Published in International Journal of Medical Microbiology 297, issue 2, 97-107, 2007
which should be used for any reference to this work

Complementation of a *Borrelia afzelii* OspC mutant highlights the crucial role of OspC for dissemination of *Borrelia afzelii* in *Ixodes ricinus*

Volker Fingerle[a,1], Gereon Goettner[a,1], Lise Gern[b], Bettina Wilske[a], Ulrike Schulte-Spechtel[a,*]

[a]*Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, der Ludwig-Maximilians-Universität München, National Reference Center for Borreliae, Pettenkoferstr. 9a, D-80336, Munich, Germany*
[b]*Institute of Zoology, University of Neuchatel, Neuchatel, Switzerland*

Abstract

Alteration of the outer surface protein (Osp) composition – especially that of OspA and OspC – seems to be important for the adaptation of *Borrelia burgdorferi* sensu lato to its endothermic hosts (mammals) and poikilothermic vectors (ticks). OspA possibly mediates adherence to tick midgut cells thus enabling the borreliae to survive in the vector, while OspC is associated with borrelial invasion of the tick salivary glands and infection of the mammalian hosts. Here we describe the first successful transformation and complementation of a *Borrelia afzelii ospC* mutant with the wild-type *ospC* in trans. To test the influence of OspC on the dissemination behavior in ticks, unfed *Ixodes ricinus* nymphs were artificially infected by capillary feeding either with *B. afzelii* wild type, the *B. afzelii ospC* mutant or the *ospC*-complemented clone. Tick midguts and salivary glands were investigated after different time intervals for the presence of borreliae and for OspA and OspC by immunfluorescence staining with monoclonal antibodies. While the *B. afzelii* wild-type strain exhibiting abundant OspC on its surface disseminated to the salivary glands, the OspC-negative mutant was only present in the tick midguts. The *ospC*-complemented clone which constitutively expresses the wild-type *ospC* was again able to colonize the salivary glands. This finding demonstrates that OspC is crucial for dissemination of *B. afzelii* from the tick midgut to the salivary glands, a prerequisite for infection of the warm-blooded host. A summary of the detailed data presented here has already been given in Goettner et al. [2006. OspC of *B. afzelii* is crucial for dissemination in the vector as shown by transformation and complementation of a European OspC-deficient *B. afzelii* strain. Int. J. Med. Microbiol. 296S1(Suppl. 40), 122–124].

Keywords: *Borrelia afzelii*; Transformation; OspC deficiency; Complementation; Tick

Introduction

Lyme borreliosis caused by spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex is the most common vector-borne disease in the United States and Europe. The *B. burgdorferi* s.l. complex comprises at least three human-pathogenic species in Europe, *B. burgdorferi*

*Corresponding author. Tel.: +49 89 5160 5242; fax: +49 89 5160 4757.
E-mail address: spechtel@mvp.uni-muenchen.de (U. Schulte-Spechtel).
[1]Volker Fingerle and Gereon Goettner contributed equally to this work.

sensu stricto (s.s.), the only species causing Lyme disease in the United States, *Borrelia afzelii*, and *Borrelia garinii* (Baranton et al., 1992; Burgdorfer et al., 1982; Wang et al., 1999). These spirochetes are maintained in their natural setting through complex enzootic cycles between warm-blooded hosts and ixodid tick vectors (Benach et al., 1987; de Silva and Fikrig, 1995; Gern et al., 1990; Leuba-Garcia et al., 1998; Ribeiro et al., 1987; Schwan and Piesman, 2000). During tick feeding, *B. burgdorferi* migrates through the gut wall, disseminates and invades various tissues, including the salivary glands wherefrom they are transmitted to the host by saliva. During borrelial dissemination from vector to host, the spirochetes have been shown to differentially regulate several lipoproteins possibly in response to the rapid environmental changes (Fingerle et al., 1998, 2002; Leuba-Garcia et al., 1998; Schwan and Piesman, 2000). In particular the outer surface protein (Osp) C is upregulated with rising temperature and decrease of pH (Carroll et al., 1999; Schwan and Piesman, 2000) and seems to be a crucial factor in the initial events of the infection process. However, the precise function of this protein in *B. burgdorferi* still remains to be elucidated. Genetic analyses of bacterial pathogens are an important tool for identification and characterization of factors involved in pathogenesis. To ascertain the role of certain gene products for disease, the Koch's molecular postulates have to be applied (Falkow, 1988). In *B. burgdorferi* s.s., the recent identification of erythromycin and kanamycin resistance as useful genetic markers, the isolation of null mutants, and the development of extrachromosomal cloning vectors allow first definitions of specific gene functions (Bono et al., 2000; Sartakova et al., 2000; Stewart et al., 2001). Concerning *ospC*, two recent studies based on genetically manipulated *B. burgdorferi* s.s. in the American vector *Ixodes scapularis* were contradictory. While in one study it was shown that *B. burgdorferi s.s.* requires OspC for infection of mice but not for dissemination to the tick salivary glands (Grimm et al., 2004), the other study showed that OspC facilitates the dissemination of the borreliae from the midgut to the salivary glands (Pal et al., 2004b).

In the present study, we wanted to elucidate the role of OspC for the European species *B. afzelii* in the European vector *Ixodes ricinus.* We previously have described a *B. afzelii* clone lacking the OspC protein because of a frame-shift mutation in the corresponding gene, which had lost its ability to disseminate from the midgut to the salivary glands of *I. ricinus* (Fingerle et al., 2000, 2002). After a positive preliminary test to transform *B. afzelii* with green fluorescent protein (GFP) as a confirmatory marker, we succeeded to complement the *ospC B. afzelii* mutant to subsequently investigate the borrelial dissemination behavior in capillary-fed *I. ricinus*. Our results presented herein indicate that OspC is required for dissemination of *B. afzelii* from the *I. ricinus* midgut to the salivary glands (Goettner et al., 2006).

Materials and methods

Ticks

All of the nymphal *I. ricinus* ticks used in this study were derived from a colony maintained at the Institute of Zoology, University of Neuchâtel, Neuchâtel, Switzerland. The laboratory-reared ticks had been free of *B. burgdorferi* s.l. infection for at least two generations. The nymphs were kept shaded at 10 °C and 95% relative humidity and were used not before 2 months post larval ecdysis at the earliest for the experiments.

