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Heat Shock Response of Spirochetes

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We examined the heat shock response of the pathogenic spirochetes *Treponema pallidum*, *Borrelia burgdorferi*, and *Leptospira interrogans* and certain saprophytic spirochetes. Cellular proteins synthesized after shifts to higher temperatures were [^{35}S]methionine labeled and analyzed by gel electrophoresis and fluorography. Only *T. pallidum* failed to exhibit an obvious heat shock response. GroEL and DnaK homologs were identified in the various species, although these proteins were not thermoinducible in *T. pallidum* or *Treponema denticola*. DNA hybridization studies indicate that spirochetal *groEL* and *dnaK* genes are highly conserved.

Heat shock proteins (Hsps) are synthesized when cells are exposed to elevated temperatures or to a variety of other stresses (18, 22). In *Escherichia coli*, genes encoding the major Hsps are expressed at a basal level at normal growth temperature but are transcribed at greater levels at higher temperatures. The heat shock response is highly conserved among procaryotic and eucaryotic cells. While the exact function of Hsps has not been precisely determined, certain Hsps have been shown to act as chaperones for the assembly of complex and oligomeric proteins (4, 14). Some Hsps are thought to protect intracellular pathogens against the hostile environment of host phagocytic cells (10). Additionally, homologs of two major Hsps, GroEL and DnaK (members of the Hsp60 and Hsp70 families, respectively [18, 22]), are of considerable importance in the immunology and pathology of various bacterial and parasitic infections (13, 20, 29, 30).

We are interested in the molecular genetics and physiology of *Treponema pallidum*, the agent of syphilis, as well as certain other pathogenic spirochetal species. Techniques have not been developed to genetically manipulate these organisms, and there currently is little information concerning spirochetal genetic organization and gene expression. In hopes of enhancing our basic knowledge of the biology of these organisms, we have investigated the ability of *T. pallidum*, *Borrelia burgdorferi*, *Leptospira interrogans*, and certain saprophytic species (*Treponema phagedenis* biotype Reiter, *Treponema denticola*, and *Leptospira biflexa*) to synthesize Hsps in response to temperature shifts.

For our studies, *T. pallidum* Nichols organisms were cultivated in rabbit testes, extracted in labeling medium at 34°C, and standardized to 6×10^8 cells per ml as previously described (26, 27). *T. phagedenis* biotype Reiter and *T. denticola* 11 organisms were grown under anaerobic conditions (GasPak; BBL Microbiology Systems, Cockeysville, Md.) at 37°C in Spirolate broth or PPLO broth (BBL), respectively, supplemented with 10% heat-inactivated normal rabbit serum and 5 μg of cocarboxylase per ml. Cells were pelleted by centrifugation and suspended in labeling medium to 3×10^8 to 5×10^8 cells per ml (26, 27). Cells from a high-passage isolate of *B. burgdorferi* HB19 were grown at 30°C in BSKII medium (1), pelleted, and suspended in modified BSKII medium lacking Neopeptone and Yeastolate (Difco Laboratories, Detroit, Mich.) to 2×10^8 cells per ml. *Leptospira interrogans* serovar hardjo and *L. biflexa* serovar patoc organisms were grown in bovine serum albumin-polysorbate 80 medium (8) at 30°C, pelleted, and suspended in fresh medium to 6×10^8 cells per ml.

Sets of 1-ml samples of each organism in sterile Eppendorf tubes were incubated at their respective normal growth temperature for 15 min before the temperature shifts. Samples of *T. pallidum* were shifted from 34 to 36, 38, 40, or 42°C; *T. phagedenis* biotype Reiter and *T. denticola* were shifted from 37 to 42 or 47°C; and *B. burgdorferi*, *L. interrogans*, and *L. biflexa* were shifted from 30 to 37 or 42°C. A control sample for each organism remained at the normal growth temperature. Fifteen minutes after the temperature shift, 70 to 80 μCi of [^{35}S]methionine (10 μCi/μl; ICN Biomedicals, Inc., Irvine, Calif.) was added to the samples and incubation was continued at the same temperature for another 60 min. The samples were chilled by submersion in an ice water bath, and the cells were pelleted by centrifugation, washed once in cold phosphate-buffered saline, resuspended in 264 μl of solubilization buffer (26), and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots of the solubilized cellular extracts from each set of samples were analyzed on 15% SDS–polyacrylamide gels. The gels were processed for fluorography as previously described (27).

