Structural and biomolecular analyses of *Borrelia burgdorferi* BmpD reveal a substrate-binding protein of an ABC-type nucleoside transporter family.

Running head: BmpD - a nucleoside-binding protein of *Borrelia*

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Abstract

*Borrelia burgdorferi* sensu lato (sl), the causative agent of the tick-borne Lyme borreliosis (LB), has a limited metabolic capacity and needs to acquire nutrients, such as amino acids, fatty acids, and nucleic acids, from the host environment. Using X-ray crystallography, liquid chromatography-mass spectrometry, microscale thermophoresis, and cellular localization studies, we show that Basic membrane protein D (BmpD) is a periplasmic substrate-binding protein of an ABC transporter system binding to purine nucleosides. Nucleosides are essential for bacterial survival in the host organism and these studies suggest a key role for BmpD in the purine salvage pathway of *B. burgdorferi* sl. As *B. burgdorferi* sl lacks the enzymes required for *de novo* purine synthesis, BmpD may play a vital role in ensuring access to the purines needed for sustaining an infection in the host. Further, we show that although human LB patients develop anti-BmpD antibodies, immunization of mice with BmpD does not confer protection against *B. burgdorferi* sl infection.

Introduction

Lyme borreliosis (LB) is a tick-borne infectious disease prevalent in North American, European, and Asian countries with moderate climates (1-3). *Borrelia burgdorferi* sensu lato (*Borrelia*) spirochetes belong to the group of LB causing bacteria consisting of about twenty different genospecies, where *B. burgdorferi* sensu stricto (ss), *B. garinii*, and *B. afzelii* are the major human pathogens (1). *B. burgdorferi* ss is prevalent in North America, whereas *B. afzelii* and *B. garinii* are common in Europe and in Asia (1, 2). LB is a multisystem and multistage infection where a skin lesion called erythema migrans (EM) is commonly the first sign of a local infection (2). The EM occurring around the tick bite site results from the host immune response against replicating bacteria in the skin (1). In the disseminated stage of LB, the bacteria have
migrated from the initial entry site in the skin to distant organs, such as the central nervous system, the heart, or the joints (2, 3).

*Borrelia* circulates between its arthropod tick vector and various vertebrate hosts. Although the host environments are different, *Borrelia* is able to survive despite its limited metabolic capacities as reviewed by Radolf and colleagues (4). *Borrelia* lacks the genes encoding components of many biosynthetic pathways (4, 5). The complex genome of *Borrelia* consists of one linear chromosome (~1 Mb) and of multiple circular and linear plasmids (in total ~0.6 Mb) (5-7). The chromosome carries the main genes essential for maintaining survival and replication in the tick and vertebrate host, but is devoid of genes encoding enzymes for *de novo* synthesis of amino acids, fatty acids, enzyme cofactors, and nucleic acids (5, 8). Using the purine salvage pathway, *Borrelia* is able to rescue purine bases, nucleosides, and deoxynucleosides from the host environments, and to incorporate the nucleotides into bacterial RNA and the deoxynucleotides into DNA after enzymatic conversion (8). In contrast to the genomes of the relapsing fever spirochetes *B. hermsii* and *B. turicatae*, the *Borrelia* genome does not encode a complete set of purine salvage pathway components as it lacks the genes of the key enzymes, ribonucleotide reductase, hypoxanthine phosphoribosyltransferase, adenylosuccinate synthase, and adenylosuccinate lyase (8).

However, several essential transporters and enzymes have been identified that are involved in the purine salvage pathway and are critical for *Borrelia* infectivity in the vertebrate host. For example, the transporter proteins BBB22 and BBB23 import purine bases (adenine, guanine, and hypoxanthine) from the host environment into the bacteria, and are necessary for *Borrelia* infection in mice (9, 10). Likewise, GuaA...
(BBB18) and GuaB (BBB17) are essential enzymes for converting purine bases to GMP and deoxyGMP, vital precursors in the synthesis of RNA and DNA (11).

In addition to purine bases, *Borrelia* also rescues (deoxy)nucleosides from the environment. The host-derived (deoxy)nucleoside monophosphates are first dephosphorylated to (deoxy)nucleosides by a nucleotidase, and then, an energy-driven transporter system (BB0677–79) translocates the nucleosides into the *Borrelia* cytoplasm (12). The transporter system, containing two permeases (BB0678–79) and one ATP-binding protein (BB0677), is one of the many ATP-binding cassette (ABC) transporters involved in nutrient transportation in *Borrelia* (5).

The ABC transporters belong to one of the largest families of transporter proteins using the hydrolysis of ATP to transport various substrates across cell membranes (13). They consist of two transmembrane domains (TMD), forming a translocation channel through the membrane, and two nucleoside-binding domains (NBD), which bind to and hydrolyze ATP (Figure 1A) (13). In bacteria, ABC transporters play a vital role in the import of nutrients and require a substrate-binding protein (SBP) to deliver the substrate to the translocation channel formed by the two permeases (14). SBPs bind to their substrates with high affinity and specificity and, using the Venus fly-trap mechanism (15), they change into a closed conformation when the substrate is bound. This closed conformation is recognized by the TMDs and triggers ATP hydrolysis and opening of the translocation channel (Figure 1A) (16). Using homology modeling, we recently showed that the four members of a paralogous Basic membrane protein (Bmp) family of *B. burgdorteri* ss, BmpA, B, C, and D (BB0383, BB0382, BB0384, BB0385, respectively), belong to the substrate-binding proteins of an ABC transporter family (Figure 1B) (17). Furthermore, we predicted that BmpA-D are likely involved in the uptake of purine nucleosides based on their
structural similarities to PnrA, a purine nucleoside-binding protein of the related spirochete *Treponema pallidum* (17, 18).

Efforts to determine the exact function of the Bmp proteins have so far given contradictory results. The BmpA-D have been described as laminin-binding proteins expressed on the outer surface of *Borrelia* (19), but, on the other hand, the same proteins have been annotated as ABC transporters for simple sugars, such as ribose or galactose (20, 21). Further, BmpA and BmpB have been suggested to regulate joint inflammation *in vivo* (22). A comprehensive protein localization study showed that BmpB, BmpC, and BmpD are expressed on the inner membrane in the periplasmic space of *Borrelia* (23) arguing against the adhesin role of the proteins (24). As these earlier studies have given discrepant results, we focused on BmpD as a representative member of the Bmp family to shed light on the role of these proteins in the physiology of *Borrelia*. Additionally, thoroughly characterized *Borrelia* proteins are potential key vaccine candidates for LB prevention.

In this study, we solved the crystal structure of BmpD and analyzed its nucleoside-binding properties. Our results indicate that BmpD functions as a substrate-binding protein of the ABC-type transporter family, importing purine nucleosides from the environment into the bacterial cell. Further, we show that human LB patients develop antibodies against BmpD, but, based on mouse immunization studies, immunity against BmpD does not protect from LB.

**Results**

*BmpD is an ABC-type substrate-binding protein*

To purify BmpD for ligand binding and crystallization experiments for X-ray structure determination, we expressed recombinant BmpD (rBmpD) in *Escherichia coli* without
the signal peptide sequence (Figure 2A) and purified it by affinity chromatography, under native conditions, and size exclusion chromatography (SEC) (Figure 2D). The calculated size of rBmpD is 39 kDa (Figure 2B,C, lane 3). The purified protein was successfully crystallized, and the BmpD structure was solved by molecular replacement. The final crystal structure of BmpD was refined to 1.43 Ångström (Å) (Figure 3A, Table 1). The structure is a monomer and consists of two domains connected by a linker region, characteristic of the substrate-binding proteins (25). The N-terminal domain consists of residues 8–115 and 243–269, and the C-terminal domain consists of residues 116–242 and 270–323. The BmpD structure is similar to the structures of the other ABC-type substrate-binding proteins, which bind specific substrate molecules and transfer them to membrane-bound ABC transporters that transport the substrates into the bacteria (26). The substrate-binding proteins are characterized by two alpha/beta domains containing a central beta sheet surrounded by alpha helices. In BmpD, the central beta sheet of both domains contains six beta strands and the N-terminal domain has four alpha helices, whereas the C-terminal domain has six (Figure 3A). The crystal structure also unexpectedly contained an endogenously bound ligand, which had not been added during the crystallization setup. The ligand is bound in the cleft between the two domains and the electron density indicated that the ligand is a purine nucleoside, which was confirmed to be an adenosine by liquid chromatography-mass spectrometry (LC-MS) analysis (Figure 4A,B).

Superimposition of the B. burgdorferi ss B31 BmpD and T. pallidum PnrA structures (PDB ID: 2FQY (18)) revealed a root mean square deviation of 1.0 Å, indicating a very high structural similarity, although the proteins share an amino acid sequence identity of only 27.8% (17). The ligand-binding site is likewise highly conserved as
described in our previous study (17). Both proteins are bound to a purine nucleoside, which forms hydrogen bonds to surrounding residues and water molecules. Furthermore, the aromatic rings of the purine base form stacking interactions with two phenylalanines. Compared to PnrA, BmpD has a more extensive water-mediated hydrogen-bonding network connecting the purine base of the nucleoside with Asp19 and Asn28 (Figure 3B,C and Figure S1).

The main differences between the adenosine-binding sites in BmpD and PnrA are found in the loops flanking the binding site (Figure 3A). In Loop 1, Ser86 and Phe87 in PnrA (Figure 3C) are exchanged for Phe76 and Arg77 in BmpD (Figure 3B). Asp27 in loop 2 of PnrA points towards the ligand and makes a hydrogen bond with the backbone nitrogen of Phe87, whereas Asp19 in BmpD is turned away from the binding site and forms ionic interaction with Arg77. Compared to BmpD (Figure 3A,B), loop 2 of PnrA is longer by one residue and, thus, intrudes deeper into the binding site (Figure 3C).

The only difference between adenosine and inosine is the amino and carbonyl groups of the base part, respectively (Figure 3C, E). To analyze how BmpD could bind inosine, we thus created a model for inosine bound BmpD (Figure 3D) based on the PnrA-inosine complex structure (PDB ID: 2fqw). Comparison of the inosine and adenosine bound structures of PnrA shows that inosine binding results in a conformational change in loop 2 (Figure 3C,E). As a result, the side chain of Ser28 turns away from the binding site and its position is replaced by water molecule 6 (w6). In addition, w4 is excluded allowing direct interaction between the carbonyl oxygen of inosine and Asp27 in PnrA (Figure 3E). In the BmpD-inosine model, loop 2 may remain unchanged as Ser28 is replaced by Gly20 in BmpD, and the carbonyl
oxygen of inosine makes hydrogen bonds with w6 and the main chain oxygen of Asp19 (Figure 3D).