Borrelia strains and clones

The OspC-negative *B. afzelii* clone PKo345 (cPKo345^{ospC-}) was derived by triple-colony selection of a reisolate from a joint of a gerbil, infected with low-passage *B. afzelii* strain PKo (a human skin isolate) (Fingerle et al., 2000; Wilske et al., 1988). As described previously, cPKo345^{ospC-} has an insertion of a guanine in the *ospC* gene at position 200, leading to a frame-shift mutation with a stop codon after position 222. This mutant does not produce OspC (Fingerle et al., 2000). A low-passage *B. afzelii* PKo wild-type strain producing OspC in culture served as positive control.

Strains and clones were cultured as previously described to a density of 10^7/ml in MKP medium at 33 °C (Preac-Mursic et al., 1991). For cultivation of the transformed cPKo345^{ospC+}, 50–200 µg/ml kanamycin was added to the media.

PCR, construction of pBSV2 derivatives, and transformation of *Escherichia coli*

Extraction of DNA of the *B. afzelii* wild-type strain PKo was performed with the High Pure Template Preparation Kit (Roche; Penzberg, Germany) according to the manufacturer's instructions and was used as template for PCR. o*spC* was amplified using standard primers from our laboratory as described previously with additional NdeI and HindIII restriction sites (underlined) (Schulte-Spechtel et al., 2006): *ospC-for* (5′-TAGTAGCATATGAAAAAGAATACATTAAG-TGCG-3′) and *ospC-rev* (5′-TAGGAGAAGCTTTTA-AGGTTTTTTTGGACTTTCTGC-3′).

The *gfp* gene was amplified from the pcDNA3.1/CT-GFP-TOPO vector (Invitrogen, Carlsbad, CA, USA) with the following primers (NdeI and HindIII restriction sites underlined): *gfp-for* (5′-TAGTAGCATATG-CGTAAAGGAGAAGAACTTTTC-3′) and *gfp-rev*

(5′-TAGTAGAAGCTTTTATTTGTATAGTTCATC-CATGCC-3′).

The pBSV2 vector derived from LGC (Teddington, England) was used as template for amplification of the *flg* promoter (P_{flg}). Forward and reverse primers of P_{flg} were constructed according to the sequence found in Genebank (accession no. U71301) (Ge and Charon, 1997), and BamHI and NdeI restriction sites (underlined) were added: *P_{flg}-for* (5′-TAGTAGGGATCC-TACCCGAGCTTCAAGGAAGATTTC-3′) and *P_{flg}-rev* (5′-TAGTAGCATATGATGGAAACCTCCCT-CATTTAAAATTGC-3′).

The amplicons of the *flg* promotor, *gfp* and *ospC* were digested with restriction endonucleases BamHI, NdeI, and HindIII (Boehringer, Mannheim, Germany), respectively, according to the manufacturer's instructions. The upstream promotor was ligated with *gfp* or *ospC*, respectively, using the NdeI restriction site. These products were ligated into the multiple cloning site (BamHI, HindIII) of pBSV2 using T4 DNA ligase (Invitrogen). After ligation, competent *E. coli* XL1 blue were transformed. Plasmid DNA was extracted with the Plasmid Maxi Purification Kit (Qiagen, Hilden, Germany) and correct cloning was checked by sequencing.

Transformation of *B. afzelii*

B. afzelii, PKo wild-type strain, and cPKo345 $^{ospC-}$ were cultured to a density of 10^7/ml in 500 ml MKP medium at 33 °C (Preac-Mursic et al., 1991) and concentrated in transformation buffers as described previously (Samuels et al., 1994). Finally the borreliae were incubated with 10–20 µg of pBSV2/P_{flg}-*gfp* for 2 min at 50 °C, an interim 1-min incubation on ice, followed by electroporation with 2.5 kV, 25 µF, and 200 Ω, producing a time constant of 4–5 ms. One ml of MKP medium was immediately added to the cuvettes and cells were transferred to 10-ml culture tubes with 7 ml MKP medium and allowed to recover at 33 °C for 24 h. Thereafter, kanamycin was added to a final concentration of 50 µg/ml. Transformed *B. afzelii* were grown to a density of 10^7 cells/ml. Then the borreliae were transferred to fresh MKP medium with 200 µg/ml kanamycin. Successful transformation was confirmed by fluorescence microscopy with a drop of borrelial cultures (see below). For the complementation experiments cPKo345 $^{ospC-}$ was transformed with pBSV2/P_{flg}-*ospC* using the described protocol.

Western blot

For detection of OspC by Western blot, whole-cell lysates of *B. afzelii* PKo wild type, cPKo345^{ospC-}, and the transformed cPKo345^{ospC+} were separated by SDS-PAGE (12.5% polyacrylamide gel) and transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell, Dassel, Germany) as described previously (Hauser et al., 1997). Equal loading was controlled by staining with Ponceau-S solution. After destaining and blocking, the membrane was incubated with the OspC-specific monoclonal antibody (MAb) 1F10 (IgG2a subclass) (Wilske et al., 1993) at room temperature overnight. Immunocomplexes were detected by horseradish peroxidase-labeled anti-mouse IgG antibodies purchased from Dakopatts (Glostrup, Denmark) with diaminobenzidine as chromogenic substrate.

Pulsed-field gel electrophoresis (PFGE) and Southern blot

B. afzelii were grown to a density of 10^7/ml, harvested, washed in phosphate-buffered saline/$MgCl_2$ and lysed in agarose blocks (1.0% ultra pure agarose) as described previously (Busch et al., 1996). Plasmids were separated by PFGE on a Bio-Rad Chef-DRII system at constant voltage (120 V/gel) for 24 h with a switch time of 0.5–3.0 s in 0.5 × Tris–borate–EDTA buffer (pH 8.3) at 14 °C. DNA was transferred to a Hybond-N membrane (Nylonbind B, Serva, Heidelberg) and UV cross-linked for 45 s (GATC-crosslink; Gesellschaft für Analysetechnik und Consulting, Konstanz). For the detection of pBSV2 derivatives in transformed borreliae, the kanamycin resistance gene PCR fragment was used as probe (after amplification of pBSV2 as template with the primers *kan-for*: 5′AGCCATATTCAACGG-GAAACG3′ and *kan-rev*: 5′TTAGAAAAACTCAT-CGAGCATCAAATG3′). Probes were labeled with digoxigenin using a Dig DNA Labeling Kit (Roche, Mannheim). Prehybridization and hybridization were done in the same solution (5 × SSC, 0.1% laurylsarcosine, 0.02% SDS, 1% blocking solution (salmon sperm) at 42 °C and accordingly at 55 °C. Probes were used at a concentration of 100 ng/ml. Blots were then washed (two washes for 5 min at 25 °C with 2 × SSC + 0.1% SDS and then two washes for 15 min each at 55 °C with 0.1 × SSC + 0.1% SDS) and visualized: membrane was washed 1 min in malate buffer (100 mM maleic acid, 150 mM NaCl), 30 min in the same buffer with 1% blocking solution, 1 min in malate buffer, 30 min anti-digoxigenin F_{ab} fragment (Roche) 1:5000, two washes for 15 min with malate buffer followed by incubation with Eco Blot® substrate (BIOSENS, Oberhaching, Germany) overnight. All steps were carried out at 25 °C.

