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Temperature-Related Differential Expression of Antigens in the Lyme Disease Spirochete, *Borrelia burgdorferi*

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Previous studies have demonstrated that *Borrelia burgdorferi* in the midguts of infected ticks shows increased expression of the antigenic outer surface protein OspC after the ticks have ingested a blood meal. This differential expression is at least partly due to a change in temperature, as an increase in OspC levels is also observed when cultures are shifted from 23 to 35°C. Immunoblotting of bacterial lysates with sera from infected mice indicated that the levels of several additional antigens were also increased in bacterial cultures shifted to 35°C; we have identified one antigen as OspE. We have also observed differential expression of OspF, which has been proposed to be coexpressed in an operon with the gene encoding OspE.

The spirochete *Borrelia burgdorferi* is the causative agent of Lyme disease, an increasingly common disease in humans and some domestic mammals (6, 25). This bacterium persists in nature through a cycle of infections between its two main hosts, mammals and several species of ticks of the genus *Ixodes*. Spirochetes pass from an infected mammal into a tick with the ingestion of a blood meal and establish an infection of the tick midgut. Subsequent feeding by the tick triggers the bacterium to migrate to the tick's salivary glands, from which it is transmitted via the saliva to the mammal, thus continuing the cycle (5, 13, 17, 18, 30). The bacterium, therefore, must sustain itself in two very different living conditions, in addition to moving between hosts. Such complexities probably require that *B. burgdorferi* sense its environment and respond accordingly by expressing the appropriate constituents.

Recent work by Schwan et al. (20) demonstrated the regulated expression of a *B. burgdorferi* outer surface protein, OspC. *B. burgdorferi* within the midguts of unfed ticks had undetectable levels of OspC, while those in ticks fed to repletion had substantial levels of this protein. The increases of OspC protein appeared to be signalled partly by changes in the temperature within the feeding tick, which rises from ambient to mammalian blood temperature: *B. burgdorferi* grown in culture medium at 23°C did not contain appreciable amounts of OspC, whereas organisms grown at 37°C contained large amounts of OspC (20). Early in the course of *B. burgdorferi* infection, host mammals develop an immune response to a small number of bacterial proteins, including OspC (1, 11, 12, 19, 29), indicating that this protein is produced by the spirochete during the primary stages of infection. These data suggest that OspC may be important for migration of the bacterium to the tick salivary glands or for establishment of infection within the mammalian host. Other pathogenic bacteria, such as *Bordetella pertussis* and *Yersinia pestis*, are known to exhibit temperature-dependent effects on virulence factors (15). We have sought to identify other differentially expressed proteins of *B. burgdorferi* in order to study the regulatory mechanisms of these proteins and their roles in the pathogenesis of Lyme disease.

Two well-characterized isolates of *B. burgdorferi* were utilized in these experiments, and both are included in the *B. burgdorferi* sensu stricto group of Lyme disease spirochetes (10). Isolate B31 was originally isolated from a tick on Shelter Island, N.Y. (6), was reisolated from a tick that had fed on an infected mouse (20), and was passaged no more than three times in culture medium. Isolate N40 was originally isolated from a tick in Westchester County, N.Y. (4), has been cloned by limiting dilution, is infectious in laboratory animals (3), and has been passaged no more than three times in culture medium. All cultures of *B. burgdorferi* were grown in BSK H medium (Sigma, St. Louis, Mo.) supplemented with 6% rabbit serum (Sigma). Both isolates B31 and N40 were maintained at 23°C, with passage into fresh medium when cultures reached mid-log phase.

Cultures (100 ml) of each isolate were grown at 23°C until the culture approached the mid-log phase of growth (approximately 2 weeks). A sample of each culture was then diluted 1:100 into 100 ml of fresh medium and grown to mid-log phase at 35°C (approximately 4 to 6 days). The cultures of each isolate were harvested by centrifugation when the spirochetes had attained the same bacterial density. Centrifuged pellets of *B. burgdorferi* were washed twice with phosphate-buffered saline (PBS) (3), resuspended in distilled water, and lysed in boiling water for 5 min. The protein contents of the *B. burgdorferi* lysates were determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.), and equal amounts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Replicate samples of each bacterial lysate were separated on each gel to permit the alignment of proteins detected by immunoblotting. One gel lane for each lysate was stained with Coomassie brilliant blue, and the intensities of the visible bands were compared to ensure that equal amounts of total protein had been loaded (Fig. 1). This staining also indicated that the culture of isolate B31 that was shifted to 35°C contained significantly greater amounts of OspC than the culture grown continuously at 23°C, as has been noted previously (20) (Fig. 1A). Differential expression of OspC was also observed in isolate N40 (Fig. 1B).