**BmpD binds to a nucleoside**

As the solved crystal structure of BmpD and the LC-MS analysis of the purified protein confirmed that BmpD binds to a purine nucleoside, we further analyzed its nucleoside-binding properties using microscale thermophoresis (MST) analyses. The endogenously bound adenosine was nearly completely removed from rBmpD by denaturation and refolding before SEC purification as only a residual amount of bound adenosine remained (Figure 2E; Figure 4C). The size of the refolded ligand-free rBmpD (LF-rBmpD) was smaller than the original protein (approximately 37 kDa; lane 4 in Figures 2B,C). The protein yield was low after the denaturation treatment. Hence, MST was chosen for ligand-binding assay as it consumes only small amounts of the protein. LF-rBmpD was mixed with either adenosine, inosine, guanosine, xanthosine, or ribose, and the diffusion in a thermal gradient was monitored as a function of ligand concentration (27). The resulting data demonstrate that LF-rBmpD binds to adenosine with a higher affinity than to inosine, while no binding could be detected to ribose, the negative control ligand (Figure 5). No MST binding curves could be obtained for LF-rBmpD and guanosine and xanthosine (data not shown).

**BmpD is located in the periplasmic space**

Substrate-binding proteins are known to be located in the periplasmic space of Gram-negative bacteria where they transfer the substrate molecules to membrane-bound transporters for passage into the cytoplasm (26). The amino acid sequence of BmpD includes a signal peptide guiding the export of BmpD outside of the bacterial
cytoplasm (Figure 2A) (28). Hence, BmpD is expressed either in the bacterial periplasm or on the outer surface. Further, BmpD is not degraded by proteinase K, whereas the known surface-exposed proteins, DbpB and OspA, are degraded as seen by the decreased Western blot signals (Figure 6, lane 3) (23, 29). However, in the presence of a detergent and proteinase K, BmpD is degraded similarly to the known periplasmic flagellin (Figure 6, lane 4). Thus, BmpD is likely to be expressed in the periplasmic space.

*BmpD is expressed during human LB as indicated by anti-BmpD antibodies in patient sera*

To evaluate the role of BmpD expression in *Borrelia* survival in the host, sera of randomly selected LB patients were tested for BmpD antibodies. The BmpD antibodies were detected in sera of LB patients (Figure 7A). Although the intensity of the signals of antibodies recognizing rBmpD varied between the samples, these results show that *Borrelia* expresses BmpD during human infection, whereas the sera of non-LB patients did not recognize BmpD.

*BmpD is immunogenic in mice, but BmpD immunization does not protect from *Borrelia* infection*

Next, we wanted to study whether BmpD immunized mice are protected against *Borrelia* infection. C3H/HeN mice were first actively immunized either with rBmpD (BmpD immunized) or adjuvant only (mock immunized) and the sera were collected. The BmpD immunized mice had developed high IgG antibody levels towards rBmpD at 28 days (Figure 7B). Then, for the passive immunization studies, the collected sera of BmpD and mock immunized mice were transferred to a second set of mice before *B. burgdorferi* ss B31 challenge. In addition, two groups of control mice
pretreated with saline were either challenged with *B. burgdorferi* ss B31 (positive control) or received a phosphate-buffered saline (PBS) injection (negative control).

During the study, the joint swelling was monitored weekly as *B. burgdorferi* ss B31 causes significant arthritis (2). As expected, the mice in the positive control group developed joint swelling starting from day 14 and persisting until day 28 (Figure 8A). Also, the mice in the BmpD and mock immunized study groups developed similar joint swelling as the positive control mice. A small increase in joint diameter was observed in the negative control mice due to the growth of the mice.

The *Borrelia* infection status of the mice was further analyzed by culturing and quantifying the bacterial load in the mouse tissue samples, and by serology. Starting from day 21 and lasting until the end of the study, all mice in the BmpD and mock immunized and in the positive control groups were culture positive (Table 2). There were no statistically significant differences between the bacterial loads in ear tissue samples of the three *B. burgdorferi* ss B31 challenged study groups at days 7, 11, 14, 21, and in the ear, bladder and joint tissues collected at day 28 (*P*=0.243; *P*=0.589; *P*=0.506; *P*=0.730, *P*= 0.182; *P*= 0.571, *P*=0.218, respectively; Figure 8B).

Although the difference was small, the bacterial load in the heart tissue was statistically significantly higher in the BmpD immunized mice compared to the mock immunized mice (*P*=0.008; Figure 8B). Also, all mice in the three *B. burgdorferi* ss B31 challenged study groups had developed IgG antibodies towards *Borrelia* whole-cell sonicate (WCS; *P*=0.467) and towards rBmpD (*P*=0.324) without statistically significant differences between the three groups (Figure 8C). The negative control mice remained uninfected as all tissue samples were negative by *Borrelia* culture and qPCR (Table 2 and Figure 8B), and no IgG antibodies towards *Borrelia* WCS or rBmpD could be detected (Figure 8C).
In summary, all BmpD immunized mice were infected by *B. burgdorferi* ss B31 as evidenced by culture, detectable *Borrelia* loads in various tissues, high antibody levels towards *Borrelia* WCS and by development of joint swelling. Thus, BmpD immunization does not protect from *Borrelia* infection.

**Discussion**

Survival and proliferation of infectious bacteria require access to host nutrients as many pathogens have a limited biosynthetic capacity and must therefore use salvage pathways to obtain the nutrients needed (12). *Borrelia* lacks the essential enzymes for *de novo* synthesis of nucleic acids and acquires purines through the purine salvage pathway (8). Here, we show for the first time that Basic membrane protein D (BmpD) is a component of a purine nucleoside transporter system of *Borrelia*. Further, we report that BmpD is expressed during *Borrelia* infection in humans. However, antibodies towards BmpD do not protect against *Borrelia* infection.

