y118W**	16.5	9	1,800	3,100	0.0003
Y118A**	1.5	970	1.8	0.7	1.4
D1	0.08	53	1.5	2.1	0.48
D2	3.8	2,880	1.3	2	0.5
D3	0.8	1,890	0.4	1.1	0.9
D4	5.2	490	10.6	14	0.07
D5	6.5	670	9.9	8.9	0.1
D6	10.4	830	12.5	20.8	0.05
D1/2	0.05	275	0.4	0.8	1.25
D3/4	n.d.			0.16	6.25
D5/6	n.d.			0.15	6.7
D1/2/3	n.d.			0.05	20
D4/5/6	n.d.			0.38	2.6
D1/2/3/4	n.d.			0.02	50
D3/4/5/6	n.d.			<0.014	>70
D1/2/3/4/5	n.d.			<0.03	>33
D2/3/4/5/6	n.d.			<0.03	>33
D1/2/3/4/5/6	n.d.			<0.015	>67

D1/2+Y118W	10.8	120	92	91	0.01
D3/4+Y118W	1.8	365	5.0	6.9	0.15
D5/6+Y118W	2.0	445	4.6	7.1	0.14
D1/2/3/4+Y118W	3.7	1,200	3.1	3.9	0.26
D3/4/5/6+Y118W	n.d.			0.11	9.1
D1/2/3/4/5/6+Y118W	0.09	1,030	0.09	0.05	20

The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 1 M KCl and about 500 ng/ml LamB mutants. *K** was derived from titration experiments similar to that shown in Figure 4.4 by using Lineweaver-Burke-plots (Figure 4.5). The data represent means of at least three individual titration experiments. The standard deviation was typically less than 10% of the mean value. The results of previous titration experiments with wild type LamB*(Benz et al., 1986) and the Y118W mutant**(Orlik et al., 2002) are given for comparison. The rate constants k_1 and k_{-1} were derived from a fit of the corner frequencies as a function of the maltopentaose concentration (compare eqn. (4)). *K* is the stability constant for maltose binding derived from the ratio k_1/k_{-1}. The data represent the mean of at least three individual noise experiments. The standard deviation of the mean value was always smaller than 20% of the mean. The parameters for *wildtype LamB (Andersen et al., 1995) and the **Y118W mutant (Orlik et al., 2002) are given for comparison. n.d. means not detectable

Titration experiments were performed with all greasy slide mutants used in this study. The results for *K* and the half saturation constant, K_S, (= *1/K*) are listed in Table 4.3. The results suggest that the affinity of maltopentaose binding decreased for all double mutations within the greasy slide. The stability constants derived for the mutants D3/4 and D5/6 were about 100 times smaller than LamB wildtype. Only the D1/2 mutant showed a somewhat higher binding constant for maltopentaose that was only a factor of about 20 smaller than that of LamB wildtype. From the mutants with four mutations only binding could be measured for the D1/2/3/4 mutant. The D3/4/5/6 did not show any maltopentaose-mediated current decrease (data not shown). This was also the case for all other greasy slide mutants with more than four mutations (see Table 4.3). This means that the half saturation constant for binding of maltopentaose, if it existed, is at least above 100 mM, which is outside the physiologically relevant concentration range. It is noteworthy that no decrease of membrane conductance was observed when maltopentaose was added to the six-fold mutant D1/2/3/4/5/6 (data not shown). Even the addition of about 14 mM of maltoheptaose (which has a much higher affinity to LamB (Andersen et al., 1995; Benz et al., 1986)) to the mutant channels of this type was not able to block the current through these mutant channels to any appreciable extent (see Figure 4.6).

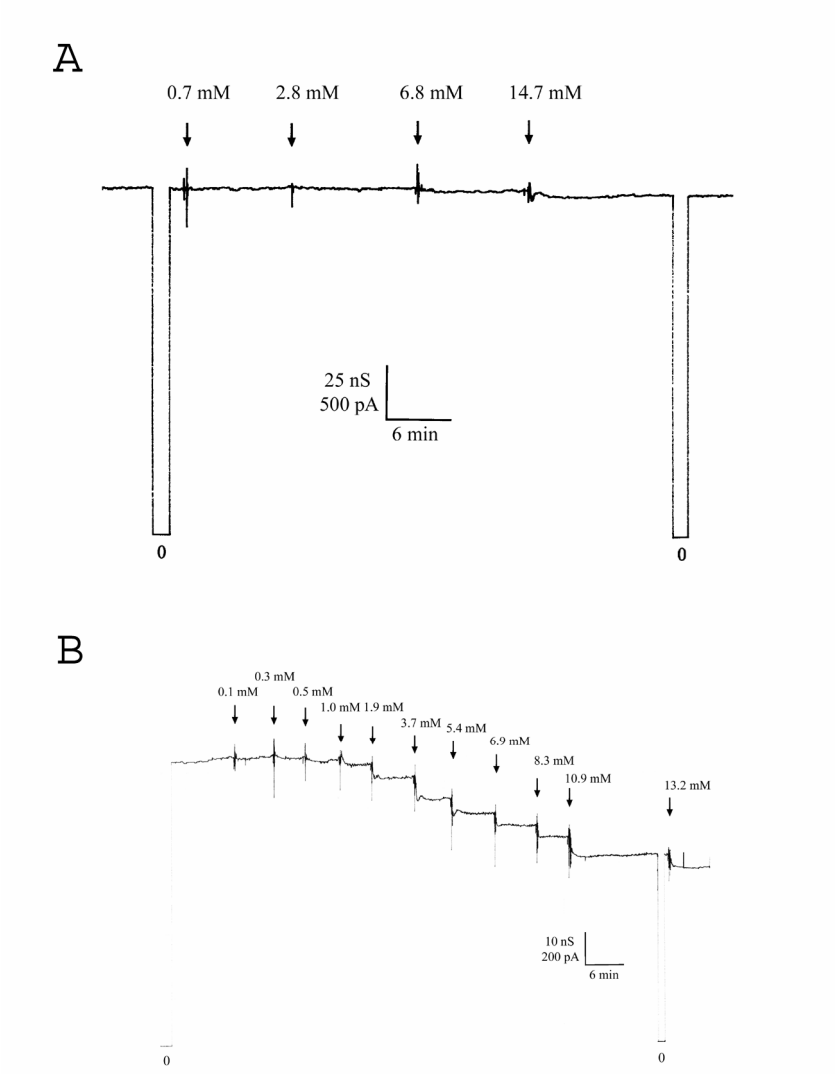


Figure 4. 6 Titration of LamB-mutant induced membrane conductance with carbohydrates. The membranes were formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 500 ng/ml protein, 1 M KCl, and maltoheptaose or maltopentaose at the concentrations shown at the top of the figure. The temperature was 20°C and the applied voltage was 20 mV.
A: The experiment was performed using the D1/2/3/4/5/6 mutant and maltoheptaose.
B: The experiment was performed using the D1/2/3/4/5/6+Y118W mutant and maltopentaose.

The LamB mutants Y118F and Y118W show increased affinity for carbohydrate binding as compared to wildtype LamB (Jordy et al., 1996; Orlik et al., 2002). To study the effect of the mutation Y118W in some more detail, greasy slide mutants were studied that contained in addition the replacement of Y118 by tryptophane (see Table 4.3). The results suggest that this mutation led to a strong increase of the affinity of maltopentaose binding to the LamB mutants (see Table 4.3). In particular, for those mutants, which did not bind maltopentaose to any appreciable extent (D1/2/3/4 and D3/4/5/6) the additional mutation Y118W resulted in

restoration of binding. The same was observed for the six-fold mutant (see Figure 4.6). The additional mutation allowed the evaluation of the binding of maltopentaose (K = 50 1/M, K_S = 20 mM). This result agrees nicely with the growth experiments. Whereas no growth could be detected for KS26 expressing the mutant D1/2/3/4/5/6, growth was reestablished when the additional mutation Y118W was introduced.

4.4.3. Measurement of current noise with the greasy slide mutants.

The equilibrium experiments do not allow the evaluation of the on- and off-rate constants for maltopentaose binding to LamB or the LamB mutants (Andersen et al., 1995; Nekolla et al., 1994). To receive some information on the binding kinetics we used the measurement of the current noise, which is based on the principle of the microscopic reversibility. Parallel to the titration measurements the frequency-dependence of the spectral density of the current noise was measured using fast Fourier transformation. The reference spectrum was taken before addition of carbohydrates to obtain the current noise of the open LamB-mutant channels, which exhibit $1/f$ noise in the frequency range between 1 and 50 Hz (Andersen et al., 1995; Bezrukov and Winterhalter, 2000; Nekolla et al., 1994; Wohnsland and Benz, 1997). An example is given in Figure 7 for the measurement of current noise of 1329 D6 mutant channels without maltopentaose (trace 1, 0 mM). At small frequencies up to about 100 Hz the spectral density was dependent on $1/f$, which is typical for open bacterial porin channels(Bezrukov and Winterhalter, 2000; Wohnsland and Benz, 1997). The increase of the spectral density at frequencies above about 200 Hz was caused by the intrinsic noise of the preamplifier that produces frequency-dependent current noise through the membrane capacity C_m. It is observed also with membranes without reconstituted LamB-channels. The time resolution of the instrumentation was approximately 10 kHz, which was limited in the experiments of Figure 4.7 and similar experiments by the bandwidth of the current amplifier and a low pass filter (0.3 ms). The reference spectrum was subtracted from each spectrum taken after the successive addition of maltopentaose in increasing concentration, which led to a considerable increase of the spectral density of the current noise as Figure 4.7 clearly indicates.

