Med Microbiol Immunol (2000) 189: 59–66

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ORIGINAL INVESTIGATION

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Differential expression of outer surface proteins A and C by individual *Borrelia burgdorferi* in different genospecies

Received: 19 July 2000

Abstract Previous studies with different *Borrelia burgdorferi* sensu stricto (s.s.) strains revealed that temperature as well as cocultivation with tick cells modulates the expression of outer surface proteins (Osp) A and C. We investigated the effects of temperature and of interaction with tick cells in culture on the expression of OspA and OspC of the *B. afzelii* clones cPKo97 and cPKo345 in comparison to the *B. burgdorferi* s.s. strain N40. To follow the dynamics of Osp expression of single borreliae we used indirect immunofluorescence microscopy with double staining of OspA and OspC. Clone PKo345 always showed expression of only OspA, regardless the conditions it was subjected to. Sequencing of the *ospC* gene disclosed a insertion leading to a stop codon after base 222 and inability to produce OspC. In cPKo97 and N40 OspC is down-regulated at lower temperatures and up-regulated at higher temperatures, which was especially pronounced on cocultivation with tick cells. Borreliae adherent to tick cells showed greater OspA expression compared to the nonadherent ones, an indication that OspA might play a role as adhesin for tick cells. Interestingly, cPKo97 and N40 displayed different patterns of Osp expression: cPKo97 simultaneously presents OspA and OspC on single borreliae, while N40 has either OspA or OspC on single cells. Adaptation of OspC expression in cPKo97 seems to occur by up- or down-regulation of this protein on single borreliae, as shown by alternating intensities of OspC expression at different temperatures. In contrast, N40 seem to consist of two subsets of borreliae one expressing only OspA and the other only OspC, and change in temperature results in growth benefit for one of these subtypes. Our findings indicate that, regarding OspA and OspC expression, response to temperature and cocultivation with tick cells of *B. afzelii* is comparable to *B. burgdorferi* s.s., but the mode of regulation seems phenotypically different. Further European isolates should be investigated for OspA and OspC regulation, especially in the face of vaccine development for the European situation.

Key words *Borrelia burgdorferi* sensu stricto · *B. afzelii* · Outer surface proteins · Temperature effect · Cocultivation with tick cells

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Introduction

The etiological agent of Lyme disease, *Borrelia burgdorferi* sensu lato (s.l.), can be divided into at least three human pathogenic species, all of which are present in Europe: *B. burgdorferi* sensu stricto (s.s.), the only species causing Lyme borreliosis in the USA, *B. afzelii* and *B. garinii* [1, 3]. This spirochete is maintained in nature by alternating host (mammals) – vector (*Ixodes* ticks) cycles. In the vector, the spirochetes restart replication during the feeding process, migrate through the gut wall and invade various tissues including the salivary glands from which they are transmitted to the host via saliva [2, 8, 11, 20, 22, 32]. The ability to survive in and change between such physiologically quite different systems requires efficient adaptation strategies. One possible mechanism is alteration in outer surface protein (Osp) expression. Several studies have investigated the expression of OspA and OspC of *B. burgdorferi* s.l.. In vitro, expression of these two proteins varies even under routine culture conditions and seems to be negatively correlated [5, 25–27]. Higher temperature and cocultivation with tick cells have been shown to induce OspC expression in *B. burgdorferi* s.s. strains [18, 21, 23]. In unfed ticks borreliae usually have OspA but not OspC on their surface [10, 21], but OspC expression seems to be up-regulated during the blood meal on the

vertebrate host, a fact that is reflected in the early human immune response, which is predominantly directed against OspC [7, 9, 22, 25, 29]. This has been taken as evidence that OspC may facilitate dissemination in the vector and enhance infectivity for the vertebrate host [2, 8, 11, 12, 14, 15, 20, 21, 32].

In the present study we investigated influence of temperature and cocultivation with tick cells on the dynamics of OspA and OspC expression by individual cells of the *B. burgdorferi* s.s. strain N40 in comparison with two *B. afzelii* clones, cPKo97 and cPKo345, derived from strain PKo. Here we report a temperature-regulated expression of OspA and OspC of PKo97 and, as shown before, for strain N40, and demonstrate that individual borreliae of the genospecies show divergent Osp-expression patterns.