Of the spirochetes examined, only *T. pallidum* failed to exhibit a discernible heat shock response (Fig. 1). Total *T. pallidum* protein synthesis decreased at successive 2°C increments above the 34°C control until, at 42°C, protein synthesis was negligible. Additional experiments with *T. pallidum* cells radiolabeled at 31 and 33°C following a temperature shift from 29°C failed to demonstrate a heat shock response. The protein profiles of organisms labeled at these lower temperatures were indistinguishable from that of organisms labeled at 34°C (data not shown). While *T. pallidum* is the only bacterial species reported to date that does not exhibit a classical heat shock response, our results appear to be consistent with the previously observed in vitro and in vivo thermolability of this organism. For example, Baseman and Hayes (3) reported that an incubation temperature of 34°C was optimal for incorporation of ^{3}H-labeled amino acids into *T. pallidum* proteins based on trichloroacetic acid-precipitable counts following a 21-h labeling period. At higher temperatures, protein synthesis decreased substantially and treponemes lost motility within a few hours. Fieldsteel et al. (11) found that temperature was one of the most restrictive conditions in achieving limited multiplica-

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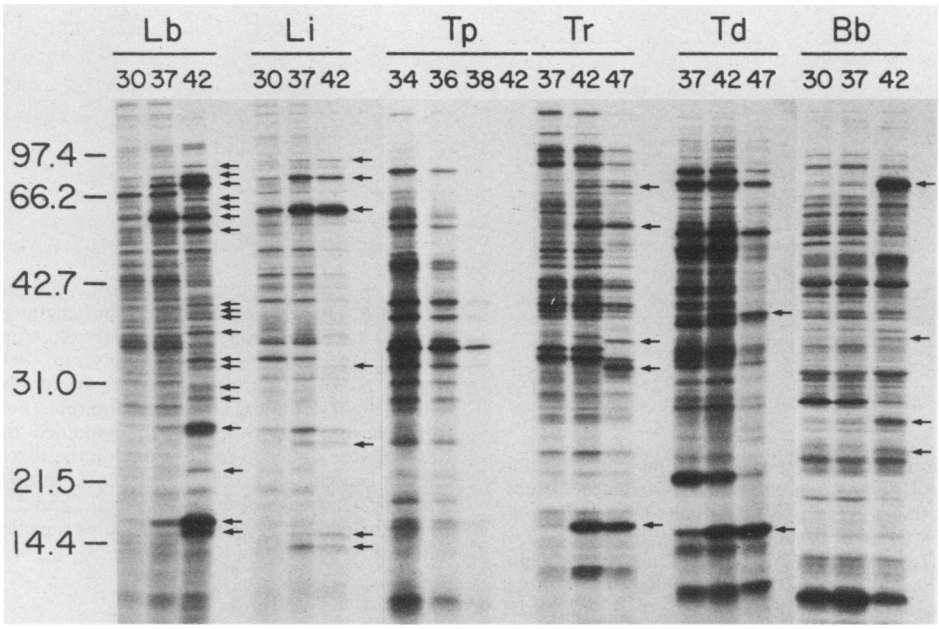

FIG. 1. Fluorogram of [^{35}S]methionine-labeled proteins of various spirochetal species before and after heat shock. Sets of 1-ml samples of cells were incubated at the normal growth temperature for the species for 15 min. A control sample for each organism was retained at this lower temperature (left lane for each set); the remaining samples were shifted to the indicated temperatures. After 15 min, 70 to 80 μCi of [^{35}S]methionine was added to each sample and the incubation was continued for an additional 60 min. For each set of samples, aliquots of solubilized cellular extracts representing total protein from an equal number of cells were run on 15% SDS-polyacrylamide gels (27). Proteins synthesized at a higher level following temperature shift are indicated by the arrows. Molecular mass standards are indicated on the left in kilodaltons. Lb, *L. biflexa*; Li, *L. interrogans*; Tp, *T. pallidum*; Tr, *T. phagedenis* biotype Reiter; Td, *T. denticola*; Bb, *B. burgdorferi*.