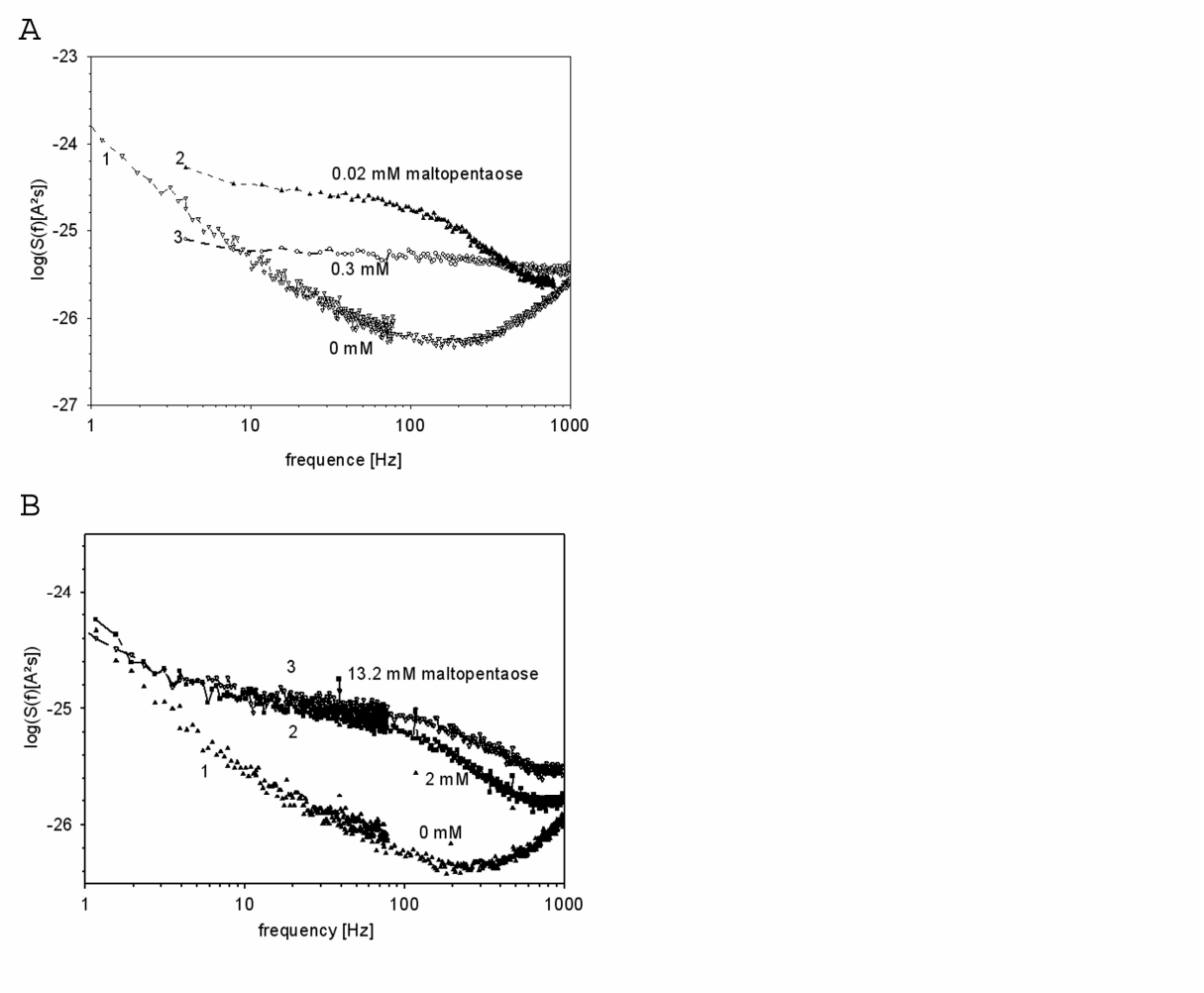


Figure 4.7 A: Power density spectra of maltopentaose-induced current noise of 1329 LamB D6 mutant channels. Trace 1 shows the control (1 M KCl). Trace 2: the aqueous phase contained 0.02 mM maltopentaose and the power density spectrum of Trace 1 was subtracted (τ = 2.0 ms; S_0 = 0.26·10^{-24} A^{2}s). Trace 3: the aqueous phase contained 0.3 mM maltopentaose and the power density spectrum of Trace 1 was subtracted (τ = 0.55 ms; S_0 = 0.05·10^{-24} A^{2}s); T = 20°C; V_m= 20 mV.

B: Power density spectra of maltopentaose-induced current noise of 215 LamB D1/2/3/4/5/6+Y118W mutant channels. Trace 1 shows the control (1 M KCl). Trace 2: the aqueous phase contained 0.5 mM maltopentaose and the power density spectrum of Trace 1 was subtracted (τ = 1.8 ms; S_0 = 0.03·10^{-24} A^{2}s). Trace 3: the aqueous phase contained 3.7 mM maltopentaose and the power density spectrum of Trace 1 was subtracted (τ = 1.4 ms; S_0 = 0.11·10^{-24} A^{2}s); T = 20°C; V_m= 20 mV.

Figure 4.7A trace 2 shows a spectrum taken after addition of maltopentaose (c = 0.02 mM; the reference spectrum of curve 1 was subtracted) to the membrane containing 1329 LamB D6 mutant channels. The current noise spectrum of the LamB-mutant channel D6 after the addition of carbohydrates could be fitted to a single Lorentzian function (see Figure 4.7A, trace 2). This result agrees well with earlier investigations with LamB (maltoporin) wildtype of *E. coli* (Andersen et al., 1995; Nekolla et al., 1994) and *Salmonella typhimurium* (Jordy et al., 1996) and also Y118 LamB-mutants (Orlik et al., 2002), where also one single Lorentzian has been observed without any exception. A spectrum taken few minutes later did not show any systematic variation compared to spectrum taken before. In further experiments the concentration of maltopentaose was increased in defined steps. At another concentration of maltopentaose (c = 0.3 mM) the power density spectrum corresponded to that of trace 3 in Figure 4.7A, which could also be fitted to a single Lorentzian. Such a type of noise is expected for a random switch with different on- and off-probabilities and can be fitted to eqn. (3) with sufficient accuracy (Conti and Wanke, 1975; De Felice, 1981; Verveen and DeFelice, 1974).

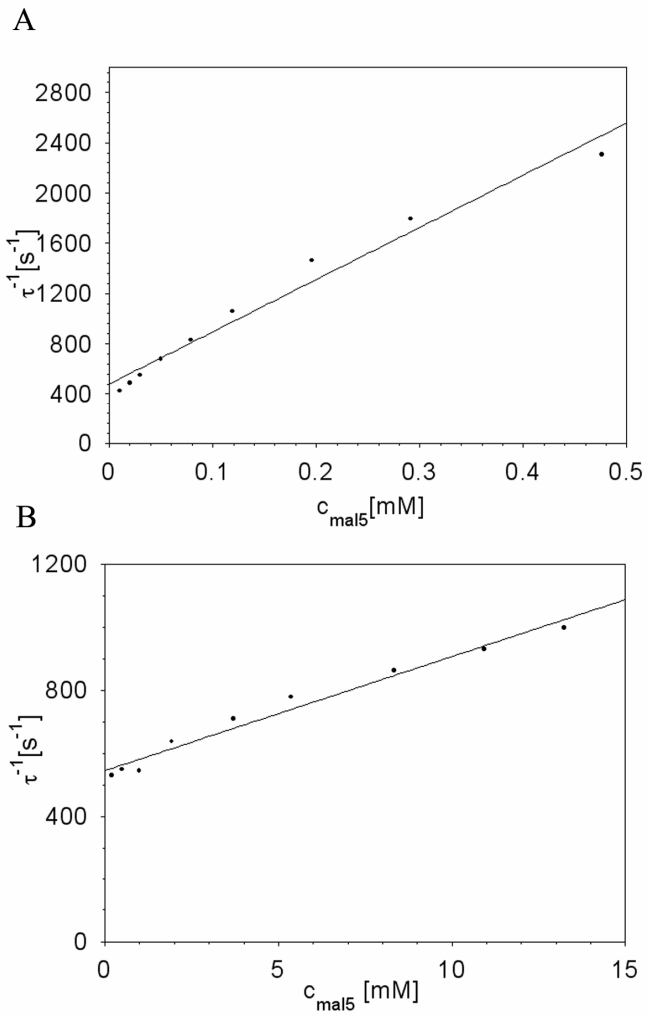


Figure 4.8 A: Dependence of the inverse reaction rate $2\pi f_c = 1/\tau$ of the maltopentaose-induced current noise of the LamB D6 mutant on the maltopentaose concentration in the aqueous phase. The data were derived from the fit of the power density spectra with Lorentzians similar to those given in Fig. 4.7A for maltopentaose concentrations ranging between 0.01 and 1.1 mM (dark circles). The aqueous phase contained 1 M KCl and about 500 ng/ml LamB D6 mutant. The straight line corresponds to k_1 = $8.3 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ and to k_{-1} = 960 1/s. The applied membrane potential was 20 mV; T = 20°C.

B: Dependence of the inverse reaction rate $2\pi f_c = 1/\tau$ of the maltotriose-induced current noise of the LamB mutant D1/2/3/4/5/6+Y118W on the maltopentaose concentration in the aqueous phase. The data were derived from the fit of the power density spectra with Lorentzians similar to those given in Fig. 4.7B for maltopentaose concentrations ranging between 0.05 and 13.3 mM (dark circles). The aqueous phase contained 1 M KCl and about 500 ng/ml LamB mutant D1/2/3/4/5/6+Y118W. The straight line corresponds to k_1 = $0.07 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ and to k_{-1} = 1090 1/s. The applied membrane potential was 20 mV; T = 20°C.

Lorentzians were also observed when the mutant D1/2/3/4/5/6+Y118W was investigated for maltopentaose binding. Figure 4.7B shows spectra obtained at 0.05 and 3.7 mM maltopentaose. The results demonstrate that the current noise increased also in these experiments and that the corner frequencies could be evaluated. The corner frequencies, f_c, of the Lorentzians are dependent on the on- and off-rate constant, k_1 and k_{-1}, for maltopentaose-binding to the binding-site inside the mutant LamB-channels according to eqs. (3) and (4).

This means that the corner frequencies, f_C, should increase with increasing maltopentaose concentration as they did in the experiments shown in Figure 4.7A and 4.7B. The reaction rate $1/\tau$ was plotted as a function of the carbohydrate concentration in the aqueous phase to evaluate the off- and the off-rate constants for maltopentaose binding. Figures 4.8A and 4.8B show the fit of the corner frequencies, f_c, of the experiments shown in Figure 4.7A and 4.7B and of other maltopentaose concentrations (data not shown) to eqn. (4). The rate constants for the binding of maltopentaose to the LamB D6 mutant channel were $k_1 = 8.3 \cdot 10^6$ 1/(M·s) and $k_{-1} = 960$ 1/s. This corresponds to stability constant, K, for the binding of maltopentaose to the binding-site inside the LamB D6 mutant-channel of 1329 1/M, which agrees well with the stability constant for the same system derived from the titration experiments (see Table 4.3). Similarly the corner frequencies of the experiments shown in Figure 4.7B were plotted as a function of the maltopentaose concentration and yielded the on- and off-rate constants for maltopentaose binding to the D1/2/3/4/5/6+Y118W mutant ($k_1 = 0.07 \cdot 10^6$ 1/(M·s) and $k_{-1} =$ 1090 1/s). We repeated these experiments several times and yielded similar results for both the rate constants of maltopentaose binding and for the stability constants. It is noteworthy that the on- and off-rate constants of maltopentaose-binding were fairly independent from the experimental conditions including the number of reconstituted channels. The rate constants describing the movement of maltopentaose to and from the binding-site inside the mutant channels are not identical to the unidirectional flux through the channel. It is possible, however, to calculate this flux through the channels from the rate constants (see Discussion).

With some of the greasy slide mutants it was not possible to evaluate the corner frequency of the spectral density. In particular, when more than 4 residues of the greasy slide were replaced by alanine-scanning mutagenesis it was not possible to fit the spectral density of the current noise to a Lorentzian function. One reason for this could be that the kinetics of maltopentaose binding was too fast. Another possibility is that the amplitude of the spectral density of the maltopentaose-mediated current noise was too small. The evaluation of the binding kinetics was possible, however, when besides the greasy slide mutations Y118 was also mutated to tryptophane. This was possible for all double mutants (D1/2, D3/4 and D5/6), for one of the mutant with four mutations (D1/2/3/4) and when all aromatic residues of the greasy slide were removed (D1/2/3/4/5/6) (see above and Table 4.3). The results of all noise measurements demonstrate that the mutations of the greasy slide preferentially affect the on-rate of

maltopentaose binding. The additional mutation Y118W led to an increase of the on-rate, which means that the kinetics could again be evaluated from the experiments (see Table 4.3). This result demonstrates again the central role of Y118 in carbohydrate transport through LamB.