Materials and methods

Borrelia strains and clones

B. burgdorferi s.s. strains and clones used were: (1) *B. burgdorferi* s.s. strain N40 (OspA positive, OspC positive), (2) *B. afzelii* clone PKo97 K37 (cPKo97; OspA positive, OspC positive), and (3) *B. afzelii* clone PKo345 II-2-3 (cPKo345; OspA positive, OspC negative). PKo clones were derived by triple colony selection of reisolates from a gerbil – cPKo345 from joint, cPKo97 from kidney – infected with low passage *B. afzelii* strain PKo (human skin isolate).

Strain and clones were grown as described [19] to a density of 10^6/ml in 100 ml MKP medium at 33 °C, tested by immunofluorescence assay (IFA) and Western blot for expression of OspA and OspC and at least 40 vials of each culture were frozen at −70 °C as a stock of cultures with identical passage and cultivation history. Before each experiment, the respective borreliae were regenerated and cultured for 1 week under routine conditions.

Influence of temperature

To determine the influence of temperature on OspA and OspC expression each strain/clone was cultured in MKP-medium for 25 days at 19 °C, 24 °C, 28 °C, 31 °C, 34 °C, and 37 °C in duplicate. To investigate Osp expression, aliquots were harvested every 2–3 days. During the experiments, the concentration of the borreliae was kept between 10^5–10^7/ml by subculturing every 7–16 days depending on the growth temperature.

Cocultivation with tick cells (ISE6)

The *I. scapularis* tick cell line ISE6 was maintained and cultured for the experiments as described previously [16–18]. Of each strain and clone 10^6 borreliae were seeded in 500 µl L15BS medium onto confluent tick cell layers in 24-well plates (total volume 1 ml/well) and incubated at 24 °C, 31 °C, 34 °C, and 37 °C, for 8 days. To determine adherence to tick cells and influence on Osp expression, two wells of each strain and clone from each temperature level were investigated by IFA at days 2, 4, 5, and 8. The cells were resuspended by pipetting and, to distinguish between cell-adherent and nonadherent borreliae, the aliquots were centrifuged three times for 5 min at 1000 rpm (184 *g*) in PBS pH 7.4 at ambient temperature. Pellet and pooled supernatants were investigated separately by IFA. As a control, ISE6 cells were cultured in parallel at 24 °C, 31 °C, 34 °C, and 37 °C, in L15BS without borreliae.

Monoclonal antibodies

The OspA-specific monoclonal antibody (mAb) L32 1F11, which recognizes an epitope conserved among *B. burgdorferi* s.l. strains, was used for OspA IFA [28], and the broadly reactive OspC-specific mAb L22 1F8 [27], reactive with OspC of strain N40 and PKo, for OspC detection.

Immunofluorescence assay

IFA was essentially done as described previously [10]. For double labeling of OspA and OspC, slides were incubated for 30 min with a mixture of anti OspA mAb L32 1F11 (IgG2a subclass, dilution 1:2) and the broadly reactive mAb L22 1F8 (IgG1 subclass, dilution 1:8) against OspC. To differentiate the two antibodies, slides were incubated for 30 min with a mixture of FITC-conjugated antibody to mouse IgG1 (Caltag, San Francisco, USA) and Cy3-conjugated antibody to mouse IgG2a (Caltag) in a final dilution of 1:100 each. The slides were finally incubated for 30 s with DAPI (1:10,000), a blue fluorescent, DNA-intercalating dye to visualize all borreliae. As positive controls we used a defined OspA- and OspC-expressing passage of the skin isolate PKo. A OspC-negative variant of the cerebrospinal fluid isolate PKa2 [30], as well as an OspA-negative variant of the skin isolate PPop [28], served as negative controls. Slides were examined with a Leitz Laborlux 12 microscope fitted for epifluorescence imaging at a magnification of ×400. Osp expression of individual spirochetes was visualized using filters suitable for FITC (green), Cy3 (red), FITC and Cy3, or DAPI (blue). To determine the percentage of borreliae expressing the respective Osp, at least 100 borreliae/well were evaluated.