*Borrelia* has a fragmented and rather small genome comprised of one linear chromosome and multiple plasmids that can be lost during long-term *in vitro* culturing (5). The linear chromosome carries genes essential for bacterial metabolism and replication (5, 30). The plasmids contain genes encoding mainly virulence factors, and are not required for bacterial growth *in vitro*, except for the circular plasmid 26 encoding the *guaAB* operon (9). *Borrelia* is an auxotrophic bacterium unable to *de novo* synthesize amino acids, fatty acids, vitamin cofactors and nucleotides (5, 20). Hence, *Borrelia* survival depends on the transportation of vital molecules from the host environment.
Previously, BmpD has been suggested to be important for *Borrelia* infection in the mammalian host, but with an unspecified function (19, 24, 31). The chromosomal localization of the *bmpD* gene, the conserved amino acid sequence of BmpD within the *Borrelia* genospecies (17), and the expression of BmpD during infection in humans suggest that BmpD is essential for *Borrelia* survival. In this study, we present the crystal structure of BmpD at a resolution of 1.43 Å, and describe the role of BmpD as a nucleoside-binding protein involved in the purine salvage pathway. The expression of BmpD in the periplasmic space of *Borrelia* further supports the notion that BmpD functions as a substrate-binding protein.

The crystal structure of BmpD revealed an endogenously bound ligand composed of a purine base and a ribose moiety resembling a nucleoside. The nucleoside was identified as adenosine by LC-MS analysis. The ligand-binding assay subsequently demonstrated that BmpD also binds to inosine. Despite the differences in the nucleoside structures (Figure 9A), similar interactions could be formed with both adenosine and inosine (Figure 3B, D). Based on our structural analysis, conformational changes and additional water molecules compensate for differences in the nucleoside structures and ensure that corresponding interactions are formed (Figure 3). Binding to a group of structurally similar substrates is also a common feature among other substrate-binding proteins (16).

During *Borrelia* infection in humans, the bacteria salvage nucleobases and nucleosides from the host environment. The physiological concentration of nucleosides in humans is 0.4–6 µM, except for inosine whose concentration is about 160 µM (32). The higher concentration of inosine reflects its pivotal role in the purine salvage pathway as a precursor of inosine monophosphate (IMP). IMP functions as a central branch point and can be converted into both adenosine monophosphate...
(AMP) and guanosine monophosphate (GMP) or to their deoxygenated forms, which are ultimately utilized for the biosynthesis of RNA and DNA, respectively. In contrast to the purine salvage pathway in humans, in *Borrelia*, the purine salvage pathway is distinct as the necessary enzymes converting IMP to AMP are missing (8). However, *Borrelia* might compensate for the lack of adenylosuccinate synthase and adenylosuccinate lyase by the uptake of adenosine via BmpD, leading to the formation of AMP, ADP, and finally of ATP for RNA incorporation. Likewise, the uptake of inosine via BmpD leads to the formation of GMP, GDP, and GTP for RNA incorporation.

In Figure 9B, we have visualized the proposed role of BmpD in the *Borrelia* purine salvage pathway. The host-derived purine nucleoside monophosphates are converted to nucleosides by a nucleotidase (12) before the nucleosides enter the periplasmic space via outer membrane porins, such as p66, allowing the diffusion of hydrophilic small molecules from the environment into the bacterial periplasmic space (33). Thereafter, BmpD, anchored by a fatty acid chain to the inner membrane of *Borrelia* (23), binds a free purine nucleoside and transports it to the ABC transporter (BB0677–79). The ABC transporter imports the nucleoside into the bacterial cytoplasm, where it is converted back to a nucleoside monophosphate by deoxynucleotide kinase (BB0239) (20). Adenylyl kinase (BB0417) and nucleoside diphosphate kinase (BB0463) add further phosphates, forming first nucleoside diphosphates, and finally nucleoside triphosphates, which can be incorporated into RNA (11).

Previously, it has been shown that BmpD is expressed during *Borrelia* infection in mice (34). Here, we show that BmpD is expressed also during *Borrelia* infection in humans as LB patients developed antibodies towards BmpD. Thus, we investigated
whether BmpD immunization would protect from *Borrelia* infection in mice. We chose the passive immunization protocol (35, 36), since young mice (4–5 weeks old) are more susceptible to *Borrelia* infection than older mice (37), and are therefore usually used in *Borrelia* mouse infection studies. Based on the immunization experiment results, we conclude that antibodies towards the periplasmic BmpD do not confer such immunity that it would protect mice from *Borrelia* infection. Also, immunization with BmpA or BmpD did not confer protection against *Borrelia* infection (22).

In conclusion, BmpD is a nucleoside-binding protein of an ABC transporter family that plays a role in the purine salvage pathway of *Borrelia*. Located in the periplasmic space, BmpD enables *Borrelia* to acquire purine nucleosides from the host environment. The importance of BmpD as a nucleoside-binding protein for *Borrelia* survival and for infectivity *in vivo* remains to be determined.

**Materials and methods**

*Escherichia coli* strains DH5α and BL21 (DE3)pLys (Novagen, Darmstadt, Germany) strains were cultured in Luria Bertani (LB) medium under appropriate antibiotic selection of kanamycin (25 µg/ml, Sigma-Aldrich, Darmstadt, Germany) and chloramphenicol (34 µg/ml, USB Corporation, Cleveland, USA) at 37°C. *Borrelia burgdorferi* sensu stricto (ss) B31 (a gift from Sven Bergström, University of Umeå, Sweden) was cultured in Barbour-Stoenner-Kelly-II medium at 33°C.