Gel-separated proteins were transferred to nitrocellulose membranes and immunoblotted by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.) as previously de-

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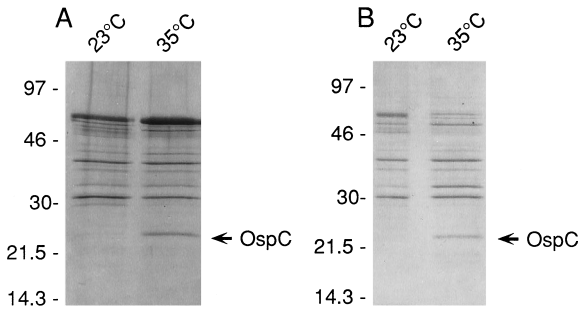

FIG. 1. Polyacrylamide gel electrophoresis of lysates of *B. burgdorferi* isolates B31 (A) and N40 (B) grown at 23 and 35°C. Equal amounts of total protein were separated on each lane and stained with Coomassie brilliant blue. Molecular mass markers (in kilodaltons) are indicated on the left. Increased amounts of OspC in the 35°C lysates are indicated with arrows.

scribed (27). *B. burgdorferi* antigens recognized during mammalian infection were detected with a 1:200 dilution of pooled sera collected from mice 30 days following intradermal inoculation with approximately 10^2 *B. burgdorferi* isolate N40 organisms (obtained from S. Barthold, Yale University). Immunoblotting with the sera (Fig. 2) produced patterns of protein expression different from those in Fig. 1. We confirmed by immunoblotting with polyclonal anti-OspC serum (21) that OspC was the protein with a size of approximately 24 kDa present only in the 35°C lysates (data not shown). Isolate B31 had six antigens in addition to OspC, with apparent molecular masses of approximately 16, 19, 37, 38, 45, and 52 kDa, in the 35°C lysate at levels significantly higher than those in the 23°C lysate (Fig. 2A). Four antigens (in addition to OspC), with apparent molecular masses of approximately 18, 20, 37, and 45 kDa, were present in the 35°C lysate of isolate N40 at higher levels than in the 23°C lysate (Fig. 2B). Three B31 antigens recognized strongly by the sera, with the apparent molecular mass of approximately 22, 39, and 41 kDa, did not appear to be affected by culture temperature; the 22-kDa antigen was not present in the N40 lysate. Sera from humans with Lyme disease infections acquired by tick bite or from laboratory animals after low-dose syringe inoculations showed immune responses to a number of *B. burgdorferi* antigens, including antigens with apparent molecular masses of 14 to 16, 37 to 38, 45, and 56 to 60 kDa (9, 11, 12, 19, 29). Engstrom et al. (11) identified human antibodies to unidentified *B. burgdorferi* antigens having molecular masses of 37, 44 to 46, and 56 kDa and posited that they are significant indicators of infection. These proteins may correspond with the differentially expressed antigens noted above. The identification of these unknown proteins may provide valuable information to our understanding of the processes involved in Lyme disease.

It has been reported that sera from both human Lyme disease patients and experimentally infected mice recognized a recombinant OspE protein (16), normally a 19-kDa surface-exposed lipoprotein of *B. burgdorferi* (14). We performed a series of immunoblotting experiments to determine whether any of the differentially expressed 18- to 20-kDa proteins seen in Fig. 2 were OspE. The *B. burgdorferi*-infected mouse sera gave a strong immunoblot signal to a purified recombinant OspE protein of isolate N40 (16) (provided by E. Fikrig, Yale University), indicating that the sera contained antibodies that recognized OspE (data not shown). We next immunoblotted lysates of isolates B31 grown at 23 and 35°C with 1:100 dilutions of a polyclonal serum directed against the recombinant OspE protein of isolate N40 (16) (provided by E. Fikrig). OspE was present at much higher levels in the cultures grown at 35°C than in those grown at 23°C (Fig. 3A). The band marked with an arrow in Fig. 3A aligned perfectly with the differentially expressed 19-kDa band seen in Fig. 2A. A similar difference in OspE concentration was also seen in isolate N40 grown at the higher temperature (Fig. 3B), with the band marked with an arrow in Fig. 3B aligning with the differentially expressed 20-