Amplification and sequencing of the *ospC* gene

Total genomic *Borrelia* DNA was extracted from cultures and the *ospC* genes were amplified by PCR with forward primer PCol-A1: CGC GGA TCC GAA TTC ATG AAT AAT TCA GGG; and reverse primer OC-3: 3′-GAG CTG CAG TTA AGG TTT TTT TGG ACT TTC TGC. Both strands of the *ospC* gene were sequenced by the Taq DyeDideoxy terminator method (377XL DNA Sequencer; Applied Biosystems, Darmstadt, Germany). Sequences were analyzed using DNAMAN for Windows 95 (Lynnon BioSoft, Quebec, Canada), and compared with the published sequences of strain PKo (GenBank accession no. X62162).

Results

Influence of temperature on expression of OspA and OspC

Of the *B. burgdorferi* s.s. strain N40 90% showed expression of only OspA, while the remainder expressed only OspC (Figs. 1a, 2). Double staining of strain N40 revealed that, under each culture conditions, the majority of the borreliae had either OspA or OspC on their surface (Fig. 2). Low temperature between 19 °C and 28 °C led to a slight increase in the proportion of borreliae that expressed only OspA, and a slight decrease in those expressing only OspC compared to the starting culture grown at 33 °C. At higher temperatures, the population expressing OspA or OspC did not change (Fig. 1a).

Of interest, the stock culture of cPKo97 predominantly (86%) comprised borreliae showing dual expression of OspA and OspC (Figs. 1b, 3). Cultivation at 24 °C and below caused the number of borreliae that displayed

only OspA to increase, and a concomitant decrease in the proportion of those expressing both proteins at once. At 19 °C the proportion of only OspA exhibiting borreliae even exceeded the number of double expressing ones. On the other hand, temperatures between 28 °C and 37 °C showed no clear effect on the ratio of OspA/C expression. Although the percentage of OspC-expressing borreliae showed no remarkable change, we observed that the intensity of OspC expression, as shown by the intensity of the red fluorescence, changed. We therefore developed a rating scheme (Fig. 3) ranging from 1 (low OspC) to 3 (high OspC expression).

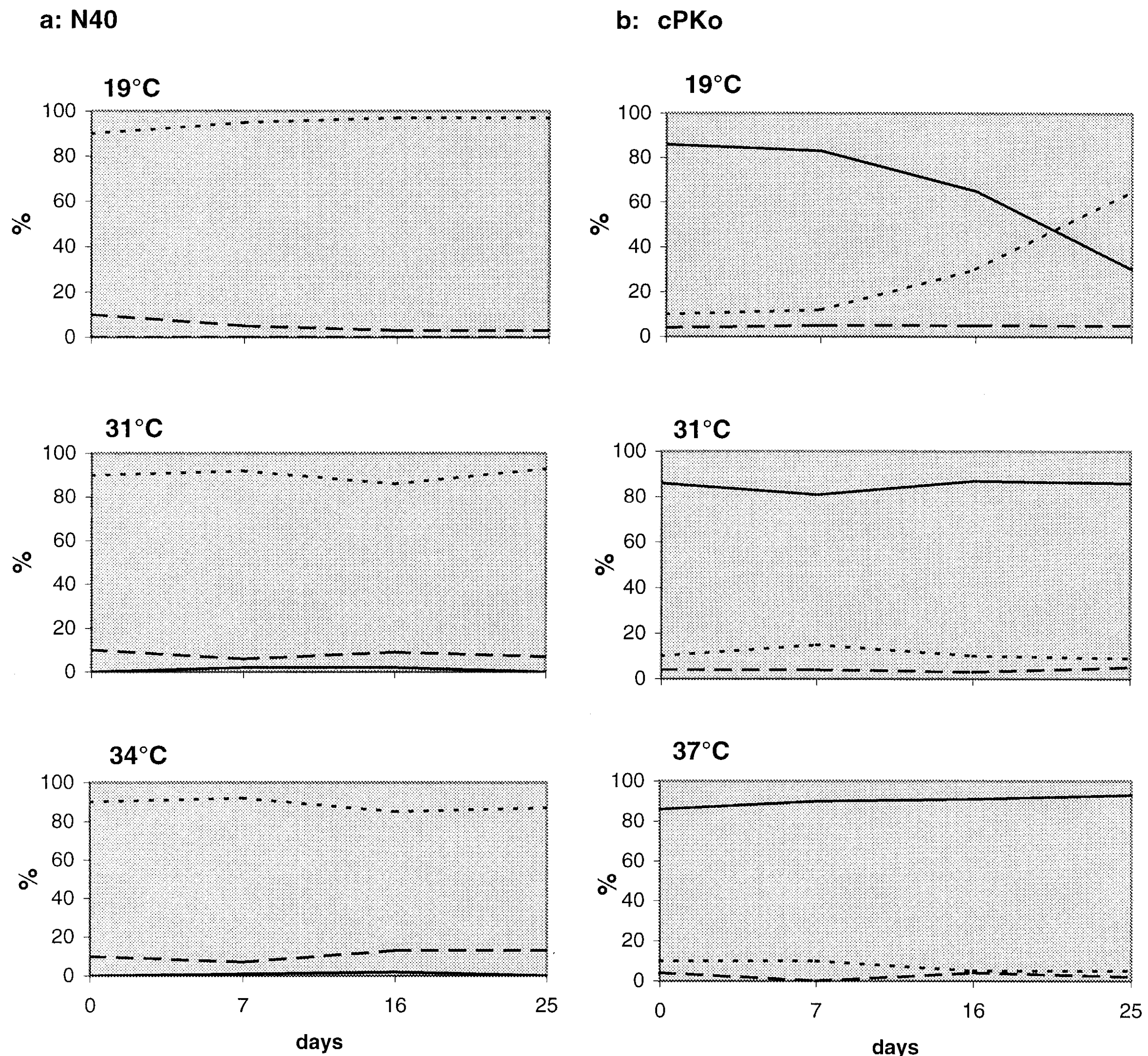
Using this scheme we were able to show that higher temperature (34 °C, 37 °C) led to up-regulation of OspC on individual borreliae dually expressing both, OspA and OspC, and vice versa for lower temperatures, as demonstrated by the intensity of OspC expression (Fig. 4).

