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Coordinate Synthesis and Turnover of Heat Shock Proteins in *Borrelia burgdorferi*: Degradation of DnaK during Recovery from Heat Shock

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The synthesis and turnover of heat shock proteins (Hsps) by *Borrelia burgdorferi*, the Lyme disease spirochete, was investigated by radiolabeling of whole spirochetes and spheroplasts, comparison of one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and use of immunochemistry. The ≈72-kDa DnaK homolog and three additional Hsps of 39, 27, and 21 kDa increased in amount by 3- to 15-fold between 2 and 6 h following temperature upshift from 28 to 39°C. Temperature downshift experiments following the transfer of spirochetes from 40 to 28°C showed that within 15 to 30 min, synthesis of most of the major Hsps returned to levels seen in spirochetes statically maintained at the lower temperature. Spheroplasts of *B. burgdorferi* produced by treatment with EDTA and lysozyme were radiolabeled, and specific Hsps were localized to either the cytoplasm or membrane fraction. Further analysis by two-dimensional electrophoresis demonstrated three constitutively expressed DnaK isoforms with pIs near 5.5. A pattern suggestive of DnaK degradation was observed following recovery from heat shock but not in spirochetes maintained entirely at a low temperature. Some of these putative degradation products were recognized by monoclonal antibodies directed against the *B. burgdorferi* DnaK protein. These data suggest that following a period of peak synthesis, DnaK is actively degraded as the spirochete reestablishes its metabolic thermometer. These findings provide a new interpretation of previous work suggesting that 10 to 15 *B. burgdorferi* polypeptides, including DnaK have a common epitope.

Lyme disease is a complex multisystem disorder caused by the tick-borne spirochete *Borrelia burgdorferi*. The initial stage of Lyme disease is typically characterized as a flu-like episode with or without local skin lesions. Systemic secondary and tertiary symptoms include lymphocytic meningoradiculitis (Bannwarth's syndrome), arthritis, myocarditis, and acrodermatitis chronica atrophicans (5, 6, 42). Studies attempting to implicate specific borrelial factors responsible for damage to host tissue have prompted the suggestion that an autoimmune response to the spirochete may play a central role in the host-pathogen interaction (51). Autoimmunity-mediated pathology may result in part from the recognition and response to *B. burgdorferi* heat shock proteins (Hsps) following infection (30), especially since Hsps with similar molecular masses are highly conserved and immunologically cross-reactive. Supportive evidence is provided by earlier studies that associated the immunological response to self stress proteins with autoimmune disease, notably rheumatoid arthritis (16) and systemic lupus erythematosus (37). In addition, the major *B. burgdorferi* Hsp of ≈72 kDa, the DnaK homolog, is immunoreactive and is commonly recognized by sera from Lyme disease patients (3). Conversely, the antigenic similarity of bacterial and host Hsps may protect the pathogen via immunological mimicry. Konga et al. suggest that during mycobacterial infections, the similarity of some mycobacterial Hsps to those produced by host macrophages interferes with efficient recognition by sensitized T cells (24).

B. burgdorferi has a biphasic life cycle. Introduction of the spirochete to either its arthropod vector, the *Ixodes* tick, or a homeothermic host almost certainly involves metabolic adaptation. Schwann et al. established that the *B. burgdorferi* outer surface protein OspC (24 kDa) is specifically induced during tick engorgement while the synthesis of OspA is shut down, seemingly to prepare the spirochete for productive infection (44). In a study examining the survival of *B. burgdorferi* in larval *Ixodes dammini* ticks, maintenance *B. burgdorferi*-infected ticks at temperatures above 27°C rendered them noninfective for mice (48). Investigators from several laboratories (12, 14, 50) have modeled the temperature differences commonly encountered by *B. burgdorferi* during transfer from ambient temperatures found in the ectothermic arthropod vector to the temperatures found in homeothermic hosts and have independently demonstrated the production of Hsps.

The major Hsps of most organisms fall into size classes of approximately 80 to 90, 68 to 75, and 15 to 30 kDa (18, 28). One of the best characterized and most prominent of these classes is the DnaK family (68 to 75 kDa) (36). These proteins have several well-described biological activities; they serve as chaperones, and they participate in protein assembly and stabilization involving an associated ATPase activity. DnaK also facilitates the degradation of denatured proteins by forming a recognition complex specific for one or several proteases, typically the Lon (or La) protease or the Clp protease (34). Sherman and Goldberg demonstrated that DnaK is required for the rapid degradation of a mutant protein by the La protease in *E.*

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coli (47), emphasizing its central role in the process. The chaperones GroEL and GroES also participate in degradation of abnormal proteins (23). The initial study investigating the biochemical activity of the *B. burgdorferi* DnaK suggested that chaperoning activity is limited to the 41-kDa protein flagellin (45); recently, the gene for the *B. burgdorferi* Lon protease, itself an Hsp, was cloned (13).

In this study, the kinetics of the *B. burgdorferi* heat stress response was evaluated by characterizing intrinsic protein synthesis following temperature shift. The *B. burgdorferi* DnaK homolog is rapidly induced following heat shock and then degraded following the period of peak synthesis. The degradation products had similar molecular masses to those previously shown by others to contain a highly reactive, conserved epitope (2). This study also establishes that spheroplasts of *B. burgdorferi* may be used in the study of Hsp metabolism and can serve as an alternative detergent-based solubilization (2, 31, 45) in efforts to localize spirochete proteins, particularly to the cytoplasm or inner membrane.