Capillary feeding and preparation of nymphs

Nymphal ticks were artificially infected with the PKo wild-type strain, cPKo345^{ospC-} and the transformed cPKo345^{ospC+} by the capillary feeding method as previously described (Fingerle et al., 2002; Gern et al.,

1990). In short, the ticks were adapted to room temperature 1 h before start of the experiments and allowed to feed for 3–4 h at room temperature in a humid chamber on capillaries containing MKP with 10^7–10^8 borreliae/ml. Indications of successful feeding were the excretion of droplets via the anus and a more transparent appearance of the tick body and midgut. The infected nymphs were kept at room temperature in a chamber at 95% relative humidity until dissection at different time intervals after feeding (0, 12, 24, 48, 96 h). Dissection of ticks and preparation of midgut and salivary glands were performed as previously described (Fingerle et al., 2002).

Immunofluorescence assay (IFA) and microscopy

The IFA protocol for simultaneous staining of OspA and OspC was essentially as described previously (Fingerle et al., 2002). OspA-specific MAb 14G7 (IgG1 subclass) and OspC-specific MAb 1F10 (IgG2a subclass) both derived from mouse were used for protein detection in all IFAs (Wilske et al., 1993, 1996). To differentiate between the two Osp-specific MAbs a mixture of a fluorescein isothiocyanate (FITC)-conjugated antibody to mouse IgG1 and a Cyanine dye (Cy3)-conjugated antibody to mouse IgG2a (both from Caltag Laboratories, Burlingame, CA, USA) were used in a final dilution of 1:100 each. The slides were finally incubated for 60 s with 4′,6′-diamidino-2-phenylindole (DAPI) in a dilution of 1:10,000 to visualize all borreliae. The entire individual spots of the IFAs were examined under the microscope as described in detail previously (Fingerle et al., 2002) and all spirochetes found in the smears of midguts and salivary glands, respectively, were counted.

Results

Transformation of *Borrelia afzelii* and complementation of the *B. afzelii ospC* mutant

Our main goal was to complement the *B. afzelii ospC* mutant with its original *ospC* gene to investigate the behavior of these mutants in ticks. For this purpose, we first created two derivatives of the shuttle vector pBSV2, one containing the *gfp* gene (pBSV2/P_{flg}-*gfp*) and the other containing the *ospC* gene from *B. afzelii* PKo wild type (pBSV2/P_{flg}-ospC) (Fig. 1b). As *gfp* and *ospC* are under the control of the borrelial P_{flg}, these genes are supposed to be expressed constitutively in the borreliae. At first we tried to transform the *B. afzelii* wild-type strain PKo and the OspC-negative clone cPKo345^{ospC-} with pBSV2/P_{flg}-*gfp*. In first transformation experiments, no *B. afzelii* transformants were obtained using

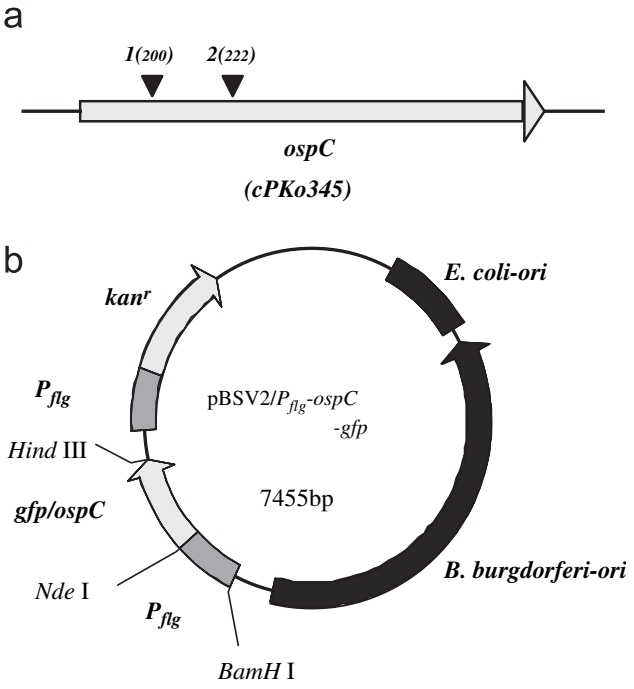

Fig. 1. Mutation and complementation of the *ospC* gene in the *B. afzelii* clone PKo345. (a) Spontaneous mutation in the *ospC* gene: 1, insertion of guanine; 2, premature stop codon. Nucleotide positions are indicated in parentheses. (b) Diagram of the shuttle vectors used for transformation of *B. afzelii* wild type PKo and mutant clone PKo345.

the protocol for transformation of the *B. burgdorferi* s.s. strain B31 as described by Stewart et al. (2001). To develop a successful procedure, we tested a number of alterations in which it turned out that an additional heat shock at 50 °C for 2 min was crucial for successful transformation of *B. afzelii* wild-type strain PKo as well as for clone cPKo345^{ospC-}. Using this modified procedure kanamycin-resistant transformants could be seen after 6–8 weeks cultivation in liquid culture under a kanamycin concentration of 50 µg/ml. After a cell density of 10^7/ml had been reached it was possible to raise the kanamycin concentration up to 200 µg/ml. Under the microscope it could be seen that nearly 100% of the borreliae were covered with GFP on their surface.