By contrast, cPKo345 uniformly and strongly expressed exclusively OspA at any temperature tested and no OspC was detected (data not shown). Therefore, we sequenced the entire *ospC* gene. Comparison with the known sequence of the *ospC* of strain PKo revealed the insertion of a G at position 200, resulting in a frame shift and stop codon (TAA) after base 222, corresponding to the end of the first third of the gene.

Cocultivation with tick cells

We separated tick cell-adherent and nonadherent borreliae by centrifugation and examined Osp expression of the borreliae in sediment and supernatant. Counts using a Petroff Hausser bacteria counting chamber revealed that the vast majority (>95%) of the borreliae were present in the culture supernatant. Osp expression patterns of single borreliae were again either OspA or OspC

Fig. 1 Influence of different temperatures on expression of OspA and OspC during 25 days of cultivation. Aliquots were taken every 2–3 days and the presence of these proteins on individual borreliae was investigated using double immunofluorescence with mAbs specific for OspA or OspC (see also legend Fig. 2) (- - - -) only OspA, (– – – –) only OspC, (——) OspA and OspC (*mAb* monoclonal antibody, *Osp* outer surface protein)



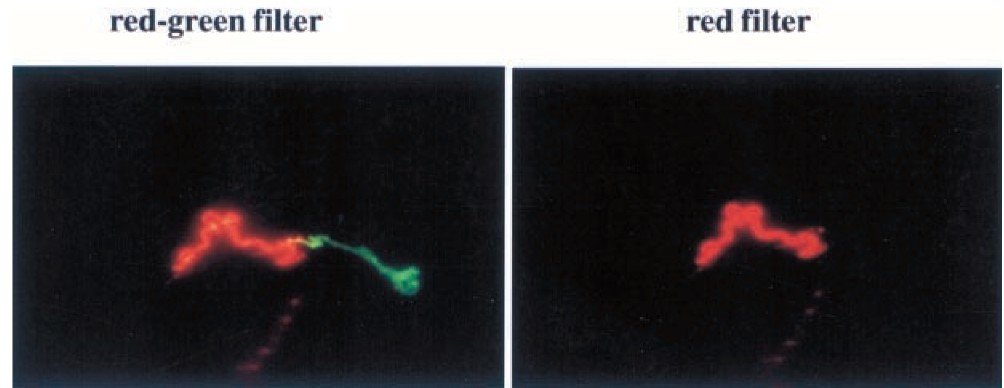


Fig. 2 Expression of either OspA or OspC of strain N40. Anti-OspC mAb L22 1F8 (IgG1) is labeled in *red* (Cy3-conjugated anti-mouse IgG1) and anti-OspA mAb L32 1F11 (IgG2a) is labeled in *green* (FITC-conjugated anti-mouse IgG2a)