MATERIALS AND METHODS

***Borrelia burgdorferi* strains and culture conditions.** *B. burgdorferi* B31 (type strain, ATCC 35210) was originally provided by Robert Lane, Department of Entomological Sciences, University of California, Berkeley, Calif. Two low-passage, pathogenic strains of *B. burgdorferi* (B31 and HB19) were supplied by Steven Barthold, Section of Comparative Medicine, Yale University, New Haven, Conn. Strain B31 was originally isolated from an *Ixodes* tick pool (11). Strain HB19 is a human isolate (8). The cultures were maintained in modified Barbour-Stoenner-Kelly (BSK II) medium as described by Barbour (7), except that the proportion of bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) was reduced to 1.25% (wt/vol).

Cultures of *B. burgdorferi* B31 (type strain) for analysis of heat stress responses and for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were grown at 28°C to cell densities of 8.5×10^7 to 1.4×10^8 cells per ml, harvested by centrifugation at $10{,}000 \times g$ for 20 min, and immediately resuspended to a concentration of 6×10^8 to 1×10^9 cells per ml in BSK II medium containing [^{35}S]methionine and [^{35}S]cysteine (ICN Biomedicals, Irvine, Calif.) at 100 to 200 μCi/ml. Aliquots of the resuspended spirochetes were incubated between 28 and 42°C for periods ranging from 15 min to 6 h. These labeled cells were then harvested by centrifugation at $10{,}000 \times g$ for 20 min at 6°C. In pulse-chase experiments, labeled spirochetes were washed three times in phosphate-buffered saline (PBS) prior to temperature shift. Labeling experiments with whole spirochetes or spheroplasts (described below) were performed at least three times. Hsp synthesis was determined as a percentage of total spirochete ^{35}S-labeled protein detected by scanning laser densitometry of audioradiographic film. The amount of individual Hsp synthesis varied by approximately 15% from experiment to experiment.

Synthesis of Hsps following temperature shift. For temperature upshift experiments, spirochetes were cultivated either at 28 or 34°C, resuspended in BSK II medium, and divided into aliquots. One portion was shifted to 40°C in the presence of [^{35}S]methionine and [^{35}S]cysteine, and labeled spirochetes were removed at designated times between 15 min and 6 h after the start of incubation. A second portion was radiolabeled at the lower temperature, and organisms were removed after 15 min and 2 h. Labeling was terminated by chilling on ice. The spirochetes were then harvested by centrifugation, washed, and prepared for SDS-PAGE and autoradiography. In temperature downshift experiments, spirochetes were initially incubated at 40°C for 2 h. Aliquots were shifted to 28°C or kept at 40°C, and protein synthesis was monitored over time by radiolabeling as described above.

Preparation of *B. burgdorferi* spheroplasts. Spirochetes were converted to spheroplasts by the method of Bruck et al. (10), an adaptation of earlier methods designed for *Escherichia coli* and *Salmonella typhimurium* (9, 40). Briefly, *B. burgdorferi* was propagated in BSK II medium at 34°C to mid-logarithmic phase (1×10^7 to 5×10^7 cells per ml) and harvested at $10{,}000 \times g$ for 10 min. The pellet was then gently washed in an equal volume of 0.01 M Tris HCl (pH 7.95) and centrifuged as described above. Slight alterations in the pH of the Tris HCl buffer drastically reduced the conversion of spirochetes to spheroplasts. This pellet was resuspended in 0.5 M sucrose–10 mM Tris-HCl (pH 7.82) to approximately 10^8 cells per ml. Lysozyme (2 mg/ml) was added to achieve a final concentration of 40 μg/ml. After 10 min at room temperature, 1 mM EDTA (pH 7.5) was slowly added to bring the suspension to the original volume. Preparations were then examined by dark-field microscopy, and those containing more than 90% spheroplasts were used in radiolabeling experiments. Spheroplasts were recovered by centrifugation at $10{,}000 \times g$ for 10 min, washed gently with PBS, and radiolabeled with [^{35}S]methionine and [^{35}S]cysteine at 100 μCi/ml in BSK II medium. Whole spheroplasts were then recovered by centrifugation, and membrane and soluble fractions of each were obtained by disruption by freeze-thaw and brief sonication followed by centrifugation at $110{,}000 \times g$ for 2 h.

SDS-PAGE, fluorography, and scanning laser densitometry. Pelleted bacteria were resuspended in 50 to 100 μl of 0.5 M Tris HCl (pH 6.8). The protein concentration of each sample was determined either by the method of Lowry et al. (29) or with bicinchoninic acid (Pierce, Rockford, Ill.), and the samples were stored at −80°C. For SDS-PAGE, the samples were thawed, suspended in sample buffer with 2-mercaptoethanol (5%, wt/vol), and heated for 5 min at 95°C. SDS-PAGE was performed by the method of Laemmli (25). All reagents were electrophoresis grade and were purchased from Bio-Rad Laboratories, Richmond, Calif. Approximately 80 to 200 μg of protein was applied per lane on a discontinuous 1.5-mm-thick acrylamide gel containing a 5% stacking gel and a 10% resolving gel (19 cm long). Electrophoretic separation was done at 12 mA and 10°C overnight. Polyacrylamide gels containing radiolabeled proteins were stained with Coomassie brilliant blue R-250, treated with a fluorographic enhancer (Research Products International, Rockwell, Ill.), dried, and exposed to photographic film (XAR-5; Kodak, Rochester, N.Y.) at −80°C. Autoradiograms were quantified by scanning laser densitometry (LKB UltroScan XL, Bromma, Sweden).