**Cloning of bmpD gene**

A synthetic *bmpD* gene based on the sequence of *B. burgdorferi* ss B31 (Gene ID: 1195222; residues 18–323) was generated by a commercial vendor (Integrated DNA Technologies, Leuven, Belgium). The *bmpD* gene was designed not to include the
signal peptide (residues 1–17), and the codons were optimized for *E. coli*. The *bmpD* gene was cloned in the pET-30a(+) -vector (Novagen) resulting in a fusion construct with a hexa-histidine tag at the N-terminus of the recombinant protein. The fusion construct was verified by sequencing. The plasmid was transformed into *E. coli* DH5α for plasmid amplification and subsequently to *E. coli* BL21 (DE3)pLys for protein expression.

**Expression and purification of rBmpD**

The *E. coli* BL21 (DE3)pLys cells were grown until the cell density reached an OD$_{600}$ of 0.6. Then, the protein expression was induced by 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3–4 hours. Cells were harvested by centrifugation and lysed by ultrasound sonication. The resulting suspension was centrifuged by 8300 rpm at 4°C for 30 minutes to remove cell debris. The rBmpD was isolated from the supernatant by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany) under native conditions.

For crystallization, rBmpD was further purified by size exclusion chromatography (SEC) with ÄKTA pure chromatography system (GE Healthcare Life Sciences, Chicago, USA) and Superdex™ 75 10/300 GL column (GE Healthcare Life Sciences) equilibrated with 50 mM Tris-HCl (pH 8.0). The peak fractions were analyzed by SDS-PAGE as described below. The fractions containing purified rBmpD were pooled, concentrated with 10 kDa molecular weight cut-off Amicon ultracentrifugal filters (EMD Millipore, Burlington, MA, USA) and the protein concentration was determined spectrophotometrically as 9.6 mg/ml. The hexahistidine tag was not removed prior to crystallization.

**Crystallization and X-ray diffraction data collection**
The crystals of rBmpD were obtained by the sitting drop vapor diffusion method. After five days, the crystals were observed in 2:1 reservoir solution of 0.2 M sodium chloride, 0.1 M Tris, 20% (w/v) PEG 6000 (pH 8.0) supplemented with 15% MPD (2-methyl-2,4-pentanediol) as cryoprotectant. The crystals diffracted to 1.43 ångström (Å) resolution at the beamline ID30A-3 European Synchrotron Radiation Facility (ESRF, Grenoble, France). Datasets were collected and processed with XDS (38).

Structure determination and refinement

The structure of BmpD was solved by molecular replacement with Phaser (39) using *T. pallidum* PnrA (PDB entry, 2FQY (18)) as search model, without a ligand. The model building of the amino acid residues corresponding to BmpD was performed in Coot (40) and the automated refinement cycles were carried out using *phenix.refine* (41). Additional electron density for adenosine as validated by mass spectrometric analysis was observed in the substrate-binding cleft in the initial refined model. The adenosine coordinates of the 2FQY structure (18) were further added to the model and included in the next refinement steps. The refinement statistics for the final refined model are listed in Table 1.

Preparation of ligand-free BmpD

To remove the endogenously bound ligand, rBmpD was denaturated and refolded (18). *E. coli* BL21 (DE3)pLys cells expressing rBmpD were lysed, centrifuged and the supernatant containing rBmpD was allowed to adhere to Ni-NTA agarose as described above. Then, the Ni-NTA bound protein was denaturated with 10 ml of buffer A (8 M urea/100 mM Tris-HCl; pH 8.5) at RT for one hour, washed with 20 ml of buffer A, 20 ml buffer A diluted 1:1 and 1:3 with buffer B (20 mM Tris-HCl/20 mM NaCl/20 mM imidazole, pH 8.5), and with 20 ml buffer B, and finally refolded by
incubating with 10 ml of buffer B at RT for one hour. The refolded protein was eluted with 5 ml of buffer C (20 mM Tris-HCl/ 20 mM NaCl/ 200 mM imidazole, pH 8.5), concentrated, and purified by SEC as described above. The refolded protein was designated as ligand-free BmpD (LF-rBmpD).

**Liquid chromatography-mass spectrometry analysis**

The rBmpD and LF-rBmpD samples were heated, centrifuged and the supernatant was directly used for liquid chromatography-mass spectrometry (LC-MS) analysis with an Agilent 1100 series liquid chromatography system. The analytical method was modified from the method described by Ren and colleagues (42). Separations were conducted using gradient elution on a SunFire™ C18 analytical column (2.1 × 150 mm, particle size 3.5 µm, Waters, Milford, MA, USA). Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient conditions were 5% of B (0–12 min), from 5% to 80% (12–13 min), 80% (13–18 min), from 80% to 5% (18–18.5 min) and 5% (18.5–25 min). The flow rate was 0.25 ml/min. Retention times for adenosine and inosine were 4.7 and 7.7 min, respectively (Figure 4A).

Mass spectrometric detection was performed in selected-ion monitoring mode (SIM) with a single quadrupole mass spectrometer (HP 1100 LC/MSD). Ionization was based on electrospray ionization in positive mode. The capillary voltage was 4.0 kV and the drying gas temperature 350 °C. The selected ions for adenosine and inosine were m/z 268.0 and 269.0, respectively (Figure 4A). These masses correspond to their protonated molecules [M+H]^+. Adenosine was also detected as m/z 269.0 due to its isotopic distribution.