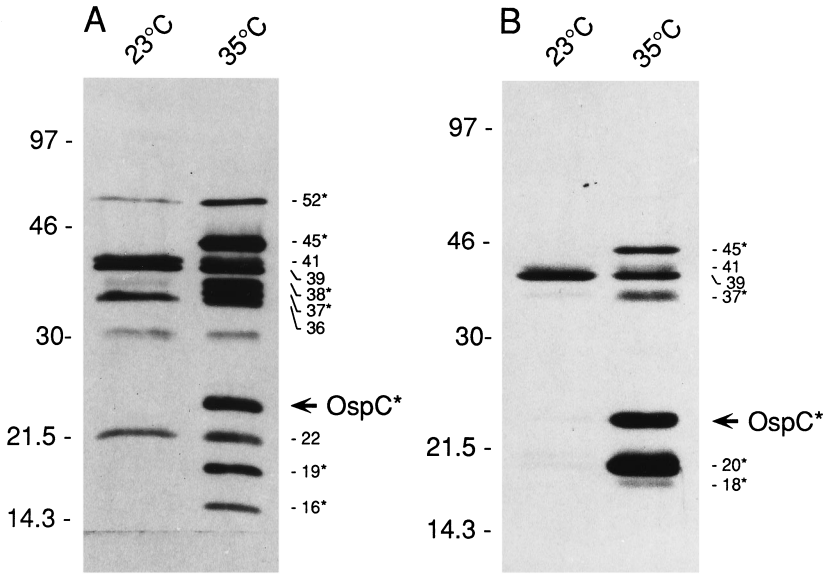

FIG. 2. Immunoblot analysis of antigens expressed by *B. burgdorferi* isolates B31 (A) and N40 (B) grown at 23 and 35°C. Lysates were immunoblotted with pooled sera collected from mice 30 days after infection with approximately 10^2 *B. burgdorferi* isolate N40 organisms. The molecular masses (in kilodaltons) of antigens recognized by the sera are indicated on the right of each panel; molecular mass markers (in kilodaltons) are indicated on the left. Several antigens were present in much greater quantities in the lysates of *B. burgdorferi* grown at 35°C relative to those maintained at 23°C and are indicated with asterisks. In addition to OspC, differential expression of antigens having approximate molecular masses of 16, 19, 37, 38, 45, and 52 kDa was observed in isolate B31, and antigens having approximate molecular masses of 18, 20, 37, and 45 kDa were observed in isolate N40. Increased amounts of OspC in the 35°C lysates are indicated with arrows.