Two-dimensional SDS-PAGE. Spirochetes were grown to 0.5×10^8 to 2.0×10^8 cells per ml, pelleted, and resuspended to a concentration of 1.0×10^9 to 4.0×10^9 cells per ml in RPMI 1640 labeling medium (Sigma) containing [^{35}S]methionine (Express; New England Nuclear, Boston, Mass.) at 400 μCi/ml. The spirochetes were then incubated at 28 or 39°C for 3 h, harvested, and washed three times in PBS. The cells were resuspended in 20 to 50 μl of lysis buffer (9.5 M urea, 2% Nonidet P-40, 2% ampholines, 5% 2-mercaptoethanol) at approximately 2×10^6 cpm per sample.

Tube gels 1.5 mm thick and 10 cm long were prepared with a gel solution containing 9 M urea, 4% acrylamide, 2% NP-40, a 1:16 dilution of 5–8 Pharmolytes (Pharmacia P-L Biochemicals, Milwaukee, Wis.), 0.3% $NH_4(SO_4)_2$, and 0.1% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). After polymerization, the gels were prefocused at 200 V for 1 h before sample loading. The samples were electrofocused for 15 to 20 h at 400 V and an additional 1 h at 1,000 V. The tube gels were then electrophoresed through a 10% acrylamide SDS-PAGE gel, prepared as described above. Isoelectric points were determined by comparison with a carbamylated standard (Pharmacia P-L Biochemicals). The gels were fixed, enhanced for autoradiography, and dried. Photographic film was developed following multiple exposures to optimize the resolution of proteins of interest.

Immunoblot analysis. *B. burgdorferi* proteins were transferred from SDS-PAGE gels to polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.) in a Transblot cell (Hoefer Scientific Instruments, San Francisco, Calif.) at 100 mA (constant) at 6°C for 3.5 h. The transfer buffer consisted of Tris base (48 mM), glycine (39 mM), SDS (0.37%, vol/vol), and methanol (20%). Transfer to the membrane was confirmed by Ponceau S staining. The polyvinylidene difluoride membrane was blocked with defatted milk protein for 1 h and then probed with specific antisera. Monoclonal antibodies (MAbs) directed against OspA (H5332) and OspB (H6831) were kindly supplied by Alan Barbour. A polyclonal antiserum directed against *E. coli* DnaK was generously provided by David Nelson and used at a dilution of 1:500. Monoclonal antisera directed against the *B. burgdorferi* DnaK homolog were gifts from Michael Kramer (LA-3) and Jorge Benach (CB312). For the blots shown here, LA-3 was used at a dilution of 1:50 and CB312, provided as hybridoma supernatant, was used at a dilution of 1:2. Primary antisera were diluted in blocking buffer, filtered through a 0.22-μm-pore-size membrane, and incubated at room temperature with shaking for 60 to 90 min. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G or conjugated goat anti-rabbit immunoglobulin G (TROPIX, Inc. Bedford, Mass.) diluted 1:10,000 in blocking buffer served as the secondary antibodies. Proteins recognized by antisera were identified by chemiluminescence as specified by the manufacturer (Tropix).

RESULTS

Kinetics of Hsp synthesis following heat shock. While a number of investigators have investigated aspects of the *B. burgdorferi* heat shock response, the kinetics of Hsp synthesis following temperature shift has not been examined thoroughly. At 15 min following a shift from 28 to 40°C, DnaK levels increased 2.7-fold and accounted for 7.1% of the protein synthesized following temperature induction (Fig. 1). Between 30 min and 1 h, DnaK reached a maximum of 10.3% of the labeled cell protein, 4.3 times greater than the level at 28°C (Fig. 2). With continued labeling of *B. burgdorferi* at 40°C, the level of protein decreased to 5.3% after 4 h and to 4.7% after 6 h. When spirochetes were cultivated at 39°C for 1 to 2 weeks, DnaK was maintained at approximately twice the level observed at 28°C (43). Hsp27 and Hsp22, while not present at as high a copy number as DnaK, reached their respective maxi-

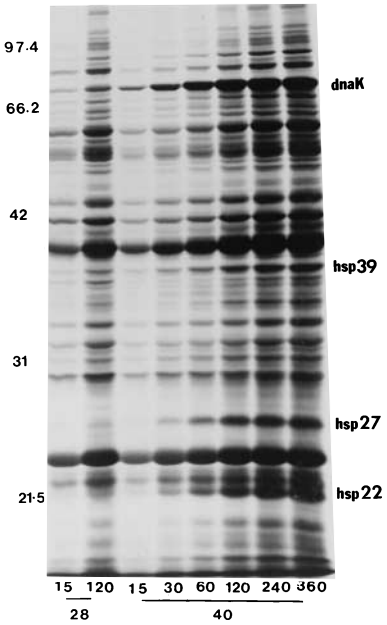

FIG. 1. Time course of *B. burgdorferi* Hsp synthesis at 40°C following a shift from 28°C. Spirochetes were incubated in labeling medium for 15 to 360 min. Lanes 1 and 2 show radiolabeled proteins synthesized by spirochetes maintained at 28°C for 15 and 120 min. Approximately 60 μg of protein was loaded per lane. Molecular mass markers, in kilodaltons, appear on the left.