For the complementation of the defect *ospC* gene, cPKo345^{ospC-} was transformed with pBSV2/P_{flg}-*ospC*. Transformants could be confirmed by PCR amplification of the kanamycin resistance cassette and the P_{flg}-*ospC* insert after the third subcultivation corresponding to 10 weeks after transformation (Fig. 2a). To prove that PCR does not simply detect a vector contamination in the culture medium, a cPKo345^{ospC-} culture spiked with 20 µg plasmid pBSV2/P_{flg}-*ospC* was processed in parallel. As shown in Fig. 2a, no amplicons were obtained from this control culture when tested in

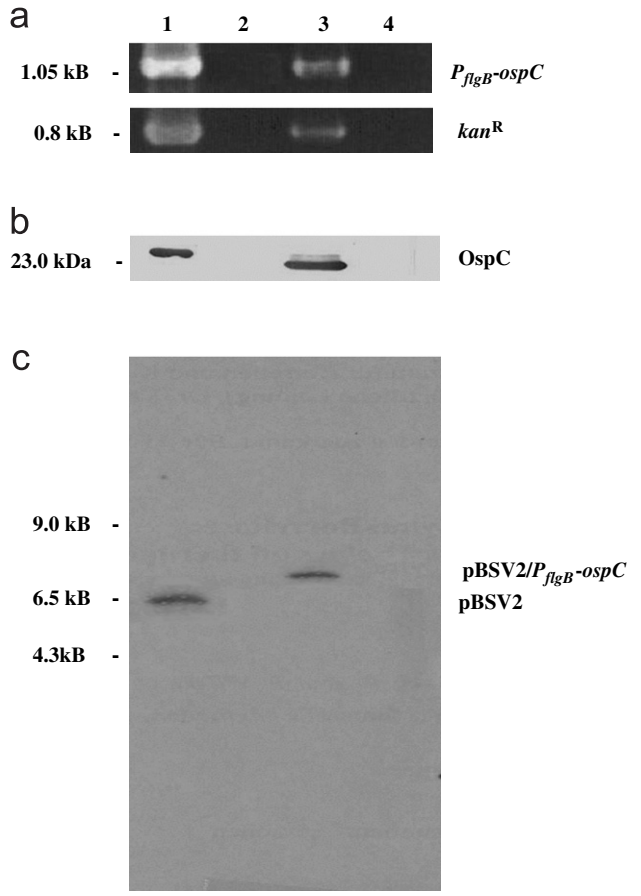

Fig. 2. Confirmation of successful transformation of *B.afzelii* and analysis of the OspC-deficient and the complemented *B. afzelii* clone. (a) PCR. Lane 1, shuttle vector pBSV2/*PflgB-ospC* as positive control; lane 2, *B. afzelii* cPKo345^{ospC-}; lane 3, *B. afzelii* cPKo345^{ospC+}; lane 4, *B. afzelii* cPKo345^{ospC-} contaminated with 20 µg of the shuttle vector pBSV2/*Pflg-ospC* when starting the experiments processed in parallel. (b) Detection of OspC with mAb 1F10 by Western blot. Lane 1, recombinant OspC protein as positive control; lanes 2–4 as in (a). (c) PFGE followed by Southern blot for detection of the shuttle vectors. Lane 1, linearized pBSV2 (BamHI) as positive control; lanes 2–4 as in (a).

parallel to the transformed clone. The transformants were also tested for OspC production by Western blot (Fig. 2b), which clearly showed OspC production of the transformed cPKo345^{ospC+}. The ability of the shuttle vector to replicate autonomously in *B. afzelii* cPKo345^{ospC+} was examined by PFGE of total genomic, BamHI-digested DNA and by Southern blot using the kanamycin resistance cassette as a probe (Fig. 2c). The unique hybridization band of the expected size in the third lane confirmed that pBSV2 efficiently replicates autonomously in cPKo345^{ospC+}. Recovery of the shuttle vector could be achieved by transforming *E. coli* XL1 blue with total genomic DNA from cPKo345^{ospC+} (data not shown). These data again indicate an autonomous replication of the shuttle vector construct pBSV2/P_{flg}-*ospC* in *B. afzelii* cPKo345^{ospC+}.

Dissemination of *B. afzelii* in *I. ricinus*

For studying the role of OspC in the dissemination process of *B. afzelii* in its vector, we used the previously described capillary feeding method for artificial infection of *I. ricinus* ticks (Gern et al., 1990). Individual groups of nymphal *I. ricinus* ticks were infected with either *B. afzelii* strain PKo, cPKo345^{ospC-}, or transformed cPKo345^{ospC+}. The infected *I. ricinus* nymphs were allowed to rest in a humid chamber until dissection, which was performed at different time intervals to assess the gut and salivary glands for the presence of stained spirochetes by IFA. Examples of midgut and salivary gland preparations with FITC-OspA- and Cy3-OspC-labeled borreliae are shown in Fig. 3. The OspC-positive *B. afzelii* PKo wild-type strain was present in the salivary glands immediately after the feeding process and throughout the investigation period (Table 1). Strain PKo showed predominantly OspC and less OspA on the surface (Fig. 4) in both, midgut and salivary glands. Notably, also only OspA-positive and borreliae that had neither OspA nor OspC were present in the salivary glands. The OspC-negative cPKo345^{ospC-} was not detectable in the salivary glands during the whole investigation period of 96 h, although this clone was always found in the midgut in high numbers (Table 1). Most of the cPKo345^{ospC-} spirochetes were only OspA positive (Fig. 4) and a minor portion had neither of the two Osps on their surface. The ability to invade the salivary glands was restored in the cPKo345^{ospC+} that was complemented in trans with the pBSV2/P_{flg}-*ospC* construct. Comparable to wild-type strain PKo, this clone was already present in the salivary glands right after feeding and throughout the investigation period (Table 1). As anticipated, the complemented mutant exhibits, similar as the wild-type strain, abundant OspC and low OspA expression. During the whole study period of 96 h, no significant changes of OspA and OspC levels in midgut or salivary glands were observed in the three investigated *B. afzelii* strains or clones.

Discussion

During its life cycle *B. burgdorferi* s.l. is confronted with two completely different, rapid changing environments: the hard tick with its ambient body temperature as vector and the warm-blooded vertebrate as host. The variety of differing factors furthermore includes pH, osmotic pressure, CO_2 tension, tick factors secreted into the blood meal, and different immune systems. Changing routinely between these environments requires