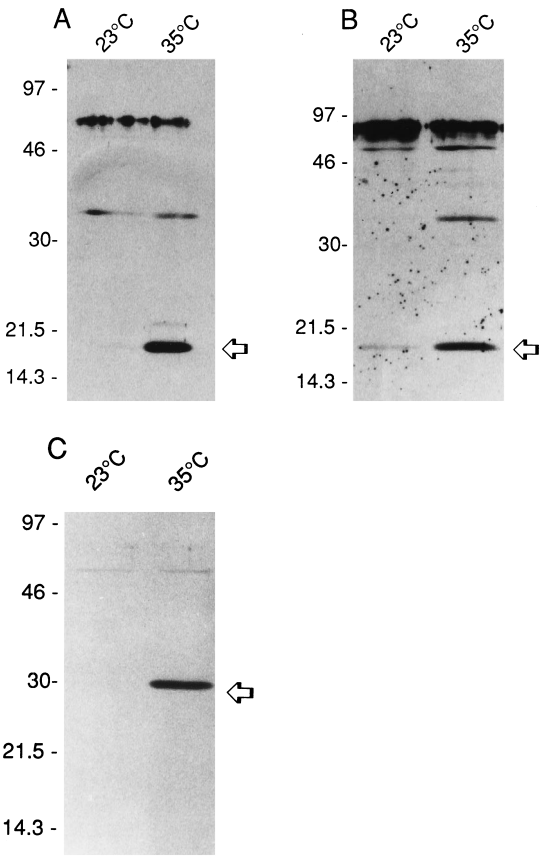

FIG. 3. Immunoblot analysis of OspE and OspF expression by *B. burgdorferi* cultured at 23 and 35°C. Isolates B31 (A) and N40 (B) were immunoblotted with a polyclonal rabbit serum directed against the recombinant OspE protein of isolate N40. (C) Isolate N40 was immunoblotted with a polyclonal rabbit serum directed against the recombinant OspF protein of isolate N40. Open arrows on the right indicate the OspE or OspF protein of each lysate. The nonregulated antigen with a size of approximately 66 kDa is probably bovine serum albumin, which is a component of BSK medium and a common contaminant of crude extracts of *B. burgdorferi* cultures (Fig. 1), and is recognized by these polyclonal rabbit antisera (data not shown). Molecular mass standards (in kilodaltons) are indicated on the left.

kDa band noted in Fig. 2B. A previous report (22) indicated variation in expression of some *B. burgdorferi* proteins as a result of culture temperature, including an unidentified 19- to 20-kDa antigen that was present in the bacterium grown at 36°C but not at 28°C, which may have been OspE. Other researchers have observed the presence of antibodies to an 18- to 20-kDa antigen in a number of patients with Lyme disease (9, 11, 12, 19). OspE may be the 20-kDa antigen identified by Engstrom et al. (11) as inducing a specific immune response that is diagnostic of human Lyme disease. It is also possible that there are additional differentially expressed 19-kDa antigens comigrating with OspE that we have not identified.

The anti-OspE polyclonal serum was also reactive with a small number of additional, unknown proteins, some of which were also differentially expressed. An apparently temperature-regulated antigen with a size of approximately 22 kDa was detected in isolate B31. Another differentially expressed antigen, approximately 35 kDa in size, was present in isolate N40. This 35-kDa band did not align with any of the variably expressed antigens visible in Fig. 2B.

The *ospE* gene is directly upstream from the gene for OspF, a 27-kDa lipoprotein, and it has been proposed that these two genes are cotranscribed as an operon (14). Equal amounts of total protein from cultures of isolates N40 and B31, grown at both temperatures, were immunoblotted with 1:100 dilutions of a polyclonal antiserum raised against a recombinant OspF protein of isolate N40 (16) (provided by E. Fikrig). As was the case with OspE, OspF was expressed at higher levels at the higher temperature in isolate N40 (Fig. 3C). The antiserum used, which was raised against the recombinant OspF of isolate N40, failed to produce a strong signal with isolate B31 (data not shown), so it was difficult to determine if there was variable expression of OspF in isolate B31. A possible explanation for this lack of detection is that isolate B31 does not produce an OspF protein. Alternatively, it may be that the sequence of the OspF protein of B31 is very different from that of isolate N40, such that antibodies directed against the N40 OspF protein are unable to recognize the B31 OspF. It has been observed that OspC protein sequences differ greatly between isolates of *B. burgdorferi*, as does the recognition of this protein by antibodies (26).

In contrast to OspE, the OspF proteins produced by the isolates studied in this work were not readily recognized by the *B. burgdorferi*-infected mouse sera, as immunoblots of B31 and N40 probed with this sera did not indicate significant recognition of a protein of the expected size of OspF (27 kDa) (Fig. 2). This finding is especially striking in the case of isolate N40, as the *B. burgdorferi*-infected mouse sera were obtained from mice infected with this same cloned isolate. Nguyen et al. (16) also observed this discrepancy of immune recognition, with anti-OspF antibodies not detected until 90 days postinfection in experimentally infected mice.