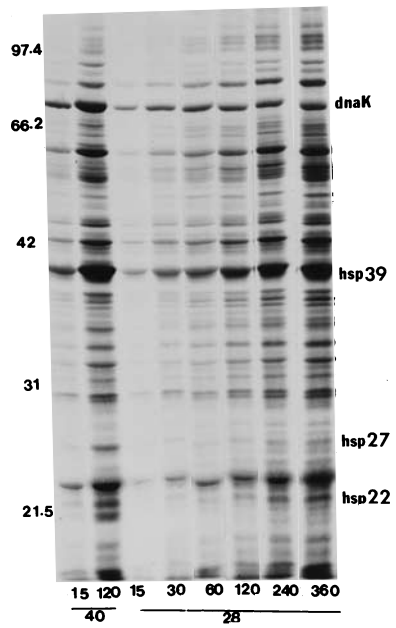

FIG. 3. *B. burgdorferi* protein synthesis following temperature downshift from 40 to 28°C. Spirochetes were labeled at 28°C for 15, 30, 60, 120, 240, or 360 min. Lanes 1 and 2 contain radiolabeled proteins from spirochetes maintained at 40°C for 15 and 120 min. Approximately 70 μg of protein was loaded per lane. Molecular mass markers, in kilodaltons, appear on the left.

mum concentrations at 2 h, and the levels were relatively constant to 6 h. In contrast, Hsp39 was weakly induced and its level declined slightly after 2 h (Fig. 1 and 2). Protein synthesis was negligible when spirochetes were radiolabeled above 40°C (43).

As shown previously (14), the synthesis of other well-studied *B. burgdorferi* proteins, notably OspA, OspB, and flagellin, was not thermoregulated. The level of OspB was relatively constant throughout the 6-h labeling period and rose slightly after the first hour, to approximately 2.5% of the total protein at 6 h. In a control culture labeled at 28°C for 2 h, OspB represented 2.4% of the total *B. burgdorferi* protein, as determined by scanning laser densitometry.

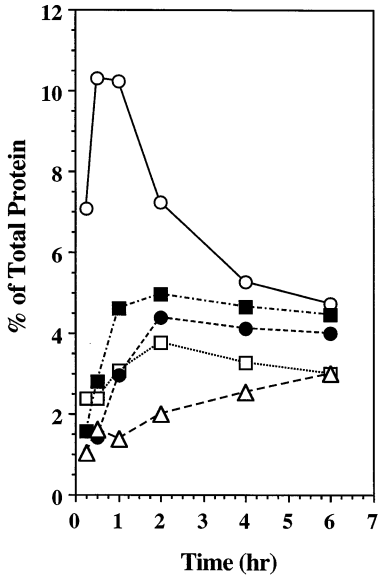

FIG. 2. Synthesis of the various *B. burgdorferi* proteins as percent total protein based on [^{35}S]methionine and [^{35}S]cysteine incorporation. Shown is a quantification of Fig. 1 by scanning laser densitometry for the designated proteins at 0.25, 0.5, 1, 2, 3, 4, and 6 h. Symbols: open circles, DnaK; open triangles, OspB; solid circles, Hsp27; solid squares, Hsp22; open squares, Hsp39.

Kinetics of Hsp synthesis following recovery from heat shock. The synthesis of individual Hsps following temperature downshift from 40 to 28°C was then monitored. Spirochetes were heat shocked for 2 h at 40°C and then radiolabeled at 40 or 28°C (downshift). In response to temperature downshift, the DnaK content of strain B31 decreased steadily from 13.6% of the total spirochete protein at 15 min to 3.8% at 4 h (Fig. 3 and 4). In comparison, DnaK represented 8.3% of the total spirochete protein in the parallel culture labeled for 4 h at 40°C. In this experiment, the synthesis of Hsp27 and Hsp22 was consistently low throughout the downshift. However, these Hsps were induced two- to threefold in the parallel 40°C culture, labeled for an additional 2 h, at levels similar to those shown in Fig. 2 after 4 h at 40°C.

Localization of DnaK and a heat-sensitive 29-kDa protein. Two major thermoregulated *B. burgdorferi* proteins were localized to the membrane and cytosolic compartments of whole spirochetes and spheroplasts. Whole spirochetes and spheroplasts were radiolabeled at 28 or 38°C, fractionated, and prepared for SDS-PAGE in the same manner. Spheroplasts were capable of de novo protein synthesis, as indicated by the differential incorporation of [^{35}S]methionine and [^{35}S]cysteine into thermoregulated proteins. DnaK was induced at the higher temperature in the whole-spheroplast preparation (Fig. 5, compare lane 4 with lane 3) and preferentially associated with the spheroplast cytoplasm rather than with the membrane (lanes 7, 8, 11, and 12). This difference was primarily due to the depletion of OspA, OspB, and flagellin in the spheroplast cytosolic fraction. A 29-kDa protein observed in an earlier