4.5. DISCUSSION

4.5.1 The replacement of the aromatic residues of the greasy slide by alanine increases ion flux through LamB

Alanine scanning mutagenesis of the amino acids of the greasy slide resulted in increased ion flux through LamB. Highest effect for the single mutation was observed for the mutants where the aromatic residues opposite Y118 were removed. This result appears to be reasonable because tyrosine 118 is localized in the central constriction at the external loop 3 and controls there the flux of ions as previous experiments with the mutant Y118A have demonstrated (Orlik et al., 2002). Experiments with multiple greasy slide mutants tend to support this view, because for additional mutation the single-channel conductance increases. Highest conductance was observed for the mutant where all aromatic residues were removed (D1/2/3/4/5/6; see Table 4.2). Nevertheless its conductance was with 600 pS in 1 M KCl still lower than that of the mutant Y118A (850 pS). Introduction of the mutation Y118W in addition to the alanine scanning mutagenesis of greasy slide amino acids always decreased ion flux through LamB, which agrees well with the situation within the cross section of the monomers (see Figure 4.1). This means that aromatic residues within the channel lumen control ion transport through LamB and probably also permeability for neutral solutes. It is noteworthy that the sucrose-specific ScrY-channel of enteric bacteria (sucroseporin) representing a natural mutant of LamB (Schmid et al., 1991), has besides a carbohydrate binding site also general diffusion properties (Schulein et al., 1991). It has a high single-channel conductance of about 700 pS in 1 M KCl similar to those of some of the LamB mutants described here. In position 201 of ScrY (corresponding to Y118 in LamB) is an aspartate. This is the reason for the increased size of ScrY wildtype as compared to LamB wildtype (see Figure 4.1).

4.5.2 The mutation of greasy slide residues has a major impact on maltopentaose binding affinity

In the preceding study by Orlik et al. (2002)(Orlik et al., 2002) and above we have demonstrated that tyrosine 118 and the aromatic residues of the greasy slide have a major effect on ion permeation through LamB of *E. coli*. The effect on ion conductance is definitely caused by the size of the bulky side chain of tyrosine, phenylalanine or tryptophane, which protrudes in the channel lumen and limits its size. LamB functions in the outer membrane of enteric bacteria as a channel for carbohydrates in particular for the uptake of maltooligosaccharides although it is induced by maltose (Schwartz, 1987). In the growth experiments we could demonstrate that this is also the case for most of the mutants (D1/2, D1/2/3/4/5/6, D1/2/3/4/5/6+Y118W) and LamB wildtype used in this study. All with the exception of D1/2/3/4/5/6 were able to confer to the porin-deficient KS26 strain the growth on maltopentaose and the growth rate was very similar to that when the strain KS26 contained a plasmid for the expression of wildtype LamB. This result means that some of the mutant channels have the same function as wildtype LamB with the exception of D1/2/3/4/5/6. The latter mutant is not able to bind maltopentaose or maltoheptaose to any appreciable extent, which suggests that these carbohydrates are also not transported (see below).

Alanine scanning mutagenesis of the single amino acids of the greasy slide suggests that they all contribute to maltopentaose binding. Highest impact on binding was observed, however, when the three aromatic residues (D3, D4 and D5) localized opposite Y118 were mutated. The stability constant for binding dropped in these cases by at least a factor of 10 (D3 and D5) and at maximum a factor of 30 (D4). These results are in qualitative agreement with another investigation of greasy slide mutants (Van Gelder et al., 2002). In this study maltose and maltohexaose binding to the same mutants was also drastically decreased as compared to wildtype LamB. These results suggest that the central part of the LamB channel has an important function in carbohydrate binding. The aromatic residues in the central part form a binding pocket about three glucose residues long that allows together with hydrogen bonds to other residues within the channel lumen (D116, E43, R8, R109, R82 and R33) optimum interaction between carbohydrates and the channel interior (Dutzler et al., 1996; Jordy et al., 1996; Meyer et al., 1997; Orlik et al., 2002; Orlik et al., 2002). The other three amino acids of the greasy slide (D1, D2 and D6) have only a relatively small effect on maltopentaose binding.

The affinity for maltopentaose binding decreased further when greasy slide mutants with two mutations were studied. Highest affinity was observed for the D1/2 mutant (K = 790 1/M). The stability constants for maltopentaose binding were for the D3/4 and the D5/6 even smaller and did not exceed about 160 1/M. From all other greasy slide mutants including three, four, five and all six residues only maltopentaose binding could be detected for the D1/2/3/4 mutant (K = 20 1/M). For the other mutants only a lower limit for maltopentaose binding could be evaluated from the titration experiments (K<10 1/M). This means that in all these cases the half saturation constant was much higher than 100 mM, if there was any binding at all.

4.5.3 The additional mutation Y118W results in an increase of the binding affinity of the greasy slide mutants

In a previous study we could demonstrate that the mutation Y118W resulted in a substantial increase of maltopentaose binding by more than a factor of 200 (see also Table 4.3). Similarly, this mutation was also able to increase the affinity of maltopentaose binding to the greasy slide mutants. Table 4.3 demonstrates that the stability constant of maltopentaose binding increased in most cases by more than a factor of 100 when Y118W was introduced in addition to the greasy slide mutations. In particular high effects were observed when Y118W was introduced in the double mutants D1/2, D3/4 and D5/6. In these cases maltopentaose binding affinity was almost the same as for LamB wildtype or considerably higher (D1/2 +Y118W). For other mutants with three, four and six greasy slide mutations the stability constant for maltopentaose binding decreased but was still much higher than without the Y118W exchange, which means that maltopentaose binding could be detected for these mutants. This result again demonstrates the importance of the central part of the LamB-channel for carbohydrate binding. The estimation of the stability constant for maltopentaose binding to the LamB greasy slide mutants allows also the calculation of the free energy for maltopentaose binding, calculated according to $\Delta G_0 = R \cdot T \ln K$. The free energy, ΔG_0, of binding for maltopentaose binding decreased considerably for all greasy slide mutants studied here from 23.2 kJ/mol for LamB wildtype to less than 5.5 kJ/mol for many of the greasy slide mutants.

4.5.4. Effect of greasy slide mutation on maltopentaose binding kinetics as derived from the analysis of carbohydrate-induced current noise.

Besides the binding affinity we also studied the effect of the greasy slide mutation on the maltopentaose binding kinetics using the analysis of the current noise. The current noise of LamB of *E. coli* had two different aspects as discussed in previous studies (Andersen et al., 1995; Nekolla et al., 1994; Wohnsland and Benz, 1997). The open channels show always *1/f*-noise up to frequencies of about 100 Hz. Major part of this noise represents the characteristics of open porin channels (Wohnsland and Benz, 1997). Another (minor) part of the *1/f*-noise is probably caused by slow closing and opening kinetics of LamB-channels (Andersen et al., 1995; Nekolla et al., 1994). We tried to minimize this part in the measurements of the current noise reported here, which means that the current recordings were only analyzed for current noise when the signals were absolutely stationary. The power spectra of the current noise of LamB and its mutant channels showed Lorentzian type of noise in the presence of maltopentaose in the aqueous phase because of the block of the channel for ion movement when the binding site is occupied. This process is controlled by a chemical reaction between maltopentaose from the aqueous phase and the binding-site (Andersen et al., 1995; Nekolla et al., 1994). Similarly as in previous studies a one-site, two-barrier model was used for the analysis of maltopentaose binding to the different LamB-mutant channels (Andersen et al., 1995; Nekolla et al., 1994; Orlik et al., 2002)'. The one-site, two-barrier model allowed the evaluation of the on- and off-rate constants for the binding of maltopentaose to the central binding-site inside the channel of a variety of LamB greasy slide mutants. For some of the mutants it was impossible to derive the rate constants from the noise measurements because the corner frequency could not be obtained from the current noise, i.e. the binding kinetics was too fast or the spectral density was too small. A symmetrical one-site two-barrier model was assumed to explain the maltopentaose transport through the LamB mutant channels, i.e. the on- and off-rate constants were the same from both sides. This has recently been questioned (Van Gelder et al., 2002). However, neither here nor in previous studies we did not find indication for much channel asymmetry concerning maltopentaose binding probably because the membrane potential was rather low in our experiments (Orlik et al., 2002). High membrane potentials tend obviously to increase the asymmetry of carbohydrate binding but this was not investigated here (Schwarz et al., 2003).

The analysis of the current noise in terms of the simple model suggested that both the on-rates

and the off-rates are influenced by the greasy slide mutations. The on-rate generally decreased, whereas the off-rate tended to increase by at maximum a factor of about 2 with one exception (D3, (Y6A)). When the single greasy slide mutations are considered k_1 decreased about 70-fold from wildtype (k_1 = $5.3 \cdot 10^6$ 1/(Ms)) to the smallest on-rate (D3 (Y6A), k_1 = $8 \cdot 10^4$ 1/(Ms)) for maltopentaose binding. This means that the on-rates became considerably smaller than those of diffusion-controlled reaction processes (Eigen et al., 1964). To explain these smaller on-rates it has to be assumed that the carbohydrates hit many times the channel before they are bound to the binding site. The carbohydrates can only be bound to LamB and its mutants when they are in a special order with respect to the binding-site. In a previous study we could demonstrate that the carbohydrates bind to LamB with the non-reducing end in advance from the surface exposed side and vice versa for the periplasmic side (Andersen et al., 1999), which agrees well with crystallographic studies (Dutzler et al., 1996).

From all the other greasy slide mutations only the kinetics of maltopentaose binding to the D1/2 mutant could be measured with sufficient accuracy. The on-rate showed in this case an even stronger decrease as compared to the single mutations D1 and D2. The off-rate was on the other hand comparable to those of the single mutations. It is possible that for all the other greasy slide mutants with more than one mutation the on-rate was so small combined with an increased off-rate that the current noise was only little increased with respect to the open channel noise. This means that it was impossible to separate the Lorentzian from the *1/f*-noise of the open channels with sufficient accuracy.

4.5.5 The additional mutation Y118W allows the derivation of the maltopentaose binding kinetics for the greasy slide mutants

Previous studies demonstrated that the mutations Y118F and Y118W had a substantial influence on the kinetics of carbohydrate binding (Jordy et al., 1996; Orlik et al., 2002). In particular, the on-rate increased slightly whereas the off-rate showed a dramatic decrease. The introduction of the additional mutation Y118W in some of the greasy slide mutants changed the binding kinetics in such a way that it was again possible to measure the current noise. This allowed for a full set of mutations to measure the influence of the greasy slide mutations on the on- and off-rate constants of maltopentaose binding, which was not possible for LamB

wildtype (see above). Even for the six-fold greasy slide mutation D1/2/3/4/5/6 it was possible to measure the binding kinetics when Y118W was additionally introduced. This allowed for many of the mutants to judge what happens to the rate constants of binding, k_1 and k_{-1}, when the aromatic residues of the greasy slide were changed to alanine. The data of Table 2 clearly demonstrate that the on-rate constants dramatically decreased while the off-rate constants increased. The data with the mutant D1/2/3/4/5/6+Y118W clearly indicate that the central tryptophane in position 118 is already sufficient for low affinity carbohydrate binding and also transport as the growth experiments with this mutant LamB channel clearly demonstrate.