tion of *T. pallidum* in an in vitro tissue culture system. Temperatures above 36°C did not permit growth of this organism. Furthermore, it is well-known that elevation of the body temperature of infected humans or experimentally infected rabbits frequently results in the amelioration of syphilitic infection (25, 28). Fever therapy induced by iatrogenic infection with *Borrelia* or *Plasmodium* species or by hypertherm cabinets was used before the advent of antibiotics as a treatment for human syphilis (2, 5, 23).

In contrast to the results obtained with *T. pallidum*, for each of the other spirochetes analyzed we were able to detect a subset of proteins that were synthesized at a distinctly higher level following the temperature shift. The *Leptospira* species synthesized the largest number of Hsps; *L. biflexa* expressed at least 19 clearly discernible Hsps, and *L. interrogans* expressed 7 Hsps (Fig. 1). Both of these organisms, in particular the free-living *L. biflexa*, are exposed to a range of environmental temperature fluctuations; thus, the Hsp response may contribute to their survivability. We found that a high-passage isolate of *B. burgdorferi* HB19 synthesized only four Hsps at 42°C (Fig. 1). These results are similar to those of Cluss and Boothby (7), who recently reported that when cells of low-passage HB19 were shifted to 39°C and then labeled with [^{35}S]methionine they synthesized four Hsps of similar molecular weights. Carreiro et al. (6) have observed by one dimensional SDS-PAGE the synthesis of five to seven Hsps in solubilized extracts of cells of strain B31 following shifts from 33 to 37 or 40°C. Further analysis by two dimensional SDS-PAGE and fluorography revealed at least five additional Hsps. Finally, we found that *T. phagedenis* biotype Reiter synthesized five Hsps at 47°C, whereas only two Hsps were discernible in solubilized cellular extracts of *T. denticola* (Fig. 1). The last two treponemal species were originally isolated from human genital and oral tissue, respectively. Despite their inclusion in the genus *Treponema*, they have virtually no DNA homology with *T. pallidum* (19).

We tested the ability of rabbit polyclonal antisera directed against two highly conserved Hsps, GroEL (of *Legionella pneumophila*; 16) and DnaK (of *E. coli*), to recognize homologs in the solubilized cellular extracts of the [^{35}S]methionine-labeled, heat-shocked spirochetal cells or, for *T. pallidum*, cells labeled at 34°C. Solubilized extracts of heat-shocked *E. coli* MC4100 cells containing plasmid pBB1 (encoding DnaK) served as the control to confirm the specificity of the antisera (Fig. 2). Radioimmunoprecipitations were performed and analyzed by SDS-PAGE and fluorography (27). We observed a GroEL homolog in all of the spirochetes examined except for *B. burgdorferi* HB19 (Fig. 2, lane B for each organism). Although Cluss and Boothby (7) identified a protein, designated common antigen (CA), in low-passage HB19 that was thought to correspond to GroEL based on its molecular weight, expression of this protein was not thermoinducible. An inducible GroEL homolog has been recently reported for strain B31 by Carreiro et al. (6). Additionally, Hansen et al. (12) have cloned the gene encoding CA (GroEL) of *B. burgdorferi* ACA-1. Houston et al. (17) have isolated and characterized a 60-kDa *T. pallidum* protein (TpN60). Antiserum to TpN60 cross-reacts with *E. coli* GroEL and with a treponemal protein expressed by a recombinant clone isolated from a *T. pallidum* genomic library (15). We found the GroEL homologs of the two *Leptospira* species to be similar in size to that of *E. coli*, whereas the homolog identified for each of the *Treponema* species was consistently somewhat smaller (Fig. 2). While the GroEL homologs of the *Leptospira* species and *T.*