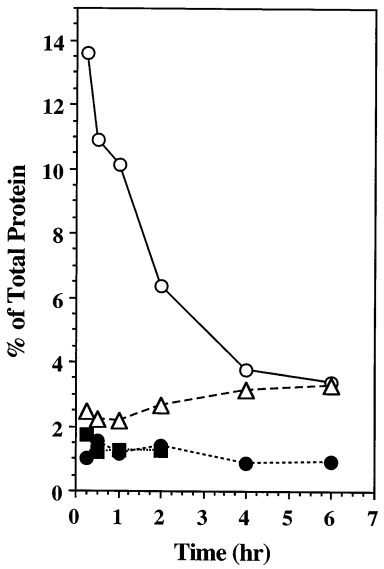

FIG. 4. Synthesis of the various *B. burgdorferi* proteins at 28°C following heat stress at 40°C based on [^{35}S]methionine and [^{35}S]cysteine incorporation. Shown is a quantification of Fig. 4 by scanning laser densitometry for the designated proteins at 0.25, 0.5, 1, 2, 4, and 6 h. Symbols: open circles, DnaK; open triangles, OspB; solid circles, Hsp27; solid squares, Hsp22.

study (14) was readily observable in spheroplasts labeled at 28°C, particularly in the cytoplasmic fraction (lane 7). This protein was much less prominent at 39°C (lane 8). A similar pattern was noted in the spirochete preparations (lane 5 versus lane 6).

Identification of Hsps by two-dimensional electrophoresis. Hsps of strains HB19 and B31 were further characterized by two-dimensional electrophoresis. Three DnaK isoforms (as distinct triplets) within a narrow pH range around 5.5 were detected. All isoforms were present at the low temperature, although the most acidic species was less prominent than the others (see Fig. 7A for the situation with strain HB19). Three DnaK isoforms were also clearly evident at 39°C when film was exposed for very brief periods (data not shown). The most interesting finding was a pattern suggestive of DnaK degradation from spirochetes labeled throughout 2 to 3 h of heat shock. A series of triplets ranging from 70 to 26 kDa with pIs around 5.5 were apparent in both strains tested, and are shown in Fig. 6B for strain HB19 and in Fig. 6D for strain B31. We were unable to obtain a similar pattern in spirochetes labeled at 28°C, despite exposing the film for prolonged periods.

Additional minor Hsps with pIs between 5 and 8, including proteins of 60, 39, 36, 22, 18, and 14 kDa, were revealed by two-dimensional electrophoresis (Fig. 6D). The basic pI of the 39-kDa Hsp compares closely to that determined for the *E. coli* DnaJ protein (41).

Reactivity of MAbs. To more conclusively determine if the triplets seen by two-dimensional SDS-PAGE were the products of proteolytic processing or limited proteolytic degradation of DnaK, we probed similar preparations with two MAbs directed against the *B. burgdorferi* DnaK. Strains HB19 and B31 were heat shocked for 2 h at 39°C to induce DnaK, and cell lysates fractionated by one-dimensional SDS-PAGE were probed with the MAbs. As expected, MAbs CB312 and LA-3 reacted most strongly against DnaK. However, protein bands with lower molecular masses were recognized equally well in both strains (Fig. 7). Most of these minor bands were between 70 and 55 kDa, with the most prominent being at 60 kDa. It was necessary to expose the film for 15 min to 1 h to strengthen the signal associated with these minor bands.

In the original report describing the reactivity of CB312 (17), purified MAb weakly recognized 10 to 12 bands between 70 and 38 kDa. The CB312 hybridoma supernatant used here reacted similarly, although the 60-kDa band was more conspicuous. With strain B31, the reactivity of the bands at and below 60 kDa increased with time following heat shock (Fig. 7B, right panel). A similar trend was obtained in a different set of experiments with strain HB19 (Fig. 7A, right panel, lanes 1 to 3). While the LA-3 antibody also recognized the band at 60 kDa, the signal was much weaker than that obtained with MAb CB312 and the additional minor bands were barely discernible (Fig. 7A and B, left panels). Control assays lacking primary antibody demonstrated that a strongly reactive band at 39 kDa, thought initially to be recognized by both MAbs, was recognized solely by the alkaline phosphatase-conjugated secondary antibody. Further studies are planned to examine how recovery from heat shock contributes to the generation of these smaller, cross-reactive proteins.

A polyclonal antibody directed against *E. coli* DnaK recognized the *B. burgdorferi* homolog but not proteins with lower molecular masses.

DISCUSSION

Thermal stress induces rapid synthesis of Hsps in *B. burgdorferi*. The genes for a number of *B. burgdorferi* Hsp homologs, including DnaK (3, 52), the 60-kDa GroEL (21), the 39-kDa DnaJ (4, 52), and the 21-kDa GrpE (52), have been cloned. The biochemical activities of these gene products are well described for *E. coli*, and investigators are beginning to conduct similar studies with *B. burgdorferi* (45, 52). One difference has been observed; *dnaJ* and *grpE* mutants of *E. coli* are complemented by the *B. burgdorferi* homologs whereas an *E. coli dnaK* mutant is not (52). Additional information regarding the roles of specific *B. burgdorferi* Hsps and related temperature-dependent events within the spirochete may provide