4.5.6 Implication of greasy slide mutations for the carbohydrate transport *in vivo*

The porin-deficient KS26 strain has a small growth rate on maltose as sole carbon source and did not grow on the medium containing maltopentaose. The expression plasmids containing the genes for wildtype LamB and some of the mutants confer to the strain KS26 the possibility to grow on maltopentaose as sole carbon sources. These experiments suggest that maltopentaose can pass through the mutant channels, which means that they are functional. This allows the calculation of maltopentaose flux through the channel using the one-site two-barrier model. The LamB channel shows only a small asymmetry if any at small voltages (Andersen et al., 1995; Benz et al., 1986; Benz et al., 1987; Orlik et al., 2002; Van Gelder et al., 2000). This means that a symmetrical channel can be assumed. The net flux of sugar molecules, Φ, through the channel under stationary conditions as the result of a concentration gradient $c'' - c'$ across the membrane is given by the net movement of sugar across one barrier of the two identical potential energy barriers (Benz et al., 1987):

$$\Phi = k_1 \cdot c'' / (1 + K') - k_{-1} \cdot K' / (1 + K') \qquad (5)$$

K' is under symmetrical conditions given by:

$$K' = K \cdot (c' + c'') / 2 \qquad (6)$$

In equation (5) the rate constants k_1 and k_{-1} are multiplied by the probabilities that the

binding site is free or occupied, respectively. The flux has in the case $c'' = c$, $c' = 0$, when carbohydrates are only present on one side of the channel the form:

$$\Phi = k_1 \cdot c / (2 + K \cdot c) \qquad (7)$$

Eqn. (7) suggest that the maximum permeability of the channel for a carbohydrate with an on-rate k_1 for its binding to the binding site is $k_1/2$, which is obtained for wildtype LamB at very small carbohydrate concentration ($c \leq 10$ µM). The flux strongly saturates at high carbohydrate concentration since the half saturation constant for the sugar flux is $K_S = 1/K$. The maximum turnover number of the channel (similar to the maximum turnover number of an enzyme that is substrate saturated) is reached at high carbohydrate concentration on one side of the membrane and is given by k_{-1}. This means that the flux through LamB is limited at high maltose and maltooligosaccharide concentration. It is noteworthy; however, that this is not a serious restriction since the substrate concentration is normally low under physiological conditions. For the effective scavenging of nutrients at very small concentrations it seems to be more important to have a high permeability (i.e. a high k_1), which is indeed given for the transport of maltooligosaccharides through the wildtype LamB channel (Andersen et al., 1995).

Our data allow a comparison of the flux of maltopentaose through wildtype and some of the LamB-mutant channels. Figure 4.9A shows the maximum flux of maltopentaose through a single LamB wildtype, D3 (Y6A) and D4 (W420A) mutant channels calculated on the basis of eqn. (7). The curves were calculated using the rate constants given in Table 4.3. Fig. 4.9A shows the substantial effect of a single amino acid mutation within the primary sequence of LamB on the transport of maltopentaose. The carbohydrate-specific porins have their maximum permeability in the linear range of the Figure. This means LamB wildtype has the highest permeability, followed by D4 and D3. The D4 mutant had the highest turnover number of 1,890 1/s as compared to wildtype (420 1/s) and it was lowest for the D3 mutant (53 1/s).

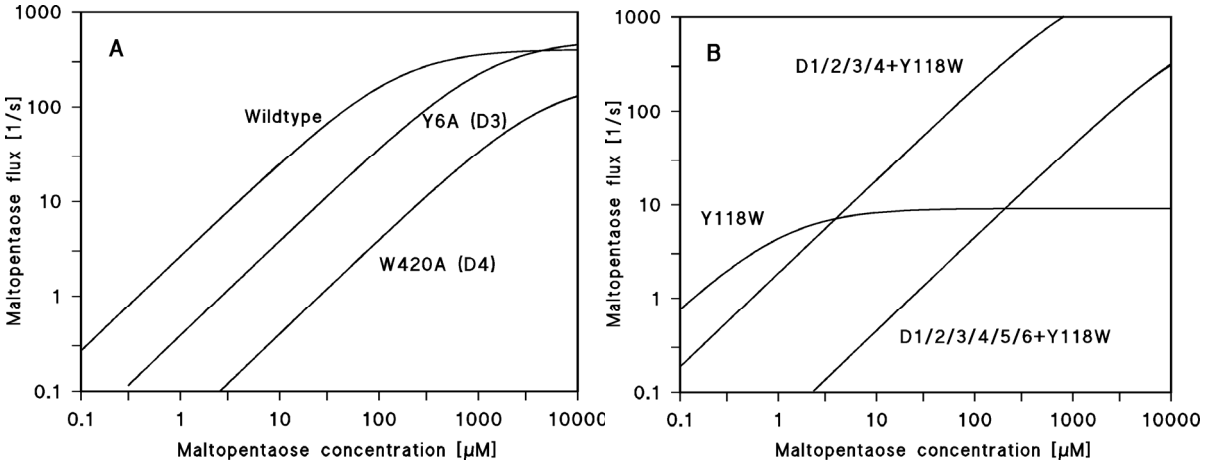


Figure 4. 9 A: Flux of maltopentaose through one single wildtype LamB, D3 (Y6A) and D4 (W420A) mutant channel (i.e. through the monomer in a wildtype, D3 and D4 trimer channel) as a function of the maltopentaose concentration on one side of the channel. The concentration on the other side was set to zero. The flux was calculated using eqn. (7) and the rate constants given in Table 4.3.

B: Flux of maltopentaose through one single Y118W, D1/2/3/4+Y118W and D1/2/3/4/5/6+Y118W channel (i.e. through the monomer in an Y118W, D1/2/3/4+Y118W and D1/2/3/4/5/6+Y118W channel) as a function of the maltopentaose concentration on one side of the channel. The concentration on the other side was set to zero. The flux was calculated using eqn. (7) and the rate constants given in Table 4.3.

Figure 4.9B demonstrates the influence of the additional mutation Y118W on the greasy slide mutants. It shows the flux of maltopentaose through Y118W, D1/2/3/4+Y118W and through D1/2/3/4/5/6+Y118W. The comparison of the different fluxes again demonstrates the advantage of a binding site for the maximum scavenging of substrates and the role of Y118 within this binding site. The Y118W mutant has the highest permeability at small carbohydrate concentration, but the flux through this mutant channel saturates already at 1 µM maltopentaose (see Figure 4.9B). Furthermore, it has also the smallest turnover number (k_{-1} rate), which would be effective at higher carbohydrate concentration. This could mean that the channel is blocked for the uptake of nutrients at somewhat higher carbohydrate concentration. This could be the reason why nature did not make advantage from the possibility to use W118 instead of Y118 in the central constriction of LamB. Nevertheless we could demonstrate here that the deletion of all aromatic residues of the greasy slide together with the mutation Y118W allows again carbohydrate binding *in vitro* and maltopentaose uptake *in vivo*. This means that W118 is sufficient for carbohydrate transport in the absence of all aromatic residues of the greasy slide.

The expression of LamB in enteric bacteria is always induced with the expression of the periplasmic maltose binding protein MBP or MalE. MalE is present in the periplasmic space in a concentration in the range of millimolar and confers this space into a sink for carbohydrates although it does not modulate LamB channel function (Benz et al., 1986; Brass et al., 1985; Schwartz, 1987). This property is an essential part of carbohydrate uptake across the outer membrane because a carbohydrate bound from the cell surface to the binding site inside the LamB channel has still two possibilities with equal probabilities for further movement because the channel is symmetric with respect to its transport properties. The maltooligosaccharides bound to LamB can move back to the cell surface or further on to the periplasmic space. In the latter case they are bound to MBP with a half saturation constant of considerably less than 10 μM, which means that they are trapped within the periplasmic space and as long as inner membrane transport functions it is unlikely that the maltooligosaccharides can be lost through the outer membrane.

The calculation of the maximum flux of maltopentaose molecules through LamB and its mutants allows also a meaningful comparison with the *in vivo* requirements of maximum growth of *E. coli* cells. The minimum carbon source supply for maximum growth rate is 20 nmol glucose/(min x 10^9 cells) (Freundlieb et al., 1988). The expression of the LamB mutants studied here is about 40% of full expression (Heine et al., 1988). This means that a single cell has about 10^4 channels ($3.3 \cdot 10^3$ trimers) in the outer membrane. The flux of maltopentaose through one single D1/2/3/4/5/6+Y118W monomer at a concentration of 4.8 mM (the conditions of the growth experiments) is 179 s^{-1}, which means that the maximum flux of maltopentaose in 10^9 cells is about $1.79 \cdot 10^{15}$ s^{-1} or $1.07 \cdot 10^{17}$ min^{-1}. This number corresponds to a maximum flux of about 172 nmol maltopentaose/(min x 10^9 cells). The carbon source supply under these conditions in the cells expressing the D1/2/3/4/5/6+Y118W mutant is 863 nmol glucose/(min x 10^9 cells). This result suggests indeed that the KS26 cells expressing the LamB mutant D1/2/3/4/5/6+Y118W can show maximum growth under the conditions of our growth experiments. On the other hand it is clear that the flux of maltopentaose through a single channel under the conditions of the D1/2/3/4/5/6 mutant should be much below 4 s^{-1}, otherwise some growth would be observed. This result clearly indicates that binding is in the case of LamB mutant channels a prerequisite for transport. The aromatic residues of the greasy slide and tyrosine 118 are both important for carbohydrate

transport through LamB.

The separation of the hydrate water and the sugar can be done only effective with the interaction of the greasy slide and the residue Y118.

CHAPTER 5

CONCLUSION

5.1 Conclusion and Outlook

In our research group the focus lies on the biophysical characterization of pore-forming proteins by the use of the black lipid bilayer assay. In this study, pore-forming proteins of gram-negative bacteria, such as *E.coli* and *Borrelia*, were studied. The strains used in this study have been studied in science with different effort and thus different amounts of background information of these strains were available. For example, *E. coli* as a model organism for genetic engineering of bacteria is completely sequenced whereas the genome of *B. duttonii* is not. Out of this, future studies of pore-forming proteins of these strains will differ from each other.

Especially in the field of pore-forming proteins of *Borrelia*, there are many studies still to be done to gain more knowledge of *Borrelia*. Neither for Lyme borreliosis nor for relapsing fever vaccines are available on the market and it is of great interest to find targets for vaccine development against these pathogens.

Although outer membrane proteins like porins are potential targets for vaccine development the aim of this thesis was not directly to identifiy a new target for a vaccine, but it could be a first step in this direction. Furthermore the characterization of porins would results in a better understanding of the uptake and efflux mechanism of gram-negative *Borrelia* across their permeability barrier, the outer membrane.

In the case of the 80 pS channel-forming protein of *B. duttonii*, which could be characterized electrophysically, the next step will be to determine parts of the amino acid sequence of the putative 80 pS channel forming protein by N-terminal sequencing (Edman degradation). By a following BLAST search homologues in other bacteria strains can be found. Further experiments make sense when more background knowledge is available. Exceedingly relevant would be the sequencing of the genome, which would bring much needed information. When the sequenced genome is available, then knock-out mutants can be generated and be investigated for their channel forming activity. And also identification of *B. duttonii* proteins by mass spectrometry would be easier.

However, the situation for the 300 pS channel of *B. burgdorferi* is different. The genome of *B. burgdorferi* B31 is fully sequenced and methods to generate knock-out mutants are established. Hence, BB0142 could be identified as a TolC homologue in *B. burgdorferi*. The next step should be the biophysical characterization of the outer membrane preparation of a *bb0142* knock-out mutant using the black lipid bilayer, to show that the 300 pS activity is gone. Furthermore the substrate spectrum of the drug efflux pump would be interesting to know for a more effective treatment of Lyme borreliosis. Also it would be very interesting to investigate the expression levels of BB0142 in the different hosts of *B. burgdorferi*. Because if BB0142 is expressed in all stages, it can be used as a putative target for vaccine development. Moreover, the expression of recombinant BB0142 in *E. coli* would help in obtaining enough protein material for crystallization of BB0142 for 3D structure determination by X-ray. With the knowledge of the BB0142 3D structure, it would be possible to generate point mutations within the BB0142 channel and perform functional studies in vitro to verify the presented theory about electrophysical properties of BB0142 in this study.

However, first studies with outer membrane protein preparations from different *Borrelia* species were done in parallel to this study and they showed a wide spectrum of pore-forming proteins. It would be interesting to get a complete profile of pore-forming proteins appearing under different growth conditions in the Lyme borreliosis and relapsing fever *Borrelia*. So we could see which porins *Borrelia* has in common and which porins are expressed under different conditions.