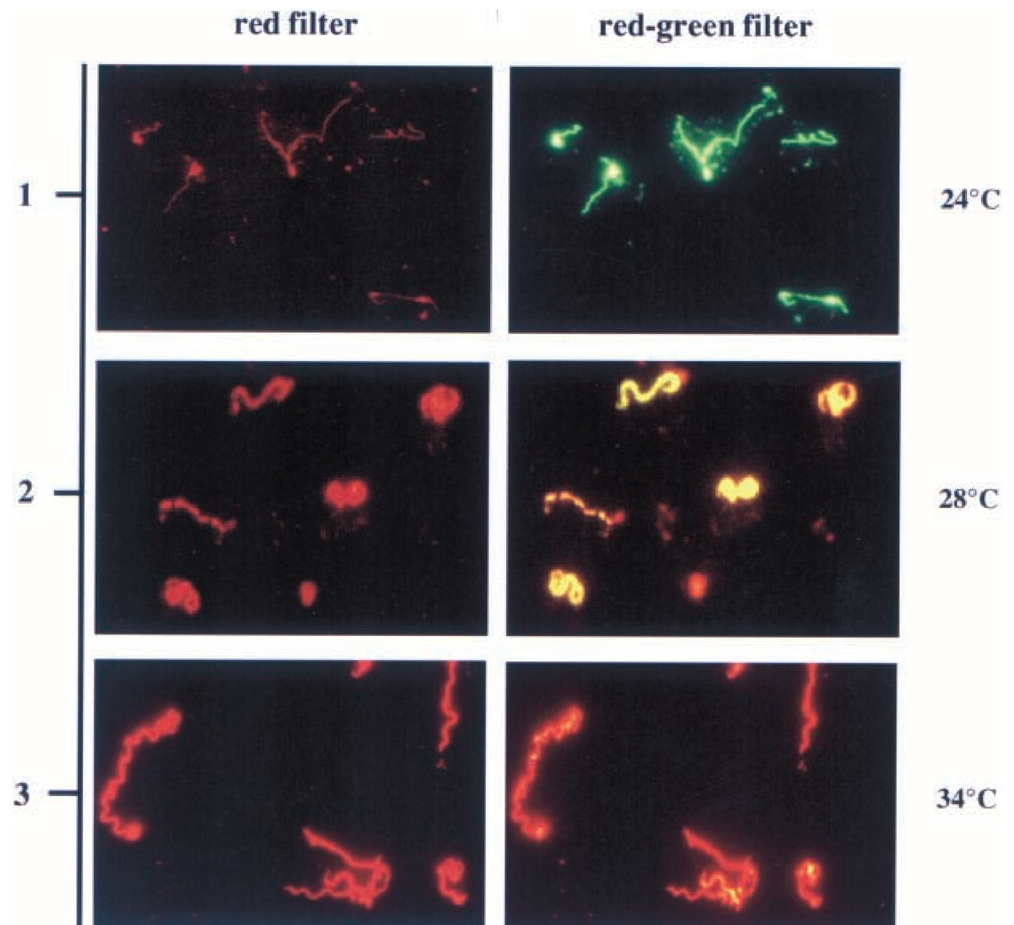


Fig. 3 Rating scale for OspC expression and simultaneous expression of OspA and OspC of cPKo97 (see also legend for Fig. 2). Strength of OspC expression was evaluated on the basis of the intensity of the red coloration, ranging from *1* (poor) to *3* (strong) OspC expression

for strain N40, and predominantly OspA and OspC for cPKo97 (Fig. 5). In strain N40 and in cPKo97 OspC expression in the supernatant increased at 34 °C and 37 °C, and decreased at 24 °C, while borreliae in the pellet – those that were adherent to tick cells – expressed primarily OspA regardless of temperature.

Discussion

During the transmission cycle between the tick vector and the mammalian host *B. burgdorferi* is subject to extremely different and rapidly changing environmental conditions. One possible adaptive mechanism promoting survival of tick-borne pathogens during their passage from one environment to the other is variation in the antigenic composition of the outer surface. Temporal changes in the composition of major surface proteins, i.e., mainly OspA and OspC, in response to environmental stimuli have been described by several authors. Temperature and cocultivation with tick cells have been shown to influence expression of OspA and OspC [18, 21, 23]. Within unfed *I. scapularis* ticks, OspA is abundantly expressed on the surface of the spirochetes,

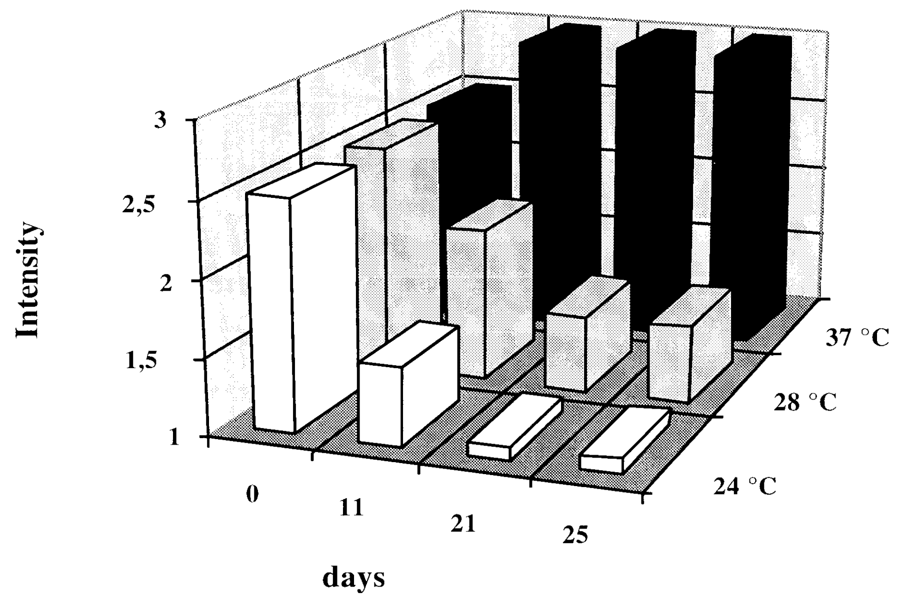


Fig. 4 Time course of intensity of OspC expression in cPKo97 at different temperatures. Single borreliae (> 100 each time point) were evaluated for OspC expression by means of the intensity of the red coloration

but later, during the feeding process, OspC production is up-regulated and the majority of borreliae clear OspA from their surface [8, 21]. Up-regulation of OspC was found to be associated with dissemination in the vector, i.e., spirochetes found in the salivary glands solely expressed OspC but not OspA [8], and has been linked to infectivity for the mammal [14, 15, 22]. These results were obtained from studies using *I. scapularis* infected with *B. burgdorferi* s.s. strains, the only human pathogenic species present in the US. In Europe at least three different human pathogenic species coexist in the vector *I. ricinus*. These borreliae display considerable genetic heterogeneity with respect to OspA and OspC [25–28, 31]. As shown for American ticks, almost all field-collected flat ticks from Germany carried borreliae that presented only OspA on their surface, but in one tick borreliae expressing OspC were present [10]. Leuba-Garcia et al. [13] found OspC-positive borreliae in eight of ten unfed nymphs infected with the *B. afzelii* strain NE496 as larvae. In a study on partially engorged *I. ricinus* ticks detached from humans, we confirmed that in general, OspC is up-regulated in feeding ticks. However, in a number of these ticks we detected spirochetes exhibiting all possible expression patterns: (OspC or OspA alone, both, or neither) could be found in ticks at different stages of engorgement as determined by tick weight [9]. These results did not conform to the current view on the sequence of events that is initiated by the blood meal. We therefore wished to compare differential expression of OspA and OspC in *B. burgdorferi* s.s. with *B. afzelii* under identical culture conditions.