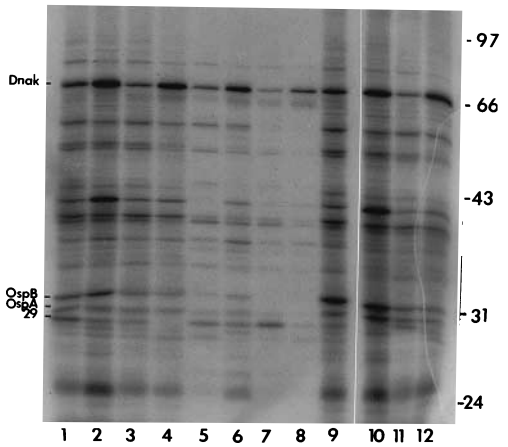

FIG. 5. Localization of *B. burgdorferi* Hsps in spheroplasts and whole spirochetes. Hsps were identified by radiolabeling either spirochetes or spheroplasts for 2 h as described in the Materials and Methods. Odd-numbered lanes contain either spirochetes or spheroplasts labeled at 28°C, and even-numbered lanes contain spirochetes or spheroplasts labeled at 39°C. Lanes: 1 and 2, whole spirochetes; 3 and 4, whole spheroplasts; 5 and 6, spirochete soluble fraction; 7 and 8, spheroplast soluble fraction; 9 and 10, spirochete membrane fraction; 11 and 12, spheroplast membrane fraction. Lane pairs contain equivalent amounts of sample. Molecular mass markers, in kilodaltons, appear on the right.

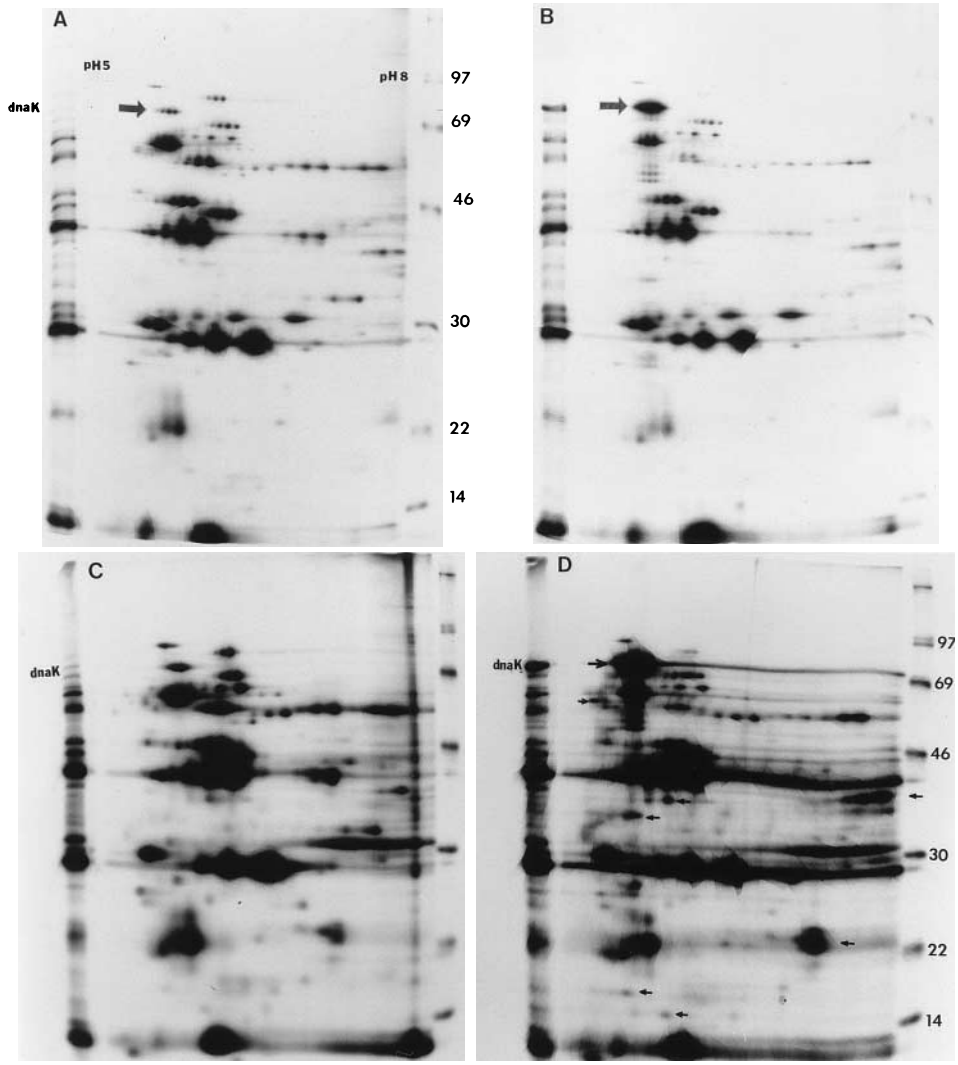

FIG. 6. *B. burgdorferi* Hsps resolved by two-dimensional SDS-PAGE and autoradiography. (A and B) Strain HB19 proteins radiolabeled for 2 h at 28°C (A) and 39°C (B), with identical counts per minute loaded per gel. The distinct triplet at the DnaK band in panel A (arrow) was also observed at 39°C in panel B, at much shorter film exposures (1 to 2 h). Note that in panel B, the series of triplets beneath DnaK have identical pIs. (C and D) Strain B31 proteins radiolabeled for 2 h at 28°C (C) and 39°C (D), with identical counts per minute loaded per gel. DnaK is indicated by a large arrow in panel D, and additional Hsps are indicated by small arrows. Molecular mass markers in kilodaltons and the pH range of the isoelectric focusing gels in panels B to D are the same as shown in panel A.

important insight into the physiological adaptation that occurs immediately following introduction into a mammalian host.