For LamB, the best studied specific pore in *E. coli*, it is hard to think of any further black lipid bilayer experiments to gain more information about the channel and its properties. In this thesis it was shown in a very detailed study of point mutants of the "greasy slide", how important the aromatic residues are for the sugar binding. All three factors (central constriction by residue Y118, "polar tracks" and the "greasy slide") together build a finely tuned system for the sugar binding, which was determined by point mutations within the channel already in several studies. For the future it would be very interesting to verify the obtained black lipid bilayer kinetic data by other approaches. One technique of choice could be isothermal titration calorimetry (ITC), which is a direct experimental access to the enthalpy of the binding process, to gain data of the affinity constant, stoichiometry of the complex and the entropy of the binding process. Another interesting technique is the surface plasmon resonance biomolecular interaction analysis (SPR-BIA). Sensorgram data are a function of analyte concentration and can be used to determine kinetic parameters and dissociation constants of the interaction. Taken together kinetic data are important for a better understanding of the specific binding and they can be used as basis background knowledge to interpret other specific bindings between molecules.

CHAPTER 6

SUMMARY

6.1 Summary

Structural differences in the cell envelope of gram-negative bacteria are a consequence of evolutionary development. One conserved feature of all gram-negative bacteria is the incidence of pore forming channels in the outer membrane for the uptake and efflux of substrates through their cell wall.

In this study pore forming proteins of the gram-negative bacteria *B. burgdorferi, B. duttonii* and *E.coli* were investigated. Therefore the study is subdivided into three parts. In the first part outer membrane preparation of three relapsing fever *Borrelia* were investigated. In the second part the putative TolC homologue BB0124 of *B. burgdorferi*, the Lyme borreliosis agent, was studied. In the last part the influence of point mutants within the greasy slide of the maltose specific porin (LamB) of *E. coli* were shown.

In the first part of this study outer membrane preparations of three *Borrelia* relapsing fever strains have been studied for pore-forming activity in the black lipid bilayer assay. Histograms of conductance fluctuations were obtained from single-channel experiments with outer membrane preparations of B. *hermsii, B. recurentis* and *B. duttonii*. All strains had a different conductance fluctuation pattern with a broad range of single-channel conductance values varying from 0.5 nS – 11 nS. Common for all three strains was a high pore-forming activity at around 0.5 nS. Furthermore the proteins of the outer membrane of *B. duttonii* were separated by chromatographic methods. Some eluate fractions contained a channel-forming

protein, which was forming stable channels with a single-channel conductance of 80 pS in 1 M KCl. Characterization of this channel showed that it is slightly anionic selective and voltage independent. The small single-channel conductance suggests that it is a specific pore. However, a substrate specificity could not be determined.

In the second part, for the *B. burgdorferi* HB19 and *p66* knock out strain HB19/K02, their outer membrane preparations were characterized in the black lipid bilayer assay. Comparing the histograms of single-channel conductions fluctuations of both strains showed no single-channel activity at 11.5 nS for the *p66* knock out strain. This verifies earlier studies that P66 is a pore-forming protein in *B. burgdorferi*. Furthermore, one fraction obtained by anion exchange chromatography of the *p66* knock out outer membrane protein preparation showed a uniform channel-forming activity with a single channel conductance of 300 pS. The electrophysically characterization of the 300 pS channel showed that it is not ionselective or voltage dependent. By mass spectrometry using peptide mass finger prints, BB0142 could be identified as the sole channel forming candidate in the active fraction. A BLAST search and a conserved domain search showed that BB0142 is a putative TolC homologue in *B. burgdorferi*. Furthermore the location of the *bb0142* gene within the chromosome is in an operon encoding a multidrug efflux pump. In this study the expression of an outer membrane component of a putative drug efflux system of *B. burgdorferi* was shown for the first time.

In the third part functional studies of the maltooligosaccharide-specific LamB channel were performed. The 3D-structure of LamB suggests that a number of aromatic residues (Y6, Y41, W74, F229, W358 and W420) within the channel lumen is involved in carbohydrate and ion transport. All aromatic residues were replaced by alanine (A) scanning mutagenesis. Furthermore, LamB mutants were created in which one, two, three, four and five aromatic residues were replaced to study their effects on ion and maltopentaose transport through LamB. The purified mutant proteins were reconstituted into lipid bilayer membranes and the single-channel conductance was studied. The results suggest that all aromatic residues provide some steric hindrance for ion transport through LamB. Highest impact is provided by Y6 and Y41, which are localized opposite to Y118, which forms the central constriction of the LamB channel. Stability constants for binding of maltopentaose to the mutant channels were measured using titration experiments with the carbohydrate. The mutation of one or several aromatic amino acids led to a substantial decrease of the stability constant of binding. The

highest effect was observed when all aromatic amino acids were replaced by alanine because no binding of maltopentaose could be detected in this case. However, binding was again possible when Y118 was replaced by tryptophane (W). The carbohydrate-induced block of the channel function could also be used for the study of current noise through the different mutant LamB-channels. The analysis of the power density spectra of some of the mutants allowed the evaluation of the on- and off-rate constants (k_1 and k_{-1}) of carbohydrate binding to the binding-site inside the channels. The results suggest that both on- and off-rate constants were affected by the mutations. For most mutants k_1 decreased and k_{-1} increased.

6.2 Zusammenfassung

Strukturelle Unterschiede in dem Zellwandaufbau von gram-negativen Bakterien sind eine evolutionäre Entwicklung. Eine konservierte Eigenschaft von gram-negativen Bakterien ist das Vorkommen von porenformenden Kanälen in der Außenmembrane für die Aufnahme und den Export von Substraten durch die Zellwand.

In dieser Studie wurden porenformende Proteine der gram-negativen Bakterien *B. burgdorferi*, *B. duttonii* und *E. coli* untersucht. Daher wurde die Arbeit in drei Teile untergliedert. Im ersten Teil wurden zuerst die Außenmembranpräparationen von drei Rückfallfieber auslösenden *Borrelien* Stämmen untersucht. Der zweite Teil der Arbeit behandelt das TolC homologe Protein BB0142 des Erreger der Lyme Borreliose, *B. burgdorferi*. Im letzten Teil wurde der Einfluss von Punktmutationen innerhalb der „greasy slide“ auf die zuckerspezifische Pore (LamB) von *E. coli* untersucht.

Die Außenmembranpräparationen der drei Rückfallfieber *Borrelien* wurden auf porenformende Aktivität im Black Lipid Bilayer untersucht. Leitfähigkeitshistograme wurden nach Einzelkanalmessungen der Außenmembranepräparation von *B. hermsii*, *B. recurrentis* und *B. duttonii* erstellt. Alle Stämme zeigten ein anderes Leitfähigkeitsfluktuationsmuster, wobei die Werte der Einzelleitfähigkeit von 0,5 nS bis 11 nS variierten. Auffällig war das alle drei Stämme eine hohe Aktivität um 0,5 nS gemeinsan hatten. Darauffolgend wurden die Proteine der Außenmembran von *B. duttonii* durch verschiedene chromatographische Methoden aufgetrennt. Einige Eluatfraktionen enthielten ein kanalformendes Protein, das

stabile Kanäle mit einer Einzelleitfähigkeit von 80 pS in 1 M KCl formt. Die Bestimmung der Kanaleigenschaften zeigte, dass er leicht anionenselektive und spannungsunabhängig ist. Die geringe Einzelleitfähigkeit suggeriert, dass der Kanal zudem eine spezifische Pore sein könnte. Bisher konnte aber keine Substratespezifität festgestellt werden.

Im zweiten Abschnitt erfolgte die Untersuchung von Außenmembranpräparationen von *B. burgdorferi* HB19 und dem *p66* knock-out Stamm HB19/K02 mit Hilfe des Black Lipid Bilayers. Im Vergleich der beiden erhalten Einzelleitfähigkeitshistogramme konnte gezeigt werden, dass die Einzelleitfähigkeit von 11,5 nS nicht mehr im *p66* knock out Stamm vorkam. Dies belegt frühere Studien, dass P66 ein poreformendes Protein von *B. burgdorferi* ist. Durch Anionenaustauscher-Chromatographie der Außenmembranpräparation des *p66* knock out Stammes wurde zudem eine Proteinfraktion mit einer einheitlichen porenformenden Aktivität von 300 pS in 1 M KCl erhalten. Die elektrophysikalische Charakterisierung der 300 pS Pore zeigte, dass der Kanal nicht ionenselektiv oder spannungsabhängig ist. Mit Hilfe von Massenspektrometrie und Peptidemassenbestimmung, konnte BB0142 als einziger möglicher kanalformender Kandidat identifiziert werde. Eine BLAST Suche sowie eine „conserved domain“ Suche zeigten, dass BB0142 ein putatives TolC homologes Protein von *B. burgdorferi* ist. Darüber hinaus ist das *bb0142* Gen in einem Operon lokalisiert, das eine putative Efflux Pumpe codiert. In der vorliegenden Arbeit konnte das erste Mal gezeigt weden, dass in *B. burgdorferi* die Außenmembrankomponente einer Efflux Pumpe expremiert wird.

Im dritten Teil wurden Funktionsstudien an dem zuckerspezifischen Kanal LamB durchgeführt. Die 3D Struktur zeigte, dass einige aromatische Aminosäuren (Y6, Y41, W74, F229, W358 und W420) innerhalb des Kanallumen in den Kohlenhydrat- und Ionentransport involviert sind. Alle aromatischen Reste wurden bei Punktmutation durch Alanin ersetzt. Zudem wurden LamB Mutanten erzeugt in denen ein, zwei, drei, vier oder fünf aromatische Reste durch Alanin ersetzt. Die aufgereinigten LamB Mutanten wurden im Black Lipid Bilayer untersucht und ihre Einzelleitfähigkeit bestimmt. Die Ergebnisse zeigen, dass alle aromatischen Reste eine gewisse sterische Hinderung beim Ionentransport durch LamB bewirken. Den größten Einfluss haben die Reste Y6 und Y41, die gegenüber dem Rest Y188 liegen, der die zentrale Verengung des LamB Kanal darstellt. Stabilitätskonstanten der Zuckerbindung in den Mutanten wurde durch Titrationsexperimente mit Maltopentaose und -

heptaose bestimmt. Mutation an einem oder mehreren aromatischen Resten führt zu einer deutlichen Abnahme der Bindungsstabilitätskonstanten. Den stärksten Effekt zeigte die Mutante, in der alle aromatischen Reste durch Alanin ersetzt wurden. Es konnte dort keine Zuckerbindung mehr festgestellt werden. Die Bindung wurde wieder hergestellt nachdem Y118 durch ein Tryptophan ersetzt wurde. Durch das zuckerinduzierte Öffnen und Schließen des Kanals ergiebt sich ein Stromrauschen. Die Analyse des Rausch Spektrum von einigen Mutanten erlaubte die Bestimmung der Dissoziations- (k_{-1}) und der Assoziationskonstaten (k_1) der Zuckerbindung an der Bindestelle innerhalb des Kanals. Die Ergebnisse zeigten, dass die Geschwindigkeitskonstanten durch die Mutationen beeinflusst wurden. Für die meisten Mutanten sank der k_1 Wert während der k_{-1} Wert anstieg.

CHAPTER 7

APPENTIX

7.1 REFERENCES

Andersen, C. (2003) Channel-tunnels: outer membrane components of type I secretion systems and multidrug efflux pumps of Gram-negative bacteria. *Rev Physiol Biochem Pharmacol*, **147**, 122-165.

Andersen, C. (2004) *Drug efflux and protein export through channel-tunnels*. WILEY-VCH Verlag GmbH, Weinheim/Germany.

Andersen, C., Bachmeyer, C., Tauber, H., Benz, R., Wang, J., Michel, V., Newton, S.M., Hofnung, M. and Charbit, A. (1999) In vivo and in vitro studies of major surface loop deletion mutants of the *Escherichia coli* K-12 maltoporin: contribution to maltose and maltooligosaccharide transport and binding. *Mol Microbiol*, **32**, 851-867.

Andersen, C., Cseh, R., Schulein, K. and Benz, R. (1998) Study of sugar binding to the sucrose-specific ScrY channel of enteric bacteria using current noise analysis. *J Membr Biol*, **164**, 263-274.

Andersen, C., Hughes, C. and Koronakis, V. (2000) Chunnel vision. Export and efflux through bacterial channel-tunnels. *EMBO Rep*, **1**, 313-318.

Andersen, C., Hughes, C. and Koronakis, V. (2001) Protein export and drug efflux through bacterial channel-tunnels. *Curr Opin Cell Biol*, **13**, 412-416.

Andersen, C., Jordy, M. and Benz, R. (1995) Evaluation of the rate constants of sugar transport through maltoporin (LamB) of *Escherichia coli* from the sugar-induced current noise. *J Gen Physiol*, **105**, 385-401.

Andersen, C., Koronakis, E., Bokma, E., Eswaran, J., Humphreys, D., Hughes, C. and Koronakis, V. (2002) Transition to the open state of the TolC periplasmic tunnel entrance. *Proc Natl Acad Sci U S A*, **99**, 11103-11108.

Andersen, C., Koronakis, E., Hughes, C. and Koronakis, V. (2002) An aspartate ring at the TolC tunnel entrance determines ion selectivity and presents a target for blocking by large cations. *Mol Microbiol*, **44**, 1131-1139.

Baranton, G., Marti Ras, N. and Postic, D. (1998) Molecular epidemiology of the aetiological agents of Lyme borreliosis. *Wien Klin Wochenschr*, **110**, 850-855.

Baranton, G., Postic, D., Saint Girons, I., Boerlin, P., Piffaretti, J.C., Assous, M. and Grimont, P.A. (1992) Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. *Int J Syst Bacteriol*, **42**, 378-383.

Barbour, A.G. (1984) Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med*, **57**, 521-525.

Barbour, A.G. (1990) Antigenic variation of a relapsing fever *Borrelia* species. *Annu Rev Microbiol*, **44**, 155-171.

Barbour, A.G. and Hayes, S.F. (1986) Biology of *Borrelia* species. *Microbiol Rev*, **50**, 381-400.

Barbour, A.G., Tessier, S.L. and Hayes, S.F. (1984) Variation in a major surface protein of Lyme disease spirochetes. *Infect Immun*, **45**, 94-100.

Barclay, A.J. and Coulter, J.B. (1990) Tick-borne relapsing fever in central Tanzania. *Trans R Soc Trop Med Hyg*, **84**, 852-856.

Benz, R. (1994) *Uptake of solutes through bacterial outer membranes*. Elsevier, Amsterdam.

Benz, R. (2001) Porins - structure and function. In Winkelmann, G. (ed.), *Microbial Transport Systems*. Wiley-VCH, Weinheim, pp. 227-246.

Benz, R. and Bauer, K. (1988) Permeation of hydrophilic molecules through the outer membrane of gram-negative bacteria. Review on bacterial porins. *Eur J Biochem*, **176**, 1-19.

Benz, R. and Hancock, R.E. (1987) Mechanism of ion transport through the anion-selective channel of the *Pseudomonas aeruginosa* outer membrane. *J Gen Physiol*, **89**, 275-295.

Benz, R., Janko, K., Boos, W. and Lauger, P. (1978) Formation of large, ion-permeable membrane channels by the matrix protein (porin) of Escherichia coli. *Biochim Biophys Acta*, **511**, 305-319.

Benz, R., Janko, K. and Lauger, P. (1979) Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli*. *Biochim Biophys Acta*, **551**, 238-247.

Benz, R., Maier, E. and Gentschev, I. (1993) TolC of *Escherichia coli* functions as an outer membrane channel. *Zentralbl Bakteriol*, **278**, 187-196.

Benz, R., Schmid, A. and Hancock, R.E. (1985) Ion selectivity of gram-negative bacterial porins. *J Bacteriol*, **162**, 722-727.

Benz, R., Schmid, A., Nakae, T. and Vos-Scheperkeuter, G.H. (1986) Pore formation by LamB of *Escherichia coli* in lipid bilayer membranes. *J Bacteriol*, **165**, 978-986.

Benz, R., Schmid, A. and Vos-Scheperkeuter, G.H. (1987) Mechanism of sugar transport through the sugar-specific LamB channel of *Escherichia coli* outer membrane. *J Membr Biol*, **100**, 21-29.

Bergström, S., Noppa, L., Gylfe, A. and Östberg, Y. (2002) *Molecular and Cellular Biology of Borrelia burgdorferi sensu lato*. CAB, International.

Beveridge, T.J. (1981) Ultrastructure, chemistry, and function of the bacterial wall. *Int Rev Cytol*, **72**, 229-317.

Bezrukov, S.M. and Winterhalter, M. (2000) Examining noise sources at the single-molecule level: 1/f noise of an open maltoporin channel. *Phys Rev Lett*, **85**, 202-205.

Blattner, F.R., Plunkett, G., 3rd, Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B. and Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science*, **277**, 1453-1474.

Blum, H., Beier, H. and Gross, H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, **8**, 93-99.

Brass, J.M., Bauer, K., Ehmann, U. and Boos, W. (1985) Maltose-binding protein does not modulate the activity of maltoporin as a general porin in *Escherichia coli*. *J Bacteriol*, **161**, 720-726.

Burgdorfer, W., Barbour, A.G., Hayes, S.F., Benach, J.L., Grunwaldt, E. and Davis, J.P. (1982) Lyme disease-a tick-borne spirochetosis? *Science*, **216**, 1317-1319.

Burman, N., Bergstrom, S., Restrepo, B.I. and Barbour, A.G. (1990) The variable antigens Vmp7 and Vmp21 of the relapsing fever bacterium *Borrelia hermsii* are structurally analogous to the VSG proteins of the African trypanosome. *Mol Microbiol*, **4**, 1715-1726.

Burman, N., Shamaei-Tousi, A. and Bergstrom, S. (1998) The spirochete *Borrelia crocidurae* causes

erythrocyte rosetting during relapsing fever. *Infect Immun*, **66**, 815-819.
Cadavid, D., Pennington, P.M., Kerentseva, T.A., Bergstrom, S. and Barbour, A.G. (1997) Immunologic and genetic analyses of VmpA of a neurotropic strain of *Borrelia turicatae*. *Infect Immun*, **65**, 3352-3360.
Canica, M.M., Nato, F., du Merle, L., Mazie, J.C., Baranton, G. and Postic, D. (1993) Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. *Scand J Infect Dis*, **25**, 441-448.
Carter, C.J., Bergstrom, S., Norris, S.J. and Barbour, A.G. (1994) A family of surface-exposed proteins of 20 kilodaltons in the genus *Borrelia*. *Infect Immun*, **62**, 2792-2799.
Casjens, S., Palmer, N., van Vugt, R., Huang, W.M., Stevenson, B., Rosa, P., Lathigra, R., Sutton, G., Peterson, J., Dodson, R.J., Haft, D., Hickey, E., Gwinn, M., White, O. and Fraser, C.M. (2000) A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. *Mol Microbiol*, **35**, 490-516.
Chimento, D.P., Kadner, R.J. and Wiener, M.C. (2003) The *Escherichia coli* outer membrane cobalamin transporter BtuB: structural analysis of calcium and substrate binding, and identification of orthologous transporters by sequence/structure conservation. *J Mol Biol*, **332**, 999-1014.
Coburn, J., Chege, W., Magoun, L., Bodary, S.C. and Leong, J.M. (1999) Characterization of a candidate *Borrelia burgdorferi* beta3-chain integrin ligand identified using a phage display library. *Mol Microbiol*, **34**, 926-940.
Coburn, J. and Cugini, C. (2003) Targeted mutation of the outer membrane protein P66 disrupts attachment of the Lyme disease agent, *Borrelia burgdorferi*, to integrin alphavbeta3. *Proc Natl Acad Sci U S A*, **100**, 7301-7306.
Conti, F. and Wanke, E. (1975) Channel noise in nerve membranes and lipid bilayers. *Q Rev Biophys*, **8**, 451-506.
Cullen, P.A., Haake, D.A. and Adler, B. (2004) Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiol Rev*, **28**, 291-318.
Cutler, S.J., Akintunde, C.O., Moss, J., Fukunaga, M., Kurtenbach, K., Talbert, A., Zhang, H., Wright, D.J. and Warrell, D.A. (1999) Successful in vitro cultivation of *Borrelia duttonii* and its comparison with *Borrelia recurrentis*. *Int J Syst Bacteriol*, **49 Pt 4**, 1793-1799.
Danelon, C. and Winterhalter, M. (2004) *Reconstitution of general diffusion pores from bacterial outer membranes*. Wiley-VCH, Weinheim.
De Felice, L.J. (1981) *Introduction to Membrane Noise*. Plenum Press, New York.
de Silva, A.M., Telford, S.R., 3rd, Brunet, L.R., Barthold, S.W. and Fikrig, E. (1996) *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. *J Exp Med*, **183**, 271-275.
Dodge, R.W. (1973) Culture of Ethiopian strains of *Borrelia recurrentis*. *Appl Microbiol*, **25**, 935-939.
Dodge, R.W. (1973) Human serological response to louse-borne relapsing fever. *Infect Immun*, **8**, 891-895.
Dutton JE, T.J. (1905) The nature of tick fever in the eastern part of the Conga Free State, with notes on the distribution and bionomics of the tick. *Br Med J*, **2**, 1259-1260.
Dutzler, R., Wang, Y.F., Rizkallah, P., Rosenbusch, J.P. and Schirmer, T. (1996) Crystal structures of various maltooligosaccharides bound to maltoporin reveal a specific sugar translocation pathway. *Structure*, **4**, 127-134.
Eckerskorn, C. and Lottspeich, F. (1993) Structural characterization of blotting membranes and the influence of membrane parameters for electroblotting and subsequent amino acid sequence analysis of proteins. *Electrophoresis*, **14**, 831-838.
Eigen, M., Kruse, W., Maass, G. and De Maeyer, L. (1964) Rate constants of protolytic reactions in aqueous solutions. *Prog. React. Kinet.*, **2**, 287-318.
Eisen, L., Eisen, R.J. and Lane, R.S. (2002) Seasonal activity patterns of *Ixodes pacificus* nymphs in