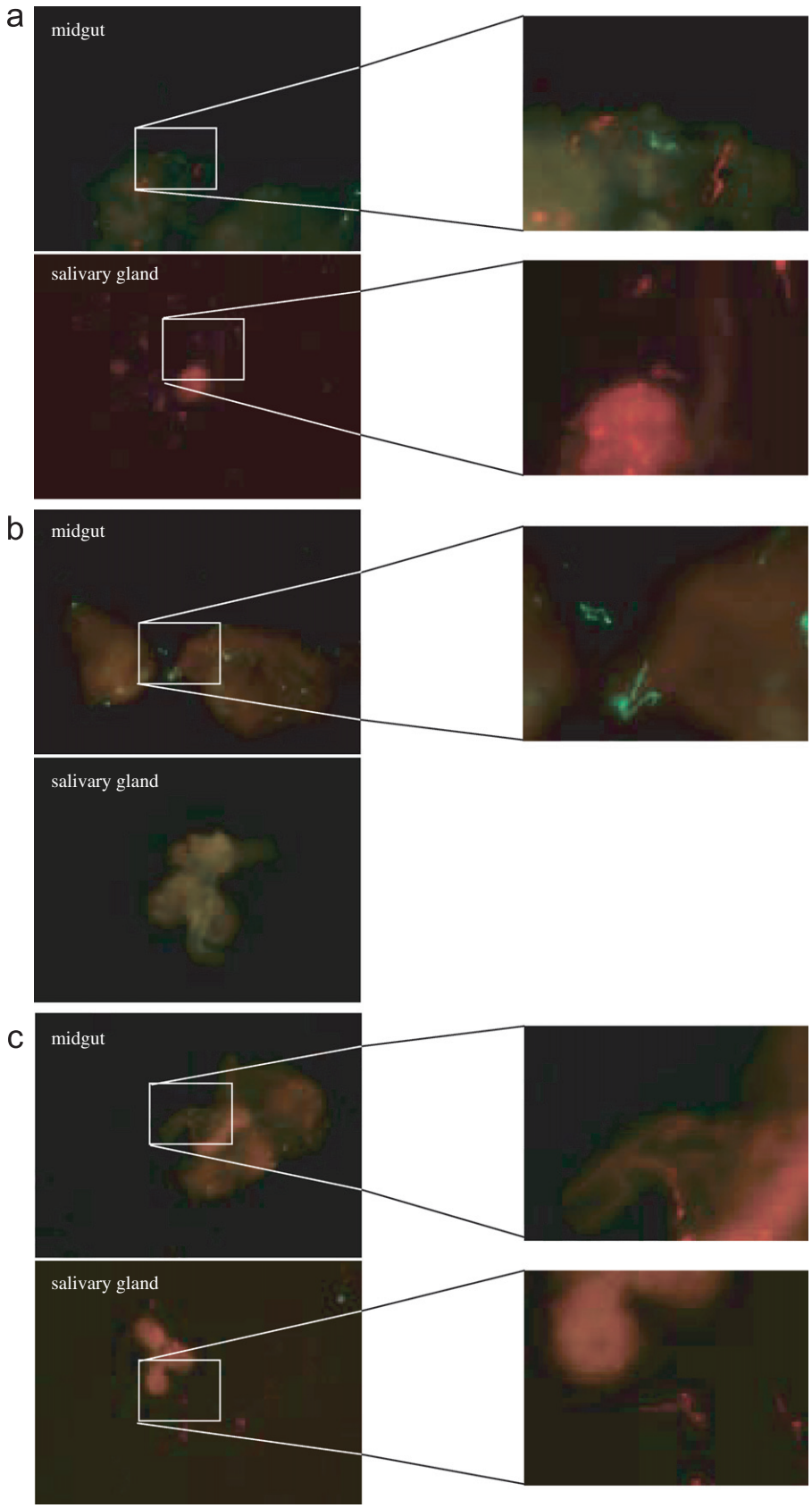

Fig. 3. *B. afzelii* detected by IFA in artificially infected *I. ricinus* nymphs: (a) PKo wild-type strain; (b) OspC- deficient cPKo345; (c) OspC-deficient cPKo345 complemented with pBSV2 carrying the *ospC* gene. *B. afzelii* proteins OspA and OspC were detected by FITC-labeled (green) and Cy3-labeled (red) secondary antibodies, respectively.

Table 1. Numbers of borreliae found in midgut and salivary glands of *I. ricinus* nymphs infected by capillary feeding

	Tick no.	Time after capillary feeding (h)									
		0 h		12 h		24 h		48 h		96 h	
		1	2	1	2	1	2	1	2	1	2
B. afzelii wild-type strain PKo	Midgut	112	88	172	316	453	>500	204	188	112	133
	Salivary glands	57	9	59	152	co[a]	387	97	122	82	141
B. afzelii cPKo345^{ospC-}	Midgut	>500	>500	>500	>500	>500	>500	184	137	145	277
	Salivary glands	0	co[a]	0	0	0	co[a]	0	0	0	0
B. afzelii cPKo345$^{pBSV2/ospC+}$	Midgut	237	121	98	197	378	>500	298	178	86	92
	Salivary glands	60	37	33	58	co[a]	308	210	121	14	27

[a]Salivary glands contaminated with midgut during dissection of the tick.