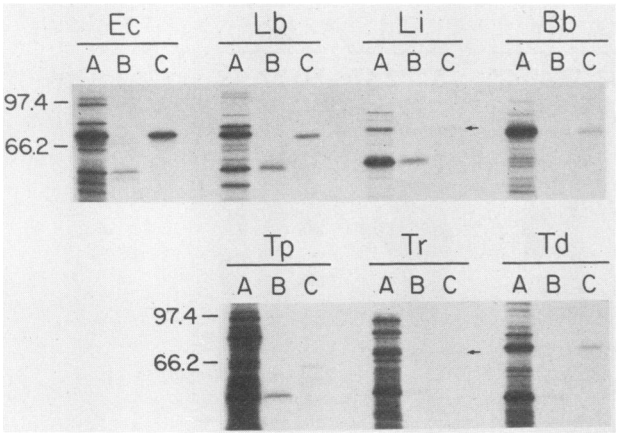

FIG. 2. Identification of GroEL and DnaK homologs in solubilized cellular extracts of *T. pallidum* and other spirochetes. Solubilized cellular extracts (lanes A) of [^{35}S]methionine-labeled *T. pallidum* (Tp, labeled at 34°C) or heat-shocked *E. coli* (Ec), *L. biflexa* (Lb), *L. interrogans* (Li), *B. burgdorferi* (Bb), *T. phagedenis* biotype Reiter (Tr), and *T. denticola* (Td) were precipitated with rabbit antisera against *L. pneumophila* GroEL (lanes B) or *E. coli* DnaK (lanes C). Precipitates were analyzed by SDS-PAGE and fluorography (27). Molecular mass standards are indicated on the left in kilodaltons. Arrows denote faint bands.

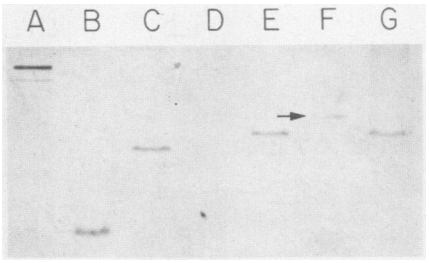

FIG. 3. Southern hybridization of spirochetal genomic DNAs with an *E. coli groEL/ES* probe. Spirochetal genomic DNAs were digested to completion with *Bam*HI, electrophoresed on a 0.8% agarose gel, and transferred to Nytran (Schleicher & Schuell, Keene, N.H.). Hybridizations were performed at low stringency with a digoxigenin-dUTP-labeled internal (*Eco*RI-*Sma*I) fragment from plasmid pOF39 (9) which contained the two *groE* genes. Results were obtained as described in the text. Lanes A through G contain genomic digests from *E. coli*, *L. interrogans*, *L. biflexa*, *B. burgdorferi*, *T. pallidum*, *T. denticola*, and *T. phagedenis* biotype Reiter, respectively. The arrow denotes the presence of a faint band in lane F.

phagedenis biotype Reiter were thermoinducible, those of *T. pallidum* and *T. denticola* were not affected by higher temperature.

We also observed a DnaK homolog in each of the spirochetes tested, including *T. pallidum* (Fig. 2, lane C for each organism). While Carreiro et al. (6) have reported the presence of a DnaK homolog in *B. burgdorferi* B31, a DnaK homolog has not been previously reported for any other spirochetal species. We observed that the DnaK homologs for the various spirochetes were either similar in size to that of *E. coli* DnaK or, in the case of *T. pallidum*, somewhat smaller than the *E. coli* protein. DnaK homologs of the *Leptospira* species, *B. burgdorferi*, and *T. phagedenis* biotype Reiter were thermoinducible, in contrast to those of *T. pallidum* and *T. denticola*.