**Microscale thermophoresis**
The binding of nucleosides to LF-rBmpD was monitored with microscale thermophoresis (MST) (43). Adenosine, guanosine, inosine, xanthosine (Sigma-Aldrich, Darmstadt, Germany), and ribose (negative control ligand, Sigma-Aldrich) were mixed with LF-rBmpD (final concentration 500 nM) in a 24-point serial dilution. The concentration of the ligands ranged from 5 mM to 1.2 nM. Samples were filled into Zero Background Treated Standard Capillaries (MO-AZ002, NanoTemper Technologies, Munich, Germany) and were measured with Monolith.NT115 LabelFree equipment (NanoTemper Technologies) using 60% LED-power and medium MST-power. The data were analyzed by MO.affinity software (Nanotemper Technologies) and GraphPad Prism (version 8.0; GraphPad Software, San Diego, CA, USA). No dissociation constants are displayed as results of only one experiment are shown.

**Proteinase K-assay**

*B. burgdorferi* ss B31 in mid-logarithmic stage were washed with phosphate-buffered saline (PBS) containing 5 mM MgCl$_2$, diluted to 2x10$^8$ bacteria/ml. In a total volume of 1 ml, 500 µl bacterial suspension were incubated with 0 or 200 µg/ml proteinase K (Sigma-Aldrich) in the absence or presence of detergent 0.05% Triton X-100 (Sigma-Aldrich) at RT for one hour. The bacteria were washed with the aforementioned buffer before analyzing the bacterial lysate samples by Western blot as described below.

**SDS-PAGE and Western blot**

The BmpD protein (0.5 µg) and bacterial lysate samples were electrophoresed though a 10% Bis-Tris polyacrylamide gel (PAGE, NuPage®, Life Technologies, Carlsbad, CA, USA) with MES running buffer containing sodium dodecyl sulfate.
The gels were either stained with Simply blue (Invitrogen, Carlsbad, CA, USA) or blotted onto a nitrocellulose membrane. The membrane was incubated at RT for one hour with polyclonal anti-BmpD serum (1:1000; custom made by Harlan Laboratories, Leicester, UK), polyclonal anti-DbpB serum (1:1000; custom made by Medprobe, Oslo, Norway), polyclonal p41 antibody (1:1000; Aviva Systems Biology, San Diego, USA), monoclonal OspA antibody (1:2500; H5332; gift from Sven Bergström, University of Umeå, Sweden) or with human serum samples (1:100) identified as *Borrelia* antibody positive (n=3) or negative (n=3) using the two-tier testing approach (44). After washing, the membrane was incubated at RT for one hour with horseradish peroxidase (HRP) conjugated goat anti-rabbit or anti-mouse IgG (1:5000 or 1:2000, Santa Cruz Biotechnology, Santa Cruz, USA) or HRP-conjugated rabbit anti-human IgG (1:1000; DAKO Agilent Technologies, Santa Clara, CA, USA). The bound antibodies were detected with WesternBright ECL HRP substrate (Advanta, San Jose, USA) and Odyssey Fc imaging system (Licor Biotechnology, Bad Homburg, Germany).

**Immunization of mice with BmpD and *B. burgdorferi* ss infection in the immunized mice**

All animal studies were approved by the National Animal Experiment Board in Finland (permission ESAVI/5507/04.10.07/2014) and performed in accordance with relevant guidelines and regulations. Four-week-old female C3H/HeN mice (Envigo, Horst, The Netherlands) (n=14) were immunized subcutaneously with 50 µg BmpD with TiterMax Gold adjuvant (Sigma-Aldrich). The control mice (n=15) were mock immunized with adjuvant only. Mice received one booster dose at day 21. The serum samples were obtained from tail veins at day 14 and by cardiac puncture at day 28.
For passive immunization studies, four-week-old female C3H/HeN mice (Envigo) were intravenously injected with 5 ml/kg mouse serum containing anti-BmpD antibodies, 5 ml/kg serum from mock immunized mice or 50 µl saline as a control pretreatment. After 48 hours, the BmpD immunized mice (n=12), mock immunized mice (n=12) and positive control mice (n=10) were infected with $10^5$ B. burgdorferi ss B31. The negative control mice (n=4) received 100 µl PBS. The course of infection was followed by collecting ear biopsy samples at days 7, 11, 14, and 21 post-infection, and measuring the lateral diameter of the hind joints in a blindfolded manner once a week. After 28 days post-infection, ear, bladder, heart, joint were collected for Borrelia culture and quantitative PCR (qPCR) analyses as described earlier (45, 46) and the serum for serological analyses.

**BmpD and Borrelia WCS serology of mouse samples**

IgG antibodies towards BmpD recombinant protein and Borrelia whole-cell sonicate (WCS) in the mouse serum samples were measured by enzyme-linked immunoabsorbent assay (ELISA) as described before (45). Briefly, wells were coated with 10 µg/ml of purified rBmpD or 20 µg/ml Borrelia WCS. After serum sample (1:100) incubation, bound IgG was detected by HRP-conjugated goat anti-mouse IgG (1:8000, Santa Cruz Biotechnologies) with ortho-phenylene-diamine substrate (OPD; Kem-En-Tec Diagnostics A/S, Taastrup, Denmark). The reaction was stopped with 0.5 M H$_2$SO$_4$. The results are expressed as OD$_{492}$ values, and the samples were measured in duplicates.

**Statistical analyses**

The data of qPCR results and serology with continuous variables of non-normality were analyzed with the Kruskal-Wallis test. Data are presented as bars representing
the medians and with range indicating the minimum and maximum of each study group. *P*-values of the comparisons were corrected using Steel-Dwass method for multiple comparisons. *P*-values below 0.05 were considered as statistically significant. Statistical analyses were performed using JMP Pro (Version 13.11, SAS Institute Inc., Cary, USA).

*Homology modeling of BmpD bound to inosine*

Inosine was modeled in the BmpD structure based on the inosine-PnrA complex structure (PDB ID: 2fqw,(18)).

*Accession number*

The coordinates for the crystal structure of *B. burgdorferi* ss B31 BmpD have been deposited in the Protein Data Bank (http://www.rcsb.org/) with the accession code 6SHU.

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**Figures legends**

**Figure 1.** ABC transporter systems consist of two transmembrane domains (TMD), two nucleotide-binding domains (NBD) and a substrate-binding protein (SBP). (A) General function of ABC transporter systems. The SBP, with the substrate molecule (purple), binds to the TMDs, which open in an ATP-dependent manner and allow the passage of the substrate through the cell membrane. (B) Genome organization of the *Borrelia* ABC transporter components.
**Figure 2.** Expression and purification of Basic membrane protein D (BmpD). (A) The amino acid sequence of BmpD of *Borrelia burgdorferi* sensu stricto (ss) B31. The amino acids marked in red indicate the signal peptide sequence not included in the recombinant BmpD (rBmpD). Detection of rBmpD by (B) Simply Blue staining and by (C) Western blot. Lanes: (1) Untransformed E. coli BL21 (DE3) pLys cells; (2) cells expressing BmpD without induction; (3) purified rBmpD (39 kDa) and (4) purified ligand free rBmpD (LF-rBmpD; 37 kDa). The molecular weight markers (MW) indicate protein size in kilodaltons (kDa). Chromatograms of (D) rBmpD and (E) LF-rBmpD after purification by size exclusion chromatography. The elution volume of the protein of correct size is indicated above the corresponding peak.

**Figure 3.** (A) Structure of *B. burgdorferi* ss B31 BmpD with adenosine solved by X-ray crystallography. The beta sheets in the center of each domain are shown in purple (1–12) and the surrounding alpha helices in light pink (A-J). The ligand (adenosine) is shown as white sticks in the cleft between the domains and the three loops (1–3) connecting the two domains are shown above the ligand. The two loops contributing to the binding site differences are shown in the close-up.

(B-E) Ligand-binding site comparison of BmpD (B), PnrA structure with adenosine (PDB ID: 2fqy) (C), model for BmpD-inosine complex (D), and PnrA structure with inosine (PDB ID: 2fqw) (E). All potential hydrogen bonds are shown. The interactions of the base part of the nucleosides are highlighted since the ribose part forms identical interactions in all the structures. Adenosine is shown as white sticks and inosine in wheat. Water molecules are shown as spheres (1-7), lighter colored...
spheres are completely conserved and darker spheres differs between the structures. Phe176 (BmpD) and Phe186 (PnrA) are omitted for clarity.

**Figure 4.** Detection of bound ligand of BmpD by liquid chromatography-mass spectrometry (LC-MS). The LC-MS chromatograms of (A) the standard solution containing both adenosine and inosine with retention times of 4.7 and 7.7 minutes, respectively, (B) the adenosine bound to rBmpD in native conditions and (C) the residual adenosine bound to LF-rBmpD after denaturation and refolding.

**Figure 5.** Ligand-binding properties of LF-rBmpD analyzed by label-free microscale thermophoresis. The binding curves are representative dose-response curves of one measurement of adenosine (black squares) and inosine (gray triangles) binding to LF-rBmpD. The concentration of LF-rBmpD was constant at 500 nM, while the concentrations of the ligands varied from 1.2 nM to 5 mM. Ribose (open spheres), the negative control, did not produce a binding curve.

**Figure 6.** Cellular localization of BmpD in *B. burgdorferi* ss B31. Detection of BmpD, Decorin binding protein B (DbpB), Outer surface protein A (Ospa), and flagellin by Western blot after incubating bacterial cells with proteinase K at concentrations of 0 or 200 µg/ml in the absence (DT-) or presence (DT+) of the detergent triton X-100 (lanes 1–4).

**Figure 7.** BmpD immunogenicity in human patients and in mice.
(A) Antibodies towards purified rBmpD (0.5 µg) were detected by Western blot in serum samples of confirmed Lyme borreliosis (LB) patients (posLB; n=3) and non-LB patients (nonLB; n=3). The correct size of BmpD (39 kDa) is shown with anti-BmpD staining as a control in the first blot on the left. The signals in the size range of 50–80 kDa originate from *E. coli* proteins. The molecular weight marker (MW) indicate the protein size in kilodalton (kDa).

(B) Levels of IgG towards BmpD in mouse serum samples after BmpD immunization at 14 and 28 days after first immunization measured by ELISA. The data are expressed as OD$_{492}$ values and are presented as bars with median and range of IgG antibody levels in each study group.

Figure 8. Passive immunization of mice with serum containing BmpD antibodies.

(A) The weekly progression of the joint swelling in mice of the different study groups. The data are expressed as the mean diameters of the joints of all mice per study group.

(B) The *Borrelia* load in tissue samples of mice in the different study groups analyzed by qPCR. The bars on the left of the dotted vertical line indicate results of the ear biopsy samples at different time points (d=days), and on the right, results from tissue samples collected at the end of the study. The data are expressed as the number of bacterial genome copies per 100 ng extracted DNA. The bars indicate the median with range of each study group. The Kruskal-Wallis test and the Steel-Dwass post-hoc test were used for statistical analyses. A $P$ value below 0.05 is considered statistically significant.

(C) The levels of IgG antibody towards rBmpD and *Borrelia* whole-cell sonicate (WCS) in mouse serum samples in different study groups measured by ELISA. The
data are expressed as OD$_{492}$ values and are presented as bars with median antibody levels and range in each study group. The Kruskal-Wallis test was used for statistical analyses. A $P$ value below 0.05 is considered statistically significant.