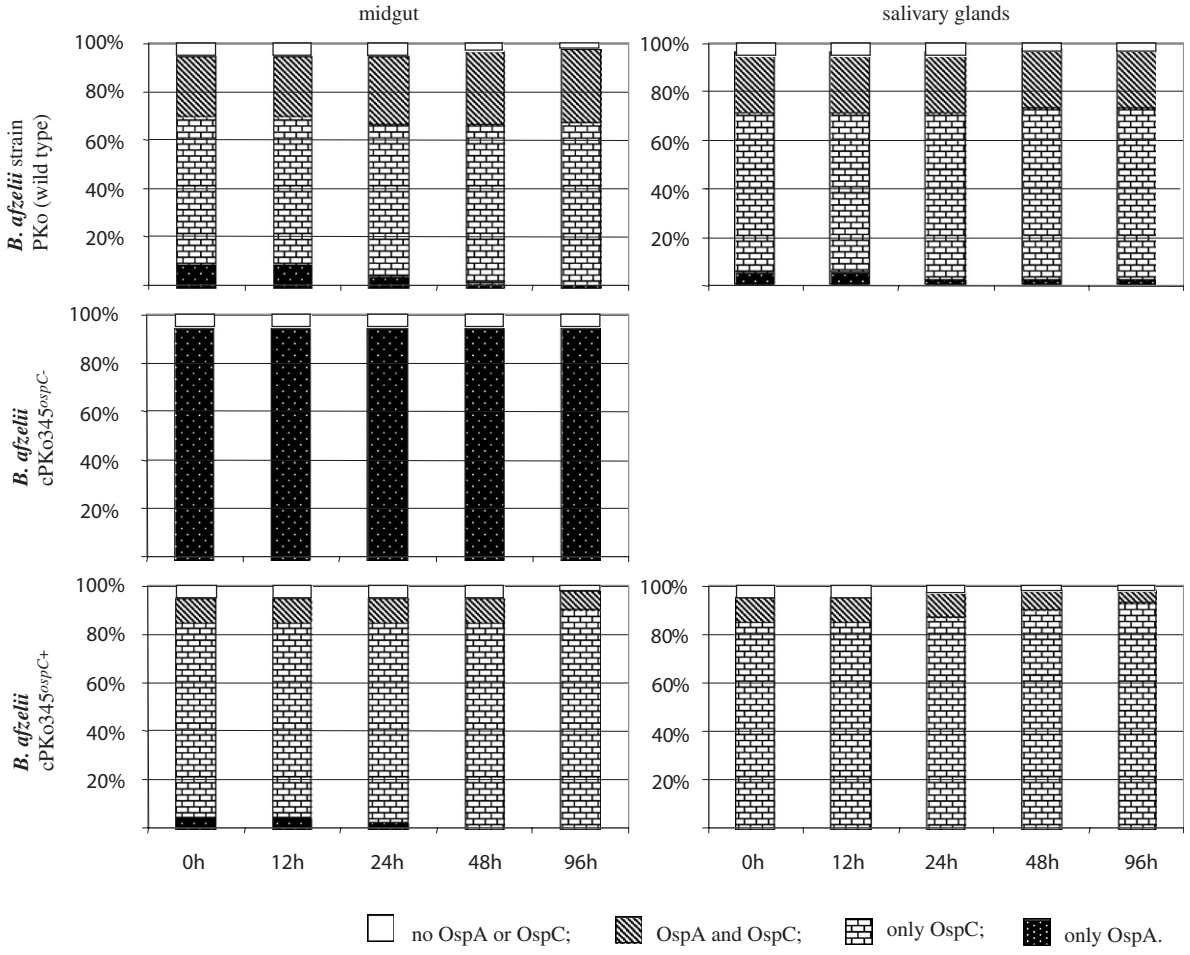

Fig. 4. Percentage of OspA- and OspC-positive borreliae in midgut and salivary glands of *I. ricinus* nymphs at different time intervals after capillary feeding. For total numbers refer to Table 1.

effective regulatory mechanisms for adaptation. Alteration of the Osp composition – especially that of OspA and OspC – seems to be crucial for this adaptation process (de Silva and Fikrig, 1995; Fingerle et al., 1995, 1998; Montgomery et al., 1996; Ohnishi et al., 2001; Schwan et al., 1995; Schwan and Piesman, 2000, 2002). In the midgut of unfed ticks the borreliae have abundant OspA on the surface, most probably mediating adherence to midgut cells and thus enabling the borreliae to survive in relative dormancy in the vector for prolonged

periods without tick feeding (Fingerle et al., 1995; Pal et al., 2004a; Schwan et al., 1995). During the next blood meal, these borreliae restart replication and upregulate OspC, which is associated with migration to the tick salivary glands and infection of the host (Fingerle et al., 1998; Gilmore and Piesman, 2000; Schwan et al., 1995; Schwan and Piesman, 2000). This protein, therefore, is thought to have a crucial role in the transmission process from the vector to the host. However, the precise function of OspC – whether it mediates the dissemination from the tick midgut to the salivary glands, or plays an important role for infection of the mammalian host, or both – is still unclear. Furthermore, the genetic heterogeneity of the *B. burgdorferi* s.l. species raises the question, whether there are differences in the infection process regarding different species or even subtypes, which might be reflected in different functions of certain proteins. To unravel such questions, genetic manipulation is widely and efficiently established in the bacterial world, especially for the fast growing *Enterobacteriaceae*. To date, genetic manipulation within the *B. burgdorferi* s.l. complex is well developed only for *B. burgdorferi* s.s., the only species causing Lyme disease in the USA, but so far not for other *B. burgdorferi* s.l. species like *B. afzelii* or *B. garinii*. Here we describe the first transformation of a European *B. afzelii* wild-type strain and the complementation of an OspC-deficient *B. afzelii* clone and show, that in this *B. afzelii* clone OspC is required for dissemination of the borreliae from the ticks midgut to the salivary glands.

For genetic manipulation of *B. burgdorferi*, significant advances have been made by the establishment of a system for the genetic manipulation of *B. burgdorferi* s.s., since Stewart et al. (2001) developed the shuttle vector pBSV2 based on the backbone of cp9 from *B. burgdorferi* s.s. strain B31. The used 3.3 kb fragment of cp9, which has been described as a cp32 deletion derivate (Casjens et al., 2000; Stevenson et al., 1996; Zuckert and Meyer, 1996), allows autonomous replication of pBSV2 in *B. burgdorferi* s.s. B31. Although the *B. afzelii* genome has been sequenced it is not known if replication and partitioning of the plasmids are comparable to those of *B. burgdorferi* s.s. (Glöckner et al., 2006). For our experiments we choose the *B. afzelii* wild-type strain PKo and the PKo clone cPKo345, an already well-characterized, OspC-deficient clone that grows well in culture. However, first transformation experiments with pBSV2/P_{flg}-*gfp* according to the protocol of Stewart et al. (2001) failed. We only succeeded when introducing a heat shock preceding transformation followed by growth of the transformants at low kanamycin concentrations for 6–8 weeks. The finally successful transformations of *B. afzelii* with pBSV2 demonstrate that the autonomous replication mechanism of this plasmid also works in *B. afzelii*. However, it still remains to be investigated whether pBSV2 could act as a universal shuttle vector for all *B. burgdorferi* s.l. species. In this context, it is also important to note that transformation procedures have to be carefully adapted, as shown in the present study, where the additional 2-min heat shock was a crucial step in the transformation of *B. afzelii* PKo. Unfortunately, it was not possible to culture any of the *B. afzelii* transformants on solid media to receive a clonal population. As nearly 100% of the observed transformed spirochetes show *gfp* or *ospC* expression in culture we assumed that the transformants were clonal. Therefore, we could not determine transformation frequencies. Beside further confirmative transformation experiments about the reproducibility of the *B. afzelii* transformation system, this would be part of additional studies. PFGE and retransformation of *E. coli* proves presence and autonomous replication of the pBSV2-derived plasmids used in our transformation experiments.

Regulation and expression of *ospC* in *B. burgdorferi s.l.* is still not completely understood on the molecular level. Therefore complementation of the mutated *ospC* gene in clone cPKo345^{ospC-} in trans with a vector using the original *ospC* promotor could not guarantee similar expression levels as in the PKo wild type because of lacking possible enhancers. For this reason, we decided to use the constitutive *flg* promotor in the complementation vector pBSV2/P_{flg}-*ospC*. A more exclusive expression of *ospC* correlated with low expression of *ospA* also reflects the situation in vivo in the tick during the blood meal. After successful complementation of *ospC* we wished to compare the dissemination behavior of PKo wild type, cPKo345^{ospC-}, and cPKo345^{ospC+} in *I. ricinus*. In a previous study, we could show by capillary feeding experiments that cPKo345^{ospC-} is able to colonize the midgut of *I. ricinus* nymphs but is unable to disseminate to the salivary glands. By contrast, an OspC-positive PKo clone rapidly disseminated to the salivary glands and was detectable there over the whole investigation period (Fingerle et al., 2002). Here we confirmed again these dissemination patterns and could show that the *ospC*-complemented clone regains the ability for dissemination to the salivary glands. These results show that the inability to colonize salivary glands of the OspC-deficient *B. afzelii* clone was due to the loss of OspC production rather than to any other potential genetic aberration. Whether or not infectivity is reinstated in the complemented mutant cPKo345^{ospC+}, remains to be investigated using infection experiments with mice and a larger number of ticks.