Although our studies with field-collected ticks had suggested earlier that European *B. burgdorferi* s.l. may not exactly mirror the events as demonstrated in American *B. burgdorferi* s.s. isolates, we were surprised to find that cPKo97 and N40 showed phenotypically very different modes of Osp expression. Except at 19 °C, cPKo97 consistently co-expressed both Osps, and changes in temperature resulted primarily in up- or down-regulation of OspC on individual borreliae. We were unable to follow any changes in the level of OspA expression, because interference from the CY3 label used for OspC prevented quantification, but not detection, of the FITC-labeled OspA. The results presented in this study suggest, that individual cells of *B. afzelii* cPKo97 respond to environmental stimuli of changing temperature or absence/presence of tick cells by adjusting the amount of OspC on their surface. By contrast, the N40 borreliae express either OspA or OspC on single spirochetes, but the proportion of different expressors changes with the culture condition. If during periods of environmental change individual spirochetes switch from one Osp to the other, cells bearing both antigens should be detectable for a certain period. However, such borreliae were not seen. Moreover, unlike with cPKo97, we were not able to document differences in the intensity of CY3 fluorescence in individual N40 cells, which would represent differences in the amount of OspC. Therefore, we assume that various temperature conditions may favor borreliae with different surface coats, such that at higher temperatures those expressing OspC gain an advantage that is reflected in faster growth. This then would lead to a shift in the composition of the spirochete population, and detection of increased or decreased amounts of one or the other Osp-expressing population. To prove this, we attempted to recover N40 clones (cloned on soft agar or by limiting dilution) that stably expressed only OspA or only OspC, but failed. Clearly, the mechanisms underlying the induction of populations of spirochetes that differ in Osp expression need to be investigated further.

In principle, our results on temperature-induced regulation of OspA and OspC expression are in agreement with previously published studies [18, 21–23]. As described for several *B. burgdorferi* s.s. strains, higher temperatures lead to up-regulation of OspC, while a