**Figure 9.** (A) 2D structures of nucleobases, nucleosides, and nucleotides (nucleoside monophosphates). The atom numbering convention of nucleobases is shown in the adenine structure. (B) Schematic view of the purine salvage pathway in *Borrelia*. 1. A nucleotidase converts nucleotides (i.e. GMP, IMP, and AMP) into nucleosides (guanosine, inosine, and adenosine) by removing the phosphate. 2.–3. The nucleosides are then transported through the outer membrane into the periplasmic space where they bind to substrate-binding proteins, like BmpD. 4. The substrate-binding protein transports the nucleoside to a membrane-bound ABC-transporter system (BB0677–79) where it is transported into the cytoplasm. 5. Inside the cytoplasm, deoxynucleotidase kinase (BB0239) adds a phosphate to the nucleoside, reforming a nucleoside monophosphate. Then, adenylate kinase (BB0417) and nucleoside diphosphate kinase (BB0463) add further phosphates, forming first nucleoside diphosphates and then finally nucleoside triphosphates (ATP, GTP) which are incorporated into RNA.
A

SBP

TMD

NBD

TMD

NBD

ATP \rightarrow ADP + Pi

B

bmpB bb0382 → bmpA bb0383 → bmpC bb0384 → bmpD bb0385

rbsA bb0677 → bb0678 → bb0679

- Substrate-binding protein (SBP)
- ATP-binding protein/Nucleotide-binding domain (NBD)
- Permeases/Transmembrane domains (TMD)
A BmpD of *Borrelia burgdorferi* ss B31
MLKKVVYFLFLFLVACSSDDGKSEAKTVSLVDGAFDDKGFNESSSSKAIRKLKADLNINIIEKASTGN
SYLGDIANLEDGNSNLIGIIGFRLSDILFQRASENSVSVNYAIIEGVYDEIQIPKNLLNIISFRSEEVAFLA
GYFASKASKTGFVGGVRVKLESFMYGEAGAKYANSNIKVVSQYVGTGDFGLGRSTASNMYRDGV
DIIFAAQSLGIGVIEAAKELGPDHYIIGVDDQDSYLAPNVIVSAVKVDLSMYSLTALKYLETGVLDGG
KTMLGLKEGLGLNLENLNSNYSEIYNKSLIGQSIMINGIKVYPDKVSYDNFVLQMN

B

C

D

E

![Image of gel electrophoresis and chromatograms](image-url)
Table 1. Data collection and refinement statistics\(^a\).

| Parameter                                    | Value                       |
|----------------------------------------------|-----------------------------|
| **Diffraction source**                       | ID30A/massif-3 - ESRF       |
| **Detector**                                 | Eiger                       |
| **Wavelength**                               | 0.9677                      |
| **Resolution range**                         | 30.7–1.43 (1.481–1.43)      |
| **Space group**                              | C 1 2 1                     |
| **Unit cell**                                | 106.78 42.9 66.38 90 117.339 90 |
| **Total reflections**                        | 343017 (12968)              |
| **Unique reflections**                       | 44953 (2652)                |
| **Multiplicity**                             | 7.6 (4.9)                   |
| **Completeness (%)**                         | 0.91 (0.51)                 |
| **Mean I/\sigma(I)**                         | 19.35 (3.86)                |
| **Wilson B-factor**                          | 11.44                       |
| **R-meas**                                   | 0.06703 (0.3546)            |
| **CC1/2**                                    | 0.999 (0.915)               |
| **Reflections used in refinement**           | 44944 (2652)                |
| **Reflections used for R-free**              | 2193 (154)                  |
| **R-work**                                   | 0.1538 (0.1856)             |
| **R-free**                                   | 0.1792 (0.2163)             |
| **Number of non-hydrogen atoms**            | 2889                        |
| **Protein**                                  | 2459                        |
| **Ligand**                                   | 19                          |
| **Ion**                                      | 2                           |
| **Water**                                    | 409                         |
| **RMS (bonds)**                              | 0.005                       |
| **RMS (angles)**                             | 0.80                        |
| **Ramachandran favored (%)**                 | 96.6                        |
| **Ramachandran allowed (%)**                 | 3.4                         |
| **Ramachandran outliers (%)**                | 0                           |
| **Average B-factor**                         | 17.28                       |
| **Protein**                                  | 14.94                       |
| **Ligand, ion**                              | 10.36                       |
| **Water**                                    | 31.76                       |

\(^a\) Statistics for the highest-resolution shell are shown in parentheses.
| Study group (n)       | Days post-infection | At the end of the study |
|-----------------------|---------------------|------------------------|
|                       | 7                   | 11                     | 14 | 21 | Ear | Heart | Bladder | Joint | Any tissue |
| Negative control (4)  | 0/4                 | 0/4                    | 0/4 | 0/4 | 0/4 | 0/4   | 0/4     | 0/4   | 0/4         |
| Positive control (10) | 0/10                | 6/10                   | 8/10 | 10/10 | 9/10 | 8/10  | 10/10   | 10/10 | 10/10       |
| Mock immunized (12)   | 0/12                | 6/12                   | 12/12 | 12/12 | 11/12 | 12/12  | 12/12   | 12/12 | 12/12       |
| BmpD immunized (12)   | 0/12                | 6/12                   | 9/12 | 12/12 | 12/12 | 12/12  | 12/12   | 12/12 | 12/12       |

Table 2. Number of positive *Borrelia* cultures among all studied tissues samples of mice by study group.