relation to climatic conditions. *Med Vet Entomol*, **16**, 235-244.
Eswaran, J., Koronakis, E., Higgins, M.K., Hughes, C. and Koronakis, V. (2004) Three's company: component structures bring a closer view of tripartite drug efflux pumps. *Curr Opin Struct Biol*, **14**, 741-747.
Ferenci, T., Schwentorat, M., Ullrich, S. and Vilmart, J. (1980) Lambda receptor in the outer membrane of *Escherichia coli* as a binding protein for maltodextrins and starch polysaccharides. *J Bacteriol*, **142**, 521-526.
Ferguson, A.D., Hofmann, E., Coulton, J.W., Diederichs, K. and Welte, W. (1998) Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. *Science*, **282**, 2215-2220.
Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., White, O., Ketchum, K.A., Dodson, R., Hickey, E.K., Gwinn, M., Dougherty, B., Tomb, J.F., Fleischmann, R.D., Richardson, D., Peterson, J., Kerlavage, A.R., Quackenbush, J., Salzberg, S., Hanson, M., van Vugt, R., Palmer, N., Adams, M.D., Gocayne, J., Weidman, J., Utterback, T., Watthey, L., McDonald, L., Artiach, P., Bowman, C., Garland, S., Fuji, C., Cotton, M.D., Horst, K., Roberts, K., Hatch, B., Smith, H.O. and Venter, J.C. (1997) Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature*, **390**, 580-586.
Freundlieb, S., Ehmann, U. and Boos, W. (1988) Facilitated diffusion of p-nitrophenyl-alpha-D-maltohexaoside through the outer membrane of *Escherichia coli*. Characterization of LamB as a specific and saturable channel for maltooligosaccharides. *J Biol Chem*, **263**, 314-320.
Fuchs, H., Wallich, R., Simon, M.M. and Kramer, M.D. (1994) The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. *Proc Natl Acad Sci U S A*, **91**, 12594-12598.
Garavito, R.M. (1994) The X-ray structures of *Escherichia coli* porin OmpF and ovine prostaglandin synthase: implications for membrane structure. *Ren Physiol Biochem*, **17**, 219-222.
Grkovic, S., Brown, M.H. and Skurray, R.A. (2002) Regulation of bacterial drug export systems. *Microbiol Mol Biol Rev*, **66**, 671-701, table of contents.
Gupta, R.S. (2000) The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiol Rev*, **24**, 367-402.
Hancock, R.E. (1981) Aminoglycoside uptake and mode of action--with special reference to streptomycin and gentamicin. I. Antagonists and mutants. *J Antimicrob Chemother*, **8**, 249-276.
Hancock, R.E., Schmidt, A., Bauer, K. and Benz, R. (1986) Role of lysines in ion selectivity of bacterial outer membrane porins. *Biochim Biophys Acta*, **860**, 263-267.
Heffernan, E.J., Wu, L., Louie, J., Okamoto, S., Fierer, J. and Guiney, D.G. (1994) Specificity of the complement resistance and cell association phenotypes encoded by the outer membrane protein genes rck from *Salmonella typhimurium* and ail from *Yersinia enterocolitica*. *Infect Immun*, **62**, 5183-5186.
Heine, H.G., Francis, G., Lee, K.S. and Ferenci, T. (1988) Genetic analysis of sequences in maltoporin that contribute to binding domains and pore structure. *J Bacteriol*, **170**, 1730-1738.
Hinnebusch, B.J., Barbour, A.G., Restrepo, B.I. and Schwan, T.G. (1998) Population structure of the relapsing fever spirochete *Borrelia hermsii* as indicated by polymorphism of two multigene families that encode immunogenic outer surface lipoproteins. *Infect Immun*, **66**, 432-440.
Hubalek, Z. and Halouzka, J. (1997) Distribution of *Borrelia burgdorferi* sensu lato genomic groups in Europe, a review. *Eur J Epidemiol*, **13**, 951-957.
Hudson, B.J., Stewart, M., Lennox, V.A., Fukunaga, M., Yabuki, M., Macorison, H. and Kitchener-Smith, J. (1998) Culture-positive Lyme borreliosis. *Med J Aust*, **168**, 500-502.
Hunfeld, K.P., Weigand, J., Wichelhaus, T.A., Kekoukh, E., Kraiczy, P. and Brade, V. (2001) In

vitro activity of mezlocillin, meropenem, aztreonam, vancomycin, teicoplanin, ribostamycin and fusidic acid against *Borrelia burgdorferi*. *Int J Antimicrob Agents*, **17**, 203-208.
Jordy, M., Andersen, C., Schulein, K., Ferenci, T. and Benz, R. (1996) Rate constants of sugar transport through two LamB mutants of *Escherichia coli:* comparison with wild-type maltoporin and LamB of *Salmonella typhimurium*. *J Mol Biol*, **259**, 666-678.
Kehl, K.S., Farmer, S.G., Komorowski, R.A. and Knox, K.K. (1986) Antigenic variation among Borrelia spp. in relapsing fever. *Infect Immun*, **54**, 899-902.
Killmann, H., Herrmann, C., Torun, A., Jung, G. and Braun, V. (2002) TonB of *Escherichia coli* activates FhuA through interaction with the beta-barrel. *Microbiology*, **148**, 3497-3509.
Killmann, H., Videnov, G., Jung, G., Schwarz, H. and Braun, V. (1995) Identification of receptor binding sites by competitive peptide mapping: phages T1, T5, and phi 80 and colicin M bind to the gating loop of FhuA. *J Bacteriol*, **177**, 694-698.
Koebnik, R. (1999) Structural and functional roles of the surface-exposed loops of the beta-barrel membrane protein OmpA from *Escherichia coli*. *J Bacteriol*, **181**, 3688-3694.
Koebnik, R., Locher, K.P. and Van Gelder, P. (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol*, **37**, 239-253.
Koronakis, V. (2003) TolC--the bacterial exit duct for proteins and drugs. *FEBS Lett*, **555**, 66-71.
Koronakis, V., Sharff, A., Koronakis, E., Luisi, B. and Hughes, C. (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature*, **405**, 914-919.
Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.
Li, H., Dunn, J.J., Luft, B.J. and Lawson, C.L. (1997) Crystal structure of Lyme disease antigen outer surface protein A complexed with an Fab. *Proc Natl Acad Sci U S A*, **94**, 3584-3589.
Li, M., Masuzawa, T., Takada, N., Ishiguro, F., Fujita, H., Iwaki, A., Wang, H., Wang, J., Kawabata, M. and Yanagihara, Y. (1998) Lyme disease *Borrelia* species in northeastern China resemble those isolated from far eastern Russia and Japan. *Appl Environ Microbiol*, **64**, 2705-2709.
Madigan, M.T., J.M., M. and Parker, J. (2003) *Biology of Microorganisms*. Pearson Education LTD, London.
Magnarelli, L.A., Anderson, J.F. and Barbour, A.G. (1989) Enzyme-linked immunosorbent assays for Lyme disease: reactivity of subunits of *Borrelia burgdorferi*. *J Infect Dis*, **159**, 43-49.
Maier, C., Bremer, E., Schmid, A. and Benz, R. (1988) Pore-forming activity of the Tsx protein from the outer membrane of *Escherichia coli*. Demonstration of a nucleoside-specific binding site. *J Biol Chem*, **263**, 2493-2499.
Maier, E., Polleichtner, G., Boeck, B., Schinzel, R. and Benz, R. (2001) Identification of the outer membrane porin of *Thermus thermophilus* HB8: the channel-forming complex has an unusually high molecular mass and an extremely large single-channel conductance. *J Bacteriol*, **183**, 800-803.
Marchler-Bauer, A. and Bryant, S.H. (2004) CD-Search: protein domain annotations on the fly. *Nucleic Acids Res*, **32**, W327-331.
Mata, M.T., Baquero, F. and Perez-Diaz, J.C. (2000) A multidrug efflux transporter in *Listeria monocytogenes*. *FEMS Microbiol Lett*, **187**, 185-188.
Meier, J.T., Simon, M.I. and Barbour, A.G. (1985) Antigenic variation is associated with DNA rearrangements in a relapsing fever *Borrelia*. *Cell*, **41**, 403-409.
Meyer, J.E., Hofnung, M. and Schulz, G.E. (1997) Structure of maltoporin from *Salmonella typhimurium* ligated with a nitrophenyl-maltotrioside. *J Mol Biol*, **266**, 761-775.
Meyer, J.E. and Schulz, G.E. (1997) Energy profile of maltooligosaccharide permeation through maltoporin as derived from the structure and from a statistical analysis of saccharide-protein interactions. *Protein Sci*, **6**, 1084-1091.
Nadelman, R.B., Nowakowski, J., Fish, D., Falco, R.C., Freeman, K., McKenna, D., Welch, P.,

Marcus, R., Aguero-Rosenfeld, M.E., Dennis, D.T. and Wormser, G.P. (2001) Prophylaxis with single-dose doxycycline for the prevention of Lyme disease after an *Ixodes scapularis* tick bite. *N Engl J Med*, **345**, 79-84.

Nekolla, S., Andersen, C. and Benz, R. (1994) Noise analysis of ion current through the open and the sugar-induced closed state of the LamB channel of *Escherichia coli* outer membrane: evaluation of the sugar binding kinetics to the channel interior. *Biophys J*, **66**, 1388-1397.

Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev*, **67**, 593-656.

Nikaido, H. and Vaara, M. (1985) Molecular basis of bacterial outer membrane permeability. *Microbiol Rev*, **49**, 1-32.

Noppa, L., Ostberg, Y., Lavrinovicha, M. and Bergstrom, S. (2001) P13, an integral membrane protein of *Borrelia burgdorferi*, is C-terminally processed and contains surface-exposed domains. *Infect Immun*, **69**, 3323-3334.

Orlik, F., Andersen, C. and Benz, R. (2002) Site-directed mutagenesis of tyrosine 118 within the central constriction site of the LamB (Maltoporin) channel of *Escherichia coli*. I. Effect on ion transport. *Biophys J*, **82**, 2466-2475.

Orlik, F., Andersen, C. and Benz, R. (2002) Site-directed mutagenesis of tyrosine 118 within the central constriction site of the LamB (maltoporin) channel of *Escherichia coli*. II. Effect on maltose and maltooligosaccharide binding kinetics. *Biophys J*, **83**, 309-321.

Ostberg, Y., Pinne, M., Benz, R., Rosa, P. and Bergstrom, S. (2002) Elimination of channel-forming activity by insertional inactivation of the p13 gene in *Borrelia burgdorferi*. *J Bacteriol*, **184**, 6811-6819.

Pal, U., de Silva, A.M., Montgomery, R.R., Fish, D., Anguita, J., Anderson, J.F., Lobet, Y. and Fikrig, E. (2000) Attachment of *Borrelia burgdorferi* within *Ixodes scapularis* mediated by outer surface protein A. *J Clin Invest*, **106**, 561-569.

Parola, P. and Raoult, D. (2001) Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clin Infect Dis*, **32**, 897-928.

Perkins, D.N., Pappin, D.J., Creasy, D.M. and Cottrell, J.S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*, **20**, 3551-3567.

Pinne, M., Ostberg, Y., Comstedt, P. and Bergstrom, S. (2004) Molecular analysis of the channel-forming protein P13 and its paralogue family 48 from different Lyme disease *Borrelia* species. *Microbiology*, **150**, 549-559.

Porcella, S.F., Raffel, S.J., Anderson, D.E., Jr., Gilk, S.D., Bono, J.L., Schrumpf, M.E. and Schwan, T.G. (2005) Variable tick protein in two genomic groups of the relapsing fever spirochete *Borrelia hermsii* in western North America. *Infect Immun*, **73**, 6647-6658.

Pos, K.M., Schiefner, A., Seeger, M.A. and Diederichs, K. (2004) Crystallographic analysis of AcrB. *FEBS Lett*, **564**, 333-339.

Purser, J.E. and Norris, S.J. (2000) Correlation between plasmid content and infectivity in *Borrelia burgdorferi*. *Proc Natl Acad Sci U S A*, **97**, 13865-13870.

Radolf, J.D., Bourell, K.W., Akins, D.R., Brusca, J.S. and Norgard, M.V. (1994) Analysis of *Borrelia burgdorferi* membrane architecture by freeze-fracture electron microscopy. *J Bacteriol*, **176**, 21-31.

Ried, G., Koebnik, R., Hindennach, I., Mutschler, B. and Henning, U. (1994) Membrane topology and assembly of the outer membrane protein OmpA of *Escherichia coli* K12. *Mol Gen Genet*, **243**, 127-135.