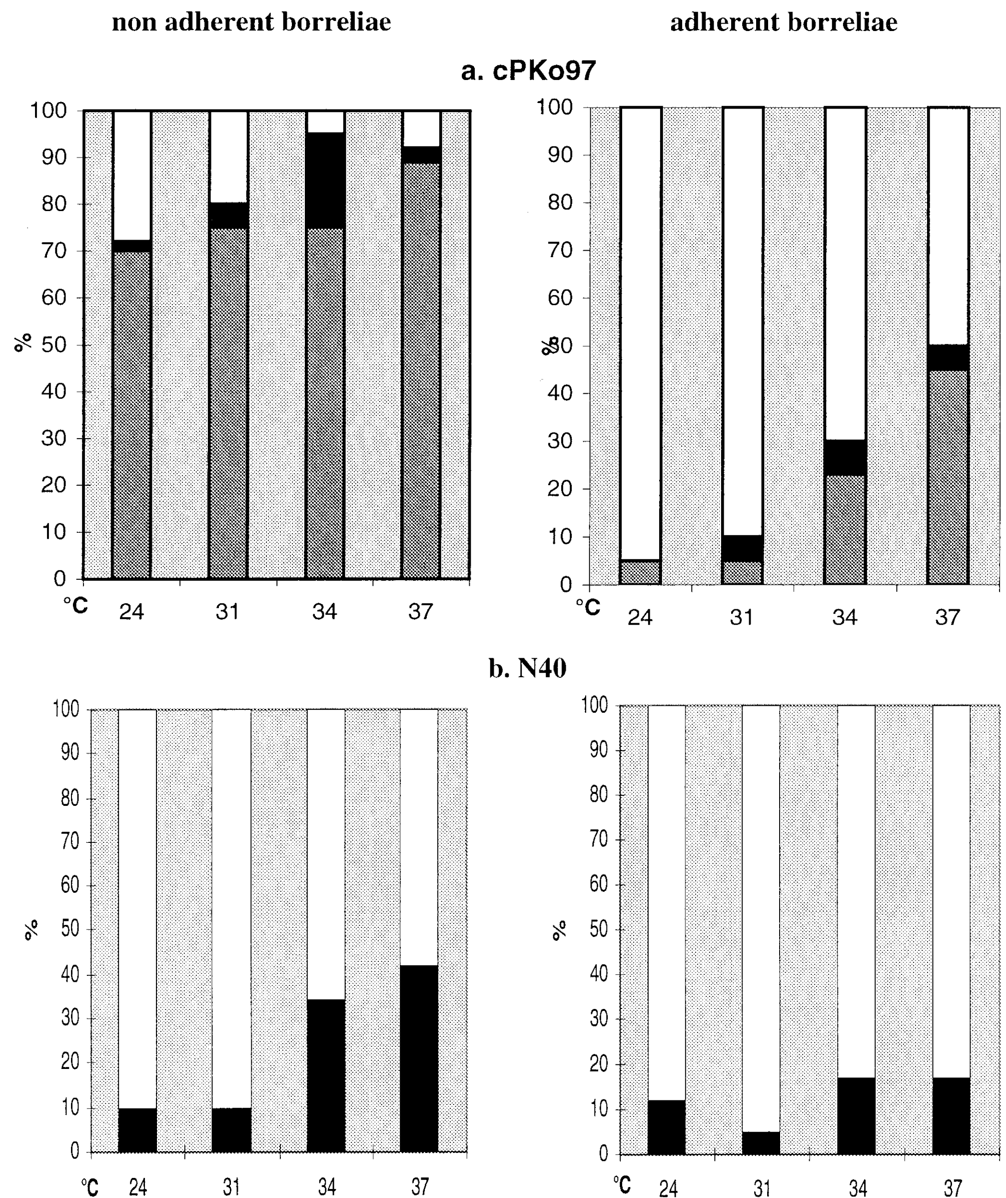


Fig. 5 OspA and OspC expression of cPKo97 and N40 after 8 days cocultivation with tick cells. Adherent and nonadherent borreliae were separated by centrifugation. Pellet (tick cell adherent borreliae) and supernatant (nonadherent borreliae) were investigated separately for OspA and OspC expression (see also legend for Fig. 2). (□) Only OspA, (■) only OspC, (▤) OspA and OspC

drop in temperature also lead to increased levels of OspA for the *B. afzelii* clone PKo97. These changes occurred faster and were more pronounced when the clone was cocultivated with tick cells. This makes sense considering that the nymphal blood meal, the stage probably most important for disease transmission to humans, usually lasts for 3–5 days. Therefore, modulation of Osps relevant for dissemination in the vector or infectivity for the host have to take place within hours to a few days in the tick midgut, as was shown for *B. burgdorferi* s.s. isolates in *I. scapularis* ticks [21, 22]. Also of note, borreliae associated with ISE6 cells after centrifugation (Fig. 5) showed greater OspA expression compared to those from the supernatant. OspA was shown to be primarily expressed by borreliae in the midgut of unfed ticks [4, 10, 21], and is considered to be an important factor for adherence in human endothelial cells [6, 24]. Our results indicate that OspA might play a role as adhesin for tick cells and further underscore the value of the tick cell culture system described by Obonyo et al. [18] as a suitable in vitro system to study the events that occur in the vector.

Following a brief period of initial up-regulation of OspC, Schwan et al. [21, 22] described substantial down-regulation of OspC during several consecutive passages at 37 °C. We were unable to detect changes in OspC expression for strain N40 during continuous culture at 37 °C, and continued up-regulation of OspC in cPKo over 25 days of culture. However, these differences might be due to the different culture media used (MKP vs BSK), or the different strains and species used in the present study, as well as the different evaluation schemes.

In contrast to the results obtained with N40 and cPKo97, cPKo345 always exhibited strong expression of

only OspA, regardless the culture conditions it was subjected to. Hypothetically, this clone should be able to produce a truncated OspC of 74 amino acids. However, we could not demonstrate a truncated OspC by SDS-PAGE, immunoblot, and IFA with mAbs recognizing epitopes in the first third of the protein [30]. In cPKo345 loss of OspC is associated with inability to down-regulate OspA expression, a further hint that OspA and OspC expression are negatively correlated. This non-OspC expressing mutant is a useful instrument for studying the influence of OspC on the sequence of infection. Presently, studies are on the way to define the ability for dissemination in the vector and infectivity for the host of this OspC-negative mutant.

In conclusion, we have shown that modulation of Osp expression of a *B. afzelii* clone by temperature and by cocultivation with tick cells is comparable to *B. burgdorferi* s.s. strains. However, modulation of Osp expression on single borreliae was different and, therefore, seems to be species or strain specific. In addition, we have shown that the tick cell culture model is also a valuable tool for investigating the adaptation process that occurs in the vector.

Further studies, especially in the face of the differing results from European studies, with different genospecies and strains are necessary to extend our knowledge on dynamics and regulation of OspA and C as well as of other variably expressed proteins. Since OspA and OspC are considered the most promising vaccine candidates, these studies will have important implications for development of a Lyme disease vaccine for Europe.

Acknowledgements We thank Khaled Taha, Gisela Lehnert, and Christiane Heimerl, for excellent technical work and Jürgen Heesemann for generous support. This work was supported by DFG grant no. Wi 894/4-1.

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