In this study, we monitored the synthesis, turnover, and localization of four major *B. burgdorferi* Hsps. In temperature upshift studies, all four Hsps reached their peak levels 1 to 2 h after the heat shock (Fig. 1 and 2). The level of the predominant Hsp, DnaK, increased fourfold within the first hour after shift to a high temperature. Thereafter, it decreased relative to total cell protein, although it was still substantial after 6 h. The synthesis of additional proteins was also induced by increased temperature. The level of Hsp22, possibly either the GrpE or Lon homolog, increased four- to fivefold. Hsp39 (most probably the DnaJ homolog) and Hsp27 were induced two- to fourfold. In addition, two-dimensional SDS-PAGE and autoradiography (Fig. 6D) showed that proteins of 36, 42, and 60 kDa (perhaps the GroEL homolog) were thermoregulated.

Experiments designed to monitor Hsp synthesis during recovery from heat shock demonstrated a rapid and continuous decrease in DnaK production during the initial 4 h, as shown in Fig. 4. In contrast, synthesis of Hsp27 and Hsp22 was essentially invariant during the same period. These results, taken together with the results of the temperature upshift experiments, establish that increased synthesis of the major *B. burgdorferi* Hsps occurs immediately in response to thermal insult and plateaus 2 h later. Studies involving cultivation of *B. burgdorferi* for 2 weeks at 28 and 38°C (two to four serial transfers) revealed that DnaK is maintained at a level two to three times greater than at the lower temperature. However, continued cultivation at 38°C resulted in markedly elongated spirochetes with decreased motility and a disintegrating outer surface (43). A possible explanation for this phenomenon is suggested by several investigators. McCarty and Walker established that in *E. coli*, the production of a mutant DnaK (nonphosphorylated, ATPase defective) at or above 34°C leads to filamentation (35). Also, if the *E. coli* Hsp SulA, which is made in response to DNA damage, is not degraded, the cells become filamentous and die (22). Perhaps the aberrant forms of *B. burgdorferi* that appear after several transfers under heat shock conditions re-

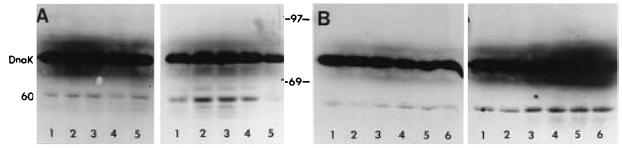

FIG. 7. Immunoblots showing *B. burgdorferi* proteins recognized by Mab directed against DnaK from strain HB19 (A) and strain B31 (B) following peak synthesis of DnaK. Blots were reacted with LA-3 (left panel) and CB312 (right panel), each MAb directed against the *B. burgdorferi* DnaK homolog. (A) Strain HB19 heat shocked at 39°C for 2 h (lane 1) and either maintained at 39°C for an additional 1 h (lane 2) or 2 h (lane 3) or shifted to 28°C for 1 h (lane 4) or 2 h (lane 5). (B) Strain B31 heat shocked at 39°C for 2 h (lane 1), not heat shocked (lane 2), heat shocked at 39°C for 2 h and shifted to 28°C for 1 h (lane 3), heat shocked at 39°C for 2 h and shifted to 28°C for 2 h (lane 4), heat shocked at 39°C for 2 h and shifted to 28°C for 3 h (lane 5), or heat shocked at 39°C for a total of 5 h. Apparent molecular mass markers are indicated in kilodaltons.

sult in part from an inability of DnaK to efficiently aid in the proteolytic degradation of irreversibly denatured proteins. Future attempts to address this question will be enhanced by the recent description of the *B. burgdorferi* Lon protease (13), which represents an important step in establishing a pathway for processing denatured proteins in the spirochete.

Numerous microorganisms and eucaryotic cells survive a shift to lethal temperatures if they are initially exposed to a sublethal heat treatment. Usually 5 to 15 min of mild heat shock is adequate to induce this state of thermotolerance (19). Despite various thermal pretreatments, we were unable to generate thermotolerance in *B. burgdorferi*. The spirochete still failed to demonstrate a heat shock response above 40°C and died rapidly. This thermal sensitivity and inability to induce thermotolerance are not readily explainable, especially since contributing evidence has established that much of the heat shock apparatus is present in *B. burgdorferi*. We believe that the spirochete physiologically behaves as an "intermediate" responder to heat shock conditions, somewhere between the responsive *E. coli*, which readily exhibits thermotolerance, and the nonresponsive *Treponema pallidum*, which is exquisitely temperature sensitive and unique in its failure to exhibit a measurable heat shock response (15, 50).

Spheroplasts of *B. burgdorferi* were useful in establishing the localization of DnaK and a heat-sensitive protein of 29 kDa, which we observed previously (14). The spheroplasting procedure may have several additional applications; it may serve as a useful tool in studies of the inner membrane, particularly if functionally active material is required, and it may be a useful surrogate in attempts to establish a genetic transfer system for the spirochete. In this study, spheroplasts were produced to limit outer surface protein contamination of the fraction containing the protoplasmic cylinder, a common problem in fractionation schemes (32). As shown by intrinsic labeling, spheroplasts retained the ability to synthesize protein. However, despite numerous attempts, spheroplasts derived by EDTA and lysozyme treatment did not divide in culture.