Two recent studies based on *B. burgdorferi* s.s. in the American vector *I. scapularis* were in part controversial (Grimm et al., 2004; Pal et al., 2004b). Both studies revealed that OspC is required for infection of the mammal. However Grimm et al. (2004) found that OspC is not required for dissemination in the tick, whereas Pal et al. (2004b) found that OspC in

B. burgdorferi s.s. is required. Possible explanations for these differences may include different dissemination behavior of different species and even strains belonging to the same species, the different modes of tick infection, and influence of different tick species. In a previous study, using capillary-fed *I. ricinus* nymphs, we showed that Osp composition patterns as well as dissemination dynamics may vary even among strains belonging to the same species (Fingerle et al., 2002). These findings together might argue for at least in part different strategies used by certain *B. burgdorferi* s.l. strains in the infection process. In the two American studies, the different outcomes may be due to the different strains used (N40 and B31, respectively). Pal et al. (2004b) infected the ticks comparable to the present study by capillary injection of the borreliae into the rectal sac, while Grimm et al. (2004) infected ticks by immersing them in a borrelial culture. Such differences might result in different dissemination abilities and subsequently in different infection patterns within the tick (e.g. systemic versus localized). In our study dissemination of borreliae was investigated after capillary feeding without a blood meal on a mammal, while in the two American studies the ticks had a blood meal prior to dissection. It is known from a study of Coleman et al. (1997) that plasmin (ogen) binding contributes to dissemination in the tick. OspA as well as OspC have been described as plasminogen receptors of the borreliae (Fuchs et al., 1994; Lagal et al., 2006). Possibly, several mechanisms, such as *ospC* upregulation or plasmin(ogen) binding and further unknown factors, promote dissemination of borreliae within the tick. Our study has shown that *ospC* upregulation in the borreliae alone without blood contact promotes dissemination in the tick. Thus OspC is at least one important factor promoting dissemination. Further investigations including more species and strains of *B. burgdorferi* s.l. are necessary to elucidate the function of OspC in the life cycle of the borreliae. These should also include a possible role of other borrelial surface proteins in this process. Identification of the proteins that participate in the transmission process might lead to new targets to interfere with the *B. burgdorferi* life cycle and to develop new therapeutic and preventive strategies against Lyme disease.

Acknowledgments

We thank Cecilia Hizo-Teufel for excellent technical work. This work was in part supported by the DFG (Graduiertenkolleg "Infektion und Immunität", head Jürgen Heesemann), Robert-Koch-Institut, Grant no. ZV2-1369-338, and is part of the thesis of Gereon Goettner.

References

Baranton, G., Postic, D., Saint, G.I., Boerlin, P., Piffaretti, J.C., Assous, M., 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.

Benach, J.L., Coleman, J.L., Skinner, R.A., Bosler, E.M., 1987. Adult *Ixodes dammini* on rabbits: a hypothesis for the development and transmission of *Borrelia burgdorferi*. J. Infect. Dis. 155, 1300–1306.

Bono, J.L., Elias, A.F., Kupko III, J.J., Stevenson, B., Tilly, K., Rosa, P., 2000. Efficient targeted mutagenesis in *Borrelia burgdorferi*. J. Bacteriol. 182, 2445–2452.

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

Busch, U., Hizo-Teufel, C., Boehmer, R., Fingerle, V., Nitschko, H., Wilske, B., Preac-Mursic, V., 1996. Three species of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* sensu stricto, *B afzelii*, and *B. garinii*) identified from cerebrospinal fluid isolates by pulsed-field gel electrophoresis and PCR. J. Clin. Microbiol. 34, 1072–1078.

Carroll, J.A., Garon, C.F., Schwan, T.G., 1999. Effects of environmental pH on membrane proteins in *Borrelia burgdorferi*. Infect. Immun. 67, 3181–3187.

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., 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.

Coleman, J.L., Gebbia, J.A., Piesman, J., Degen, J.L., Bugge, T.H., Benach, J.L., 1997. Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. Cell 89, 1111–1119.

de Silva, A.M., Fikrig, E., 1995. Growth and migration of *Borrelia burgdorferi* in *Ixodes* ticks during blood feeding. Am. J. Trop. Med. Hyg. 53, 397–404.

Falkow, S., 1988. Molecular Koch's postulates applied to microbial pathogenicity. Rev. Infect. Dis. 10 (Suppl. 2), S274–S276.

Fingerle, V., Hauser, U., Liegl, G., Petko, B., Preac-Mursic, V., Wilske, B., 1995. Expression of outer surface proteins A and C of *Borrelia burgdorferi* in *Ixodes ricinus*. J. Clin. Microbiol. 33, 1867–1869.

Fingerle, V., Liegl, G., Munderloh, U., Wilske, B., 1998. Expression of outer surface proteins A and C of *Borrelia burgdorferi* in *Ixodes ricinus* ticks removed from humans. Med. Microbiol. Immunol. (Berlin) 187, 121–126.

Fingerle, V., Laux, H., Munderloh, U.G., Schulte-Spechtel, U., Wilske, B., 2000. Differential expression of outer surface proteins A and C by individual *Borrelia burgdorferi* in different genospecies. Med. Microbiol. Immunol. (Berlin) 189, 59–66.

Fingerle, V., Rauser, S., Hammer, B., Kahl, O., Heimerl, C., Schulte-Spechtel, U., Gern, L., Wilske, B., 2002. Dynamics of dissemination and outer surface protein expression of different European *Borrelia burgdorferi* sensu lato strains in

artificially infected *Ixodes ricinus* nymphs. J. Clin. Microbiol. 40, 1456–1463.

Fuchs, H., Wallich, R., Simon, M.M., Kramer, M.D., 1994. The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. Proc. Natl. Acad. Sci. USA 91, 12594–12598.

Ge, Y., Charon, N.W., 1997. Molecular characterization of a flagellar/chemotaxis operon in the spirochete *Borrelia burgdorferi. afzelii* is crucial for dissemination in the vector as shown by transformation and complementation of a European OspC-deficient *B. afzelii* strain. FEMS Microbiol. Lett. 153, 425–431.