Riess, F.G. and Benz, R. (2000) Discovery of a novel channel-forming protein in the cell wall of the non-pathogenic *Nocardia corynebacteroides*. *Biochim Biophys Acta*, **1509**, 485-495.

Schirmer, T., Keller, T.A., Wang, Y.F. and Rosenbusch, J.P. (1995) Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution. *Science*, **267**, 512-514.

Schlegel, H.G. (1992) *Allgemeine Mikrobiologie*. Georg Thieme Verlag, Stuttgart, New York.

Schmid, K., Ebner, R., Altenbuchner, J., Schmitt, R. and Lengeler, J.W. (1988) Plasmid-mediated sucrose metabolism in *Escherichia coli* K12: mapping of the scr genes of pUR400. *Mol Microbiol*, **2**, 1-8.

Schmid, K., Ebner, R., Jahreis, K., Lengeler, J.W. and Titgemeyer, F. (1991) A sugar-specific porin, ScrY, is involved in sucrose uptake in enteric bacteria. *Mol Microbiol*, **5**, 941-950.

Schmid, K., Schupfner, M. and Schmitt, R. (1982) Plasmid-mediated uptake and metabolism of sucrose by *Escherichia coli* K-12. *J Bacteriol*, **151**, 68-76.

Schulein, K., Andersen, C. and Benz, R. (1995) The deletion of 70 amino acids near the N-terminal end of the sucrose-specific porin ScrY causes its functional similarity to LamB in vivo and in vitro. *Mol Microbiol*, **17**, 757-767.

Schulein, K., Schmid, K. and Benzl, R. (1991) The sugar-specific outer membrane channel ScrY contains functional characteristics of general diffusion pores and substrate-specific porins. *Mol Microbiol*, **5**, 2233-2241.

Schwan, T.G., Policastro, P.F., Miller, Z., Thompson, R.L., Damrow, T. and Keirans, J.E. (2003) Tick-borne relapsing fever caused by *Borrelia hermsii*, Montana. *Emerg Infect Dis*, **9**, 1151-1154.

Schwartz, M. (1987) *The maltose regulon*. ASM, Washington, DC.

Schwarz, G., Danelon, C. and Winterhalter, M. (2003) On translocation through a membrane channel via an internal binding site: kinetics and voltage dependence. *Biophys J*, **84**, 2990-2998.

Scragg, I.G., Kwiatkowski, D., Vidal, V., Reason, A., Paxton, T., Panico, M., Dell, A. and Morris, H. (2000) Structural characterization of the inflammatory moiety of a variable major lipoprotein of *Borrelia recurrentis*. *J Biol Chem*, **275**, 937-941.

Shamaei-Tousi, A., Martin, P., Bergh, A., Burman, N., Brannstrom, T. and Bergstrom, S. (1999) Erythrocyte-aggregating relapsing fever spirochete *Borrelia crocidurae* induces formation of microemboli. *J Infect Dis*, **180**, 1929-1938.

Shang, E.S., Skare, J.T., Exner, M.M., Blanco, D.R., Kagan, B.L., Miller, J.N. and Lovett, M.A. (1998) Isolation and characterization of the outer membrane of *Borrelia hermsii*. *Infect Immun*, **66**, 1082-1091.

Sickmann, A., Mreyen, M. and Meyer, H.E. (2002) Identification of modified proteins by mass spectrometry. *IUBMB Life*, **54**, 51-57.

Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H.E., Schonfisch, B., Perschil, I., Chacinska, A., Guiard, B., Rehling, P., Pfanner, N. and Meisinger, C. (2003) The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc Natl Acad Sci U S A*, **100**, 13207-13212.

Skare, J.T., Champion, C.I., Mirzabekov, T.A., Shang, E.S., Blanco, D.R., Erdjument-Bromage, H., Tempst, P., Kagan, B.L., Miller, J.N. and Lovett, M.A. (1996) Porin activity of the native and recombinant outer membrane protein Oms28 of *Borrelia burgdorferi*. *J Bacteriol*, **178**, 4909-4918.

Skare, J.T., Mirzabekov, T.A., Shang, E.S., Blanco, D.R., Erdjument-Bromage, H., Bunikis, J., Bergstrom, S., Tempst, P., Kagan, B.L., Miller, J.N. and Lovett, M.A. (1997) The Oms66 (p66) protein is a *Borrelia burgdorferi* porin. *Infect Immun*, **65**, 3654-3661.

Snijder, H.J., Ubarretxena-Belandia, I., Blaauw, M., Kalk, K.H., Verheij, H.M., Egmond, M.R., Dekker, N. and Dijkstra, B.W. (1999) Structural evidence for dimerization-regulated activation of an integral membrane phospholipase. *Nature*, **401**, 717-721.

Steere, A.C., Coburn, J. and Glickstein, L. (2004) The emergence of Lyme disease. *J Clin Invest*, **113**, 1093-1101.

Sulavik, M.C., Houseweart, C., Cramer, C., Jiwani, N., Murgolo, N., Greene, J., DiDomenico, B., Shaw, K.J., Miller, G.H., Hare, R. and Shimer, G. (2001) Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. *Antimicrob Agents Chemother*, **45**, 1126-1136.

Szmelcman, S., Schwartz, M., Silhavy, T.J. and Boos, W. (1976) Maltose transport in *Escherichia coli* K12. A comparison of transport kinetics in wild-type and lambda-resistant mutants as measured by fluorescence quenching. *Eur J Biochem*, **65**, 13-19.

Tabuchi, N., Mitani, H., Seino, S. and Fukunaga, M. (2002) The 44-kb linear plasmid molecule in the relapsing fever agent *Borrelia duttonii* strain Ly serve as a preservation of vmp genes. *Microbiol Immunol*, **46**, 159-165.

Takayama, K., Rothenberg, R.J. and Barbour, A.G. (1987) Absence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*. *Infect Immun*, **55**, 2311-2313.

Tokuda, H. and Matsuyama, S. (2004) Sorting of lipoproteins to the outer membrane in *E. coli*. *Biochim Biophys Acta*, **1694**, IN1-9.

Tommassen, J. and Lugtenberg, B. (1980) Outer membrane protein e of *Escherichia coli* K-12 is co-regulated with alkaline phosphatase. *J Bacteriol*, **143**, 151-157.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*, **76**, 4350-4354.

van den Berg, B. (2005) The FadL family: unusual transporters for unusual substrates. *Curr Opin Struct Biol*, **15**, 401-407.

Van Gelder, P., Dumas, F., Bartoldus, I., Saint, N., Prilipov, A., Winterhalter, M., Wang, Y., Philippsen, A., Rosenbusch, J.P. and Schirmer, T. (2002) Sugar transport through maltoporin of *Escherichia coli*: role of the greasy slide. *J Bacteriol*, **184**, 2994-2999.

Van Gelder, P., Dumas, F., Rosenbusch, J.P. and Winterhalter, M. (2000) Oriented channels reveal asymmetric energy barriers for sugar translocation through maltoporin of *Escherichia coli*. *Eur J Biochem*, **267**, 79-84.

Veal, W.L., Yellen, A., Balthazar, J.T., Pan, W., Spratt, B.G. and Shafer, W.M. (1998) Loss-of-function mutations in the mtr efflux system of *Neisseria gonorrhoeae*. *Microbiology*, **144 (Pt 3)**, 621-627.

Verveen, A.A. and DeFelice, L.J. (1974) Membrane noise. *Prog Biophys Mol Biol*, **28**, 189-265.

Vogt, J. and Schulz, G.E. (1999) The structure of the outer membrane protein OmpX from *Escherichia coli* reveals possible mechanisms of virulence. *Structure Fold Des*, **7**, 1301-1309.

Walker, E.M., Borenstein, L.A., Blanco, D.R., Miller, J.N. and Lovett, M.A. (1991) Analysis of outer membrane ultrastructure of pathogenic *Treponema* and *Borrelia* species by freeze-fracture electron microscopy. *J Bacteriol*, **173**, 5585-5588.

Walton, G.A. (1962) *The ornithodorus moubata superspecies problem in relation to human relapsing fever epidemiology*. Lodon Royal Zoological Society, Lodon.

Wang, I.N., Dykhuizen, D.E., Qiu, W., Dunn, J.J., Bosler, E.M. and Luft, B.J. (1999) Genetic diversity of ospC in a local population of *Borrelia burgdorferi* sensu stricto. *Genetics*, **151**, 15-30.

Wang, J., Hofnung, M. and Charbit, A. (2000) The C-terminal portion of the tail fiber protein of bacteriophage lambda is responsible for binding to LamB, its receptor at the surface of *Escherichia coli* K-12. *J Bacteriol*, **182**, 508-512.

Wessel, D. and Flugge, U.I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem*, **138**, 141-143.

Woese, C.R. (2000) Interpreting the universal phylogenetic tree. *Proc Natl Acad Sci U S A*, **97**, 8392-8396.

Wohnsland, F. and Benz, R. (1997) 1/f-Noise of open bacterial porin channels. *J Membr Biol*, **158**, 77-85.

7.2 Curriculum vitae

Name:	Katrin Denker
Address:	Hauptstrasse 1a 97218 Gerbrunn
Date and Place of birth:	03/10/1978 Lübeck/Germany
Marital status:	unmarried

Schooling:	1985-1989 Elementary school Lübeck-Schlutup 1989-1995 Secondary school Lübeck-Schlutup 1995-1998 Grammar school, Dorothea Schlözer Schule, Lübeck 06/1998 Abitur

Academical Carreer:

10/1998-12/2002	Study of chemical engineering at the University of Applied Science Lübeck Major subject: Biotechnology Diploma thesis: „Investigation to the cultivation and antimicrobial effect of a selected marine fungus“
12/2002	Diploma
07/01/2003	Start of Ph.D.-Thesis in the laboratory of Prof. Dr. R. Benz at the University of Würzburg
08-09/2003 and 11-12/2004	Research stays at the University Umeå, Sweden, in the research group of Prof. S. Bergström

ACKNOWLEDMENTS

I would like to thank…

Prof. Roland Benz, for making this thesis possible. Thanks a lot for the support and the wine lessons.
Prof. Sven Bergström, for being my second corrector and all the useful discussions in Umeå
Dr. Christian Andersen, for fruitful discussions and all the help
Marija Pinne, for being more than a collaboration partner and Christer Larsson, die Wurst
Dr. Albert Sickmann and his team, for the help with the MS analysis
EGK 587/2, for scholarship and all the extras

Furthermore I like to say thanks to:

All my dear colleagues: Marcus (to be not afraid to go the Borrelia way), Anna (for the Bergkäse) and Maite (for the Furt), Ursel (to be Ursel), Michl (what a humor), Yvonne (not a vamp) and Sree (for the international flair), Georg (my dear relaxed colleague), Joschi (beer drinking genius), Frank (for teaching me all the LamB stuff), Tobias (what a mood) Enrico (I like red shoes!), Oli and Willi, the Intech crew (the waste water treaters), Bettina Mühling (what a smile), Bettina Schiffler (football playing BMW queen), Elke (for helping me so much to find my place in front of the Bilayer and for having always good ideas to continue)

My friends, which were always there for me: Julia (my Jul), Jana (what a tuff woman), Kim ("Time for breakfast at the Remise?"), Pam und Bo (mit dem Zweiten sieht man besser;), Jennifer (since the first year in school) and Davina (my crazy sweetie noddlesauce, määh)

As promised, Klaas, this is your line and "**.**" for still being a friend

My lovely family (Mama, Papa, Kirstin und Nils) for all the support and love. DANKE SCHÖN!