Several experimental approaches have been used in efforts to localize proteins to subcellular compartments of *B. burgdorferi*. Specific and nonspecific efforts include freeze-fracturing, immunoelectron microscopy, and phase partitioning with detergent. Recently, Scorpio et al. combined several of these methods and localized DnaK almost exclusively to a soluble fraction, using spirochetes cultivated at 33 or 37°C (45). However, when *B. burgdorferi* was cultivated at 20°C, DnaK was also found in a Triton X-100-insoluble membrane fraction (45). We accounted for DnaK by differential radiolabeling rather than immunoblot analysis, and we agree that DnaK is associated preferentially with the cytoplasmic fraction. However, our data suggest that DnaK also associates with the membrane at both low and high temperatures. One caveat is that conversion of whole spirochetes to spheroplasts is itself a stressful event (1) and may result in heat shock-induced events that alter DnaK localization.

Several biochemical activities are known for DnaK (36). DnaK binds to partially unfolded and denatured proteins, protecting them and assisting in proper folding or renaturation (49). When the thermal or chemical insult is effectively overcome, the proteins are released. An alternative outcome is DnaK-mediated facilitated clearance of denatured proteins (46), a two-step process involving initial binding followed by presentation of the complex to a specific protease. Perhaps the most widely described biochemical activity of DnaK is as a chaparone to deliver bound protein to a specific cellular destination. In *B. burgdorferi*, DnaK serves as a chaperone in concert with the GroEL homolog, Hsp60, in the efficient processing of flagellin (45). However, this role appears to be limited, because DnaK did not bind additional proteins.

A combination of temperature-dependent radiolabeling and two-dimensional electrophoresis was used to unequivocally establish that no additional acidic proteins migrate with the same M_r as DnaK (Fig. 7). Closer analysis indicates that at least three species are present, each with a distinct pI. Several likely explanations are that these DnaK species (i) represent three distinct isomeric forms; (ii) represent different conformational states; (iii) may be due, in part, to posttranslational modification (notably phosphorylation); or (iv) represent differential expression and translation of *dnaK*. Supportive evidence is provided by studies of both *E. coli* and *B. burgdorferi*. While *dnaK* appears to be a single-copy gene in the spirochete, two different transcripts are produced (3). In *E. coli*, DnaK phosphorylation increases the binding affinity for polypeptide substrates (47) and, in addition, conformational changes occur in response to both protein binding and ATP hydrolysis (27).

We did not anticipate finding putative DnaK degradation products, i.e., the series of triplets with molecular masses less than 70 kDa and pIs corresponding to those of DnaK (Fig. 6B). However, in this two-dimensional SDS-PAGE analysis, the spirochetes were radiolabeled for 2 h at 40°C, the condition that induces the highest levels of DnaK, and the pH gradient of the isoelectric focusing gel was narrow enough (pH 5 to 8) to distinguish the isoforms throughout the gel. The triplets were detected in both strains examined, and most had molecular masses of 70 to 55 kDa. Recognition of similar bands by the two MAbs indicates that the respective epitope of each antibody is retained in some of the larger putative DnaK breakdown products. Also, the N-terminal domain of DnaK is highly conserved, is responsible for ATP binding, and contains numerous acidic residues (18). Since the pIs of each putative DnaK product triplet are essentially conserved, our findings imply that degradation is most probably occurring at the C terminus of the protein.

Characteristic Hsp breakdown products are well described. Those first observed for the 70-kDa Hsp of *Drosophila melanogaster* (38) were not generated by proteases but were due to an autocatalytic process. Additional studies of protein degradation in *D. melanogaster* (26) expanded these original findings and established that Hsp70 is degraded rapidly during recovery from heat shock. This also occurs in *Neurospora crassa* (39), in which Hsp70 degradation is actually inhibited during heat shock.

Degradation of the *B. burgdorferi* DnaK was initially postulated when recombinant protein from *E. coli* lysates probed with high-titer anti-*Borrelia* sera from patients with Lyme dis-

ease resulted in reactive proteins with molecular masses less than 70 kDa (3). Additional support for DnaK degradation is provided by the reactivity of MAb CB312, shown previously to recognize proteins with molecular masses less than 70 kDa (17), in a pattern very similar to that obtained by us (Fig. 7). Lastly, Anda et al. produced a MAb demonstrating broad reactivity to approximately 30 *B. burgdorferi* polypeptides, all with pIs between 5.4 and 6.2 (2). The MAb reacted to a linear epitope found in a 93-kDa protein and also recognized a proposed discontinuous epitope in the C-terminal region of DnaK. While protease inhibitors did not prevent fragmentation of p93 during the experimental procedures, proteolysis of p93 was not entirely ruled out, because smaller forms could be generated during the growth phase. Taken together with our radiolabeling and immunoblot results, it appears that some of the reactive bands of less than 70 kDa are products of DnaK degradation, not unique polypeptides as thought originally. Furthermore, as crude lysates of *B. burgdorferi* probably contain some of these highly immunogenic DnaK degradation products, they should be used with caution in serological assays.

The mechanism of DnaK degradation in *B. burgdorferi* remains to be determined. While this process may be autocatalytic, it more probably involves the participation of one or more endogenous proteases. In *E. coli*, DnaK interacts in concert with DnaJ, GrpE, and the Lon protease in the facilitated proteolytic clearance of denatured proteins (20, 33, 41). The recent identification of the last of these *B. burgdorferi* homologs (13) now permits the investigation of their respective roles in the heat shock-induced proteolytic pathway of the spirochete.

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