Gern, L., Zhu, Z., Aeschilimann, A., 1990. Development of *Borrelia burgdorferi* in *Ixodes ricinus* females during blood feeding. Ann. Parasitol. Hum. Comp. 65, 89–93.

Gilmore Jr., R.D., Piesman, J., 2000. Inhibition of *Borrelia burgdorferi* migration from the midgut to the salivary glands following feeding by ticks on OspC-immunized mice. Infect. Immun. 68, 411–414.

Glöckner, G., Schulte-Spechtel, U., Schilhabel, M., Felder, M., Sühnel, J., Wilske, B., Platzer, M., 2006. Comparative genome analysis: selection pressure on the Borrelia vls cassettes is essential for infectivity. BMC Genomics 7, 211.

Goettner, G., Gern, L., Schulte-Spechtel, U., Wilske, B., Fingerle, V., 2006. OspC of *B. afzelii* is crucial for dissemination in the vector as shown by transformation and complementation of a European OspC-deficient *B. afzelii* strain. Int. J. Med. Microbiol 296S1 (Suppl. 40), 122–124.

Grimm, D., Tilly, K., Byram, R., Stewart, P.E., Krum, J.G., Bueschel, D.M., Schwan, T.G., Policastro, P.F., Elias, A.F., Rosa, P.A., 2004. Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. Proc. Natl. Acad. Sci. USA 101, 3142–3147.

Hauser, U., Lehnert, G., Lobentanzer, R., Wilske, B., 1997. Interpretation criteria for standardized Western blots for three European species of *Borrelia burgdorferi* sensu lato. J. Clin. Microbiol. 35, 1433–1444.

Lagal, V., Portnoi, D., Faure, G., Postic, D., Baranton, G., 2006. *Borrelia burgdorferi* sensu stricto invasiveness is correlated with OspC-plasminogen affinity. Microbes Infect. 8, 645–652.

Leuba-Garcia, S., Martinez, R., Gern, L., 1998. Expression of outer surface proteins A and C of *Borrelia afzelii* in *Ixodes ricinus* ticks and in the skin of mice. Zentralbl. Bakteriol. 287, 475–484.

Montgomery, R.R., Malawista, S.E., Feen, K.J., Bockenstedt, L.K., 1996. Direct demonstration of antigenic substitution of *Borrelia burgdorferi* ex vivo: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. J. Exp. Med. 183, 261–269.

Ohnishi, J., Piesman, J., de Silva, A.M., 2001. Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. Proc. Natl. Acad. Sci. USA 98, 670–675.

Pal, U., Li, X., Wang, T., Montgomery, R.R., Ramamoorthi, N., Desilva, A.M., Bao, F., Yang, X., Pypaert, M., Pradhan, D., Kantor, F.S., Telford, S., Anderson, J.F., Fikrig, E., 2004a. TROSPA, an I*xodes scapulari*s receptor for *Borrelia burgdorferi*. Cell 119, 457–468.

Pal, U., Yang, X., Chen, M., Bockenstedt, L.K., Anderson, J.F., Flavell, R.A., Norgard, M.V., Fikrig, ., 2004b. OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. J. Clin. Invest. 113, 220–230.

Preac-Mursic, V., Wilske, B., Reinhardt, S., 1991. Culture of *Borrelia burgdorferi* on six solid media. Eur. J. Clin. Microbiol. Infect. Dis. 10, 1076–1079.

Ribeiro, J.M., Mather, T.N., Piesman, J., Spielman, A., 1987. Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodidae). J. Med. Entomol. 24, 201–205.

Samuels, D.S., Mach, K.E., Garon, C.F., 1994. Genetic transformation of the Lyme disease agent *Borrelia burgdorferi* with coumarin-resistant gyrB. J. Bacteriol. 176, 6045–6049.

Sartakova, M., Dobrikova, E., Cabello, .C., 2000. Development of an extrachromosomal cloning vector system for use in *Borrelia burgdorferi*. Proc. Natl. Acad. Sci. USA 97, 4850–4855.

Schulte-Spechtel, U., Fingerle, V., Goettner, G., Rogge, S., Wilske, B., 2006. Molecular analysis of decorin-binding protein A (DbpA) reveals five major groups among European *Borrelia burgdorferi* sensu lato strains with impact for the development of serological assays and indicates lateral gene transfer of the *dbpA* gene. Int. J. Med. Microbiol. 296S1 (Suppl. 40), 250–266.

Schwan, .G., Piesman, J., 2000. Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. J. Clin. Microbiol. 38, 382–388.

Schwan, T.G., Piesman, J., 2002. Vector interactions and molecular adaptations of Lyme disease and relapsing fever spirochetes associated with transmission by ticks. Emerg. Infect. Dis. 8, 115–121.

Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C., Rosa, P.A., 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proc. Natl. Acad. Sci. USA 92, 2909–2913.

Stevenson, B., Tilly, K., Rosa, P.A., 1996. A family of genes located on four separate 32-kilobase circular plasmids in *Borrelia burgdorferi* B31. J. Bacteriol. 178, 3508–3516.

Stewart, P.E., Thalken, R., Bono, J.L., Rosa, P., 2001. Isolation of a circular plasmid region sufficient for autonomous replication and transformation of infectious *Borrelia burgdorferi*. Mol. Microbiol. 39, 714–721.

Wang, G., van Dam, A.P., Schwartz, I., Dankert, J., 1999. Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. Clin. Microbiol. Rev. 12, 633–653.

Wilske, B., Preac-Mursic, V., Schierz, G., Kühbeck, R., Barbour, A.G., Kramer, M., 1988. Antigenic variability of *Borrelia burgdorferi*. Ann. NY Acad. Sci. 539, 126–143.

Wilske, B., Preac-Mursic, V., Jauris, S., Hofmann, A., Pradel, I., Soutschek, E., Schwab, E., Will, G., Wanner, G., 1993. Immunological and molecular polymorphisms of OspC, an

immunodominant major outer surface protein of *Borrelia burgdorferi*. Infect. Immun. 61, 2182–2191.

Wilske, B., Busch, U., Eiffert, H., Fingerle, V., Pfister, H.W., Rössler, D., Preac-Mursic, V., 1996. Diversity of OspA and OspC among cerebrospinal fluid isolates of *Borrelia burgdorferi* sensu lato from patients with neuroborreliosis in Germany. Med. Microbiol. Immunol. (Berlin) 184, 195–201.

Zuckert, W.R., Meyer, ., 1996. Circular and linear plasmids of Lyme disease spirochetes have extensive homology: characterization of a repeated DNA element. J. Bacteriol. 178, 2287–2298.