Other investigators (2, 7, 8) have observed that *B. burgdorferi* exhibits a heat shock response: the rapid induction of specific proteins as a consequence of a quick temperature shift. Carreiro et al. (7) used immunoblotting techniques to demonstrate that several *B. burgdorferi* heat shock proteins exhibited a marked increase in concentration after a 1-hour change in culture temperature. Using similar methods, we compared the levels of OspE and OspC present in *B. burgdorferi* grown at 23°C with the levels present in the bacterium shortly after shifting the culture temperature to 35°C. We divided mid-log cultures of both isolates B31 and N40, grown at 23°C, into aliquots and incubated the samples at 35°C for either 4 or 8 h and then harvested, washed, and lysed the cultures as described above. Immunoblotting of isolates B31 and N40 with antiserum directed against OspE indicated no detectable change in the OspE content as a result of shifting the cultures from 23 to 35°C for these lengths of time (data not shown). Likewise, immunoblotting with anti-OspC serum showed no detectable changes in the OspC concentration in either isolate arising from temperature shift for up to 8 h (data not shown). A more sensitive method for the analysis of protein content, such as immunofluorescence, might have indicated accumulation of OspE on individual spirochetes, but the reactivity of the polyclonal serum with additional antigens (Fig. 3A and B) would obscure the results. The differential expression that we have reported is apparently not a rapid accumulation of proteins as seen in a classical heat shock response. Instead, these data are suggestive of long-term protein accumulation due to an effect of temperature change that appears to persist through several cycles of bacterial division.

We next explored the possibility that one of the temperature-regulated antigens seen in Fig. 2, with an apparent molecular mass of approximately 37 to 38 kDa, may be P39 (BmpA), an approximately 39-kDa antigen of *B. burgdorferi* infection (23, 24). With a monoclonal antibody directed against P39, immunoblotting of B31 and N40 lysates did not indicate

any significant variation in P39 expression as a consequence of culture temperature in either isolate (data not shown). The single band detected with the anti-P39 serum aligned well with the nonregulated 39-kDa bands visible in Fig. 2. The function of P39 is at present unknown, although it is thought to be a membrane-bound lipoprotein (24). It may be that P39 is required by *B. burgdorferi* in both the unfed tick and mammals and thus is not regulated by a temperature signal. Expression of flagellin, another antigen of mammalian *B. burgdorferi* infection, was not affected by a similar shift in culture temperature (20) and probably represents the nonregulated 41-kDa protein seen in Fig. 2.

It is interesting that the sera used in this work exhibited differences in recognition of antigens of isolates B31 and N40. The sera used in the immunoblots shown in Fig. 2 were derived from mice infected with isolate N40. The sera recognized differentially expressed proteins with molecular masses of 16, 38, and 53 kDa in isolate B31 that were not present in the lysates of isolate N40 (compare Fig. 2A and B). Conversely, the infected mouse sera recognized an N40 antigen with a molecular mass of 18 kDa that was not present in isolate B31 (compare Fig. 2A and B). Differences in the recognition of additional, differentially expressed antigens by the OspE- and OspF-directed polyclonal antisera were also apparent between the two isolates (Fig. 3). It is possible that the degrees of regulation of some genes are different enough between isolates B31 and N40 to allow the expression of a protein in culture by one isolate but not the other. Suk et al. (28) have identified a 21-kDa protein of isolate N40 that did not appear to be expressed in *B. burgdorferi* grown in culture medium but to which an immune response was directed in infected mammals. This protein may be the differentially expressed 22-kDa protein of isolate B31 that was detected with the OspE-directed antiserum (Fig. 3A), as the DNA sequence upstream of the gene described by Suk et al. is very similar to that which precedes *ospE* and the protein shares 71% amino acid identity with OspE (14, 28).

Although the functions of these proteins in *B. burgdorferi* are not known, their inducibility and the apparent early immune response directed against OspE and OspC suggest that they play roles in transmission or establishment of infection. Thermoregulation of virulence factors is common in a number of other bacteria, including *Bordetella pertussis* and several species of *Yersinia* and *Shigella*. Pathogenic determinants are expressed at higher levels when these bacteria are cultured at 37°C than at cooler temperatures, the higher temperature possibly serving to indicate their presence within a warm-blooded host (reviewed in reference 15).

The mechanisms by which the expressions of OspC, OspE, and OspF are regulated remain unknown and may be different for each gene. Understanding the mechanisms by which these and other *B. burgdorferi* proteins are regulated should provide valuable information on the ability of the spirochete to respond to environmental signals as it moves through the transmission cycle between its arthropod and mammalian hosts.

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