To determine if spirochetal genes encoding GroEL and DnaK also were conserved, we probed *Bam*HI-digested spirochetal genomic DNAs isolated by the method of Saito and Miura (24), using digoxigenin-dUTP-labeled internal fragments of the cloned *E. coli groEL/ES* (9) and *dnaK* genes. The latter was derived from a plasmid (pBB1) provided by J. McCarty, Massachusetts Institute of Technology, Boston. Hybridization was detected with the Genius nonradioactive labeling and detection kit (Boehringer Mannheim, Indianapolis, Ind.). Under conditions of low stringency (55°C; 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% SDS), the *E. coli groEL/ES* probe (*Eco*RI-*Sma*I internal fragment of plasmid pOF39; 9) hybridized to a discrete fragment in each of the spirochetal genomic digests, except for the digest of *B. burgdorferi* (Fig. 3, lane D). As expected, the *groE* probe hybridized to two fragments in the control *E. coli* digest due to the presence of a *Bam*HI site within the *groEL* gene (Fig. 3, lane A). Under conditions of higher stringency (65°C; 0.1× SSC and 0.1% SDS), the *E. coli dnaK* probe (*Pvu*I-*Sal*I internal fragment of plasmid pBB1) hybridized to a single fragment of *Leptospira* DNA or to two or three fragments of *B. burgdorferi* or *Treponema* DNA, presumably due to *Bam*HI sites within the *dnaK* genes of the last two organisms (data not shown).

Our failure to detect the *groEL* gene in *B. burgdorferi* HB19 is consistent with our inability to demonstrate the presence of GroEL in this organism (Fig. 2). This may reflect a difference between strain HB19 and the relatively few other strains studied in this regard (see above). Alternatively, our finding may be due to the loss of genetic information by cells of strain HB19 during prolonged in vitro passage. This will be investigated further.

In conclusion, we have demonstrated that two species of pathogenic spirochetes, *B. burgdorferi* and *L. interrogans*, as well as certain saprophytic spirochetal species (*L. biflexa*, *T. phagedenis* biotype Reiter, and *T. denticola*), are capable of a heat shock response. In contrast, *T. pallidum* does not appear to respond in a similar manner. To our knowledge, *T. pallidum* is the only organism reported that does not exhibit a heat shock response. We have identified homologs of two major Hsps, GroEL and DnaK, in the various spirochetal species. While a GroEL homolog has been previously reported for *T. pallidum* and *B. burgdorferi* and a DnaK homolog has been reported for the latter organism, GroEL and DnaK homologs have not been previously identified for any of the other spirochetes. Although GroEL and DnaK homologs were present in *T. pallidum* and *T. denticola*, these proteins were not thermoinducible. Houston et al. have suggested that GroEL (TpN60) appears to be present in *T. pallidum* in amounts larger than the normal level found in *E. coli* (17). They have speculated that constitutively higher levels of this protein are required by *T. pallidum*. While the stress encountered during purification of treponemes from rabbit tissues could potentially induce a heat shock response, Houston et al. found the same quantity of TpN60 in freshly extracted cells as in Percoll-purified cells (17). We have shown that the failure of *T. pallidum* to exhibit a true heat shock response is not due to the lack of GroEL or DnaK. The apparent inability of the *T. pallidum* protein synthesis apparatus to function at higher temperatures may preclude such a response.

In *E. coli*, the promoters of heat shock genes are recognized by a specific RNA polymerase containing the σ^{32} subunit (18, 22). This product of the *rpoH* gene is required

for increased transcription of heat shock genes following a temperature shift up. Studies have shown that if σ^{32} is inactive or present in reduced amounts, the induction of Hsps is prevented and cells exhibit a temperature-sensitive phenotype (21, 31). Since it is not known whether *T. pallidum* possesses an equivalent of the *E. coli rpoH* gene, it is difficult to speculate about the regulation of the GroEL and DnaK homologs of this organism. Our data indicate that either *T. pallidum* Hsp homologs are maximally expressed in vivo and cannot be further induced in vitro or that *T. pallidum* lacks a regulated stress response. DNA sequence analysis of the *T. pallidum groE* and *dnaK* genes may provide further insight.

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