Profiling of Temperature-Induced Changes in *Borrelia burgdorferi* Gene Expression by Using Whole Genome Arrays

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*Borrelia burgdorferi* is the etiologic agent of Lyme disease, the most prevalent arthropod-borne disease in the United States. The genome of the type strain, B31, consists of a 910,725-bp linear chromosome and 21 linear and circular plasmids comprising 610,694 bp. During its life cycle, the spirochete exists in distinctly different environments, cycling between a tick vector and a mammalian host. Temperature is one environmental factor known to affect *B. burgdorferi* gene expression. To identify temperature-responsive genes, genome arrays containing 1,662 putative *B. burgdorferi* open reading frames (ORFs) were prepared on nylon membranes and employed to assess gene expression in *B. burgdorferi* B31 grown at 23 and 35°C. Differences in expression of more than 3.5 orders of magnitude could be readily discerned and quantitated. At least minimal expression from 91% of the arrayed ORFs could be detected. A total of 215 ORFs were differentially expressed at the two temperatures; 133 were expressed at significantly greater levels at 35°C, and 82 were more significantly expressed at 23°C. Of these 215 ORFs, 134 are characterized as genes of unknown function. One hundred thirty-six (63%) of the differentially expressed genes are plasmid encoded. Of particular interest is plasmid lp54 which contains 76 annotated putative genes; 31 of these exhibit temperature-regulated expression. These findings underscore the important role plasmid-encoded genes may play in adjustment of *B. burgdorferi* to growth under diverse environmental conditions.
ture are plasmid encoded, suggesting that plasmids are an important repository for genes integral to *B. burgdorferi* transmission to the mammalian host (2, 3, 25–27, 49, 53, 55, 66). Furthermore, *B. burgdorferi* also expresses some plasmid-encoded genes exclusively during mammalian infection, which are thought to be important in disease establishment and pathogenesis (1, 3, 13, 19, 20, 27, 57, 60). Consistent with these notions, it has been demonstrated that the loss of specific plasmids correlates with *B. burgdorferi* attenuation or loss of virulence in mice (31, 34, 43, 50). However, it has not been experimentally determined if the absence of specific plasmids or genes also can arrest the migration of *B. burgdorferi* at different points within the tick as it is transmitted to the mammalian host. To determine which genes facilitate borrelial migration through the tick and establishment in the mammalian host, a logical first step is to identify molecules induced by signals encountered in the tick and mammalian environments, such as temperature.

Global gene expression profiling is a powerful tool for assessment of bacterial gene expression under different physiological conditions (58, 62) and has the potential to identify unknown virulence genes (11). Here, we describe the construction of a *B. burgdorferi* genome array and its application for profiling global changes in *B. burgdorferi* gene expression resulting from the environmental cue of increased temperature.

### MATERIALS AND METHODS

#### ORF selection and PCR primer design

**ORF selection for a *B. burgdorferi* whole genome array** poses a unique problem, especially for the plasmids, due to the presence of a substantial number of paralogous sequences. There are 175 whole genome array poses a unique problem, especially for the plasmids, due to substantial number of paralogous sequences. This resulted in 1,697 “gene features” (see supplementary Table 1 [www.nymc.edu/irschwartz/borreliaarrays]). Primers were designed by Sigma-Genosys (Houston, Tex.) based on the following criteria: (i) a minimum length of 18 bases, (ii) a G+C content that was within the range of 30 to 70%, (iii) a melting temperature of at least 55°C, and (iv) a G or C present in one of the last three base positions. In this fashion, suitable primers were found that would amplify a significant portion of the ORF in the majority of cases. For longer ORFs, sequences were truncated from the 5’ end, such that most PCR products would not exceed 2,000 bp. For ORFs of less than 250 bp, primers were designed to amplify the entire coding sequence, from start to stop codons. Hence, these shorter ORFs would represent the maximal hybridizable domain for array studies.

**Strains and growth conditions.** B31 is the type strain of *B. burgdorferi*, and B31-M1 is the designation given to the B31 derivative for which the genomic sequence was determined (7, 22). B31-M1 was passed through an experimental mouse-tick infectious cycle and reisolated from a mouse that was infected by needle inoculation (18). This B31-M1 reisolate was directly moved to a plate, and a clone (B31-M1 P1.1) that contained all the plasmids identified in the genomic sequence was derived from an individual colony picked and cultivated in liquid Barbour-Stoenner-Kelley II (BSK-II) medium. Frozen glycerol stocks were made directly from the primary outgrowth of this clone. Plating of B31-M1 and determination of plasmid content were performed as previously described (16). *B. burgdorferi* was grown in liquid and solid BSK-II medium prepared in the laboratory; commercial BSK-H medium obtained from Sigma did not support growth of B31-M1 P1.1 at a comparable rate or to the same final density.

B31-M1 P1.1 was inoculated directly to BSK-II medium from the glycerol stock and grown to a moderate density at 35°C. This culture was diluted to 10^5 bacteria/ml and grown at 23°C. When this culture reached 2.5 × 10^7 bacteria/ml, it was diluted to a density of 2.5 × 10^6 bacteria/ml and separate cultures were incubated at 23°C and at 35°C. The latter cultures were diluted at mid-exponential phase to a density of 5 × 10^8/ml, and the incubation continued at 35°C. This was done to extend the period of growth at 35°C. Cell densities were measured daily, and at the point of harvest for RNA isolation, both 23 and 35°C cultures were in mid-exponential phase to a density of 5 × 10^8/ml, and the incubation continued at 35°C. This was done to extend the period of growth at 35°C. Cell densities were measured daily, and at the point of harvest for RNA isolation, both 23 and 35°C cultures were in mid-exponential phase and at the same density (5 × 10^8 bacteria/ml). The pH of the culture medium remained neutral (~7.4) throughout growth. Although *B. burgdorferi* grows more slowly at 23°C than at 35°C (relative doubling times of 33 versus 8 h, respectively), spirochetes at both temperatures exhibited similar morphologies and attained comparable densities of >2 × 10^9 bacteria/ml in stationary phase. Aliquots were taken of all cultures for protein analysis.

**RNA and DNA isolation.** For each condition (23 and 35°C), RNA was extracted from spirochetes in mid-exponential growth phase (5 × 10^8 bacteria/ml) from four separate 100-ml cultures, using the Ultraspec-II RNA isolation system (BIOTECX, Houston, Tex.). RNA-free chemical and material (tubes, barrier pipette tips, gloves, diethyl pyrocarbonate-water, RNase-Zap [Ambion, Austin, Tex.]) were used throughout the process. Subsequently, RNA from each

### Table 1. Oligonucleotides used for real-time quantitative RT-PCR

<table>
<thead>
<tr>
<th>ORF</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
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<tbody>
<tr>
<td>BB0147</td>
<td>TCAAGGCGTTTGGACTTTAGATT</td>
<td>CACCCAGAACATTTGACACCATTA</td>
</tr>
<tr>
<td>BB0240</td>
<td>AAGTCCGGAAATACCGAGAATA</td>
<td>TTCTGGCTGTTGTAATACCAAA</td>
</tr>
<tr>
<td>BB0241</td>
<td>AAGTGGGAAAGCTATGATCTTA</td>
<td>CGTCAATTTCATATGGGCAA</td>
</tr>
<tr>
<td>BB0242</td>
<td>GGGTGCCTCAAAATGAGTGTTT</td>
<td>TGTATATTTTGATGTTTT</td>
</tr>
<tr>
<td>BB0647</td>
<td>CCACTATTTCAACCTGACGATTA</td>
<td>TGCATGAGCTAGGCTTTT</td>
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<td>ATTTTCTTTGTATCCAAAAAATCCAA</td>
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<td>BB0838</td>
<td>AGGACACTATTTGCCATCGATGTT</td>
<td>TTCATTTTGCTTATTAAGTAATT</td>
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<tr>
<td>BB1A55</td>
<td>ATGTGAGCGGCTCGCCAGAGAA</td>
<td>GATCGCTTGGCCCTTGTGGTTT</td>
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<td>AGCTGATATTTTCAATTTAGCAGAGTAA</td>
<td>TGTTGATGCTGATCACCTGATAT</td>
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<tr>
<td>BB1B</td>
<td>CAGTGGCAAAAGGCACTTTTTAA</td>
<td>TTAGAAAGCCTTTATTTTTAGACAAGACGTT</td>
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<td>BBE19</td>
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</tr>
<tr>
<td>BBE22</td>
<td>TGCAATTTCTAATGCTATGAATT</td>
<td>TGCAATTTCTAATGCTATGAATT</td>
</tr>
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<td>BBG05</td>
<td>AATTATCATCACAATACAGGTCGTATGGT</td>
<td>TCTTAGATGCGATTTTAATTTAGTAC</td>
</tr>
<tr>
<td>BBG40</td>
<td>ATTTCCAGGGCCTGATATGATGATTTTCT</td>
<td>AGCTTCCCTAATCCCTGCTGTTTA</td>
</tr>
<tr>
<td>BBG41</td>
<td>ACAGAAAATACATTAGGCAATATGA</td>
<td>AAAACCGACCTTCTGGAATAAGTTAAT</td>
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</table>

Then, all overlapping ORFs (including the pseudogenes) were compared, and among any overlapping ORF set, only the largest was retained. Finally, primer sets were included for the largest of several unique plasmid-encoded regions several kilobase pairs in length that appear to contain no ORFs. This resulted in a list of 1,697 “gene features” (see supplementary Table 1 [www.nymc.edu/irschwartz/borreliaarrays]).
culture or condition was treated with DNase I (DNA-free kit; Ambion). Quality and concentration of the RNA, as well as the absence of contaminating DNA, were determined with the Agilent 2100 Bioanalyzer (Agilent Technologies). The 26S/18S rRNA ratio of all samples was approximately 1, which indicates high-quality RNA. No contaminating DNA was detected. Cultures at 23°C yielded approximately 30% less RNA than the same number of spirochetes grown at 35°C.

**TABLE 2. Most highly expressed genes in *B. burgdorferi* cultivated at 35°C**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description of gene product (homologous gene)</th>
<th>Relative transcript level</th>
<th>Rank at temp (°C)</th>
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</thead>
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<tr>
<td>BB0440</td>
<td>Ribosomal protein L34 (rpmH)</td>
<td>104.42</td>
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<td>BBA59</td>
<td>Lipoprotein</td>
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<td>1</td>
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<td>BB0804</td>
<td>Ribosomal protein S15 (psO)</td>
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<td>Ribosomal protein S20 (psT)</td>
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</tr>
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<td>4</td>
</tr>
<tr>
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</tr>
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<td>Lipoprotein</td>
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<td>5</td>
</tr>
<tr>
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<td>hbbU protein</td>
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<td>BB0274</td>
<td>Flagellar basal-body rod protein (flgB)</td>
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<tr>
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<td>PTS* system, glucose-specific IIA component (crr)</td>
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<td>BBA15</td>
<td>Outer surface protein A (osp4)</td>
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<td>BB0300</td>
<td>Cell division protein (ftsA)</td>
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<td>Ribosomal protein L21 (psU)</td>
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<td>Outer surface protein C (ospC)</td>
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<td>Ribosomal protein L23 (psW)</td>
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<td>Ribosomal protein L29 (psM)</td>
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<td>BB0704</td>
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<td>BB074</td>
<td>Outer membrane porin (oms28)</td>
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<td>12.33</td>
<td>34</td>
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<tr>
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<td>tRNA pseudouridine 55 synthase (truB)</td>
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<td>Hypothetical protein</td>
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<td>BB0190</td>
<td>Translation initiation factor 3 (infC)</td>
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<td>Conserved hypothetical protein</td>
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<td>Hypothetical protein</td>
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<td>BB0386</td>
<td>erpK protein (erpK)</td>
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<td>BB0441</td>
<td>Ribonuclease P protein component (mpA)</td>
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<td>Conserved hypothetical protein</td>
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</tr>
<tr>
<td>BB0664</td>
<td>Hypothetical protein</td>
<td>9.90</td>
<td>47</td>
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</table>

*PTS, phosphotransferase.*
sented on the arrays. These ORFs are among the 61 listed in supplementary Table 2.

Preparation of DNA array. The B. burgdorferi membrane arrays described here were produced by Sigma-Genosys. Ten nanograms of PCR-amplified ORFs was spotted in duplicate onto positively charged nylon membranes. Following spotting, each array was cross-linked with UV light. Other technical aspects related to array printing and layout have been described elsewhere.

Probe synthesis. A mixture of all 3′ ORF-specific primers was used to generate the hybridization probes by standard cDNA synthesis. Five micrograms of RNA was mixed with dCTP, dGTP, and dATP (final concentration, 0.4 mM each). Primers were annealed to the RNA hybridized at 65°C for 10 min by heating to 65°C and cooling to 42°C. Two hundred units of Superscript II (Life Technologies), 10 U of RNase inhibitor, and 20 μCi of [α-33P]dATP (Amersham) were added in a final volume of 20 μl of first-strand buffer. The final cDNA synthesis reaction mixture was incubated at 42°C for 2 h. The labeling efficiency was determined by trichloroacetic acid precipitation. Under the above conditions, specific activities of input RNA of 2 × 106 to 5 × 106 cpm/μg were achieved.

Hybridization. The membrane array was placed in a prehybridization solution (ExpressHyb; Clonetch) and incubated at 55°C for 1 h. The 33P-labeled cDNA was added to 10 ml of ExpressHyb solution and was used to probe the membrane array by incubation overnight at 55°C. After hybridization, the membrane was washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.05% sodium dodecyl sulfate (SDS) for 30 min at room temperature and then with 0.1× SSC–0.1% SDS at 65°C for 30 min. After washing, the arrays were exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, Calif.) for 48 h. For comparison of samples from cells grown at different temperatures, the same membranes were probed reciprocally (i.e., switching the membranes) after stripping in alkaline stripping solution.

Array data analysis. The procedures for array data analysis have been described in detail elsewhere (38), and a brief description is provided here. The exposed PhosphorImager screen was scanned with a pixel size of 50 or 100 μm on a Storm 840 PhosphorImager (Molecular Dynamics). The image files were analyzed using ArrayVision software (Imaging Research, St. Catharines, Ontario, Canada). A template which contains the spot layout of the array was overlaid on the phosphorimage, and the pixel intensity of each spot on the array was determined. The background-corrected pixel intensities for each spot were exported from ArrayVision into ArrayStat (Imaging Research) for statistical analysis. These values were log transformed, common error was determined, and outliers were removed. The values were then normalized by the mean across conditions by an iterative process, and a false-positive error correction is achieved by application of the false-discovery rate method (4). The z test for two independent conditions using the false-discovery rate method (nominal alpha, P ≤ 0.01) was used as the basis for computing confidence intervals.

Alternatively, ArrayVision data was analyzed using macro functions found within the Microsoft Excel spreadsheet program as described previously (38). Significance of differential expression was determined by a two-tailed, unpaired Student t test at a P < 0.01.

Real-time quantitative RT-PCR. To independently confirm the differential expression data generated by microarray analysis, real-time quantitative reverse transcription (RT)-PCR was utilized to determine the relative expression of specific genes at the two different temperatures. Primers were designed using PRIMER EXPRESS software (PE Biosystems, Foster City, Calif.) (Table 1). All primer pairs produced a single amplicon of the expected size when B. burgdorferi total genomic DNA was used as the template for PCR. cDNA was generated using 2 μg of total RNA and 3′–specific primers as described above. Real-time PCR was performed in triplicate on 5 ng of cDNA with the SYBR Green master mix using a Perkin-Elmer ABI Prism 7700 sequence detection system following the manufacturer’s instructions (PE Biosystems). To ensure that there were no genomic contamination of cDNA and no primer dimer artifact, reactions containing cDNA generated without reverse transcriptase and reactions containing primers alone also were included. The constitutively expressed flaB gene was used to normalize input cDNA for all experiments. Differential expression was determined by calculating the change in threshold cycle (ΔΔct) of each ORF with RNA isolated from cells grown at the different temperatures.

Supplementary data. Supplementary data can be found in supplementary Tables 1 to 4 posted at www.nymc.edu/iraschwartz/borreliaarrays.

RESULTS AND DISCUSSION

Array description. A list of 1,697 gene features was generated as described in Materials and Methods, and primers were designed to amplify these regions (see supplementary Table 1). Amplified DNAs were spotted in duplicate on positively charged nylon membranes. The array also included 29 control spots derived from PCR mixtures that either lacked DNA template or contained only spotting buffer. Sheared genomic DNA was labeled by random priming and was used to probe the arrays in triplicate. The noise level was determined by averaging the pixel values in each of the control spots after subtraction of background levels (62). Sixty-one ORFs did not yield a pixel value exceeding the noise level by a factor of 2 because they either failed PCR or did not bind sufficient probe in the hybridization step. Thus, the final array can yield informative data for 1,628 (96.4%) of the putative ORFs. The 23 chromosomal and 38 plasmid ORFs which are not represented on the array are listed in supplementary Table 2.

Generation of a B. burgdorferi strain B31-MI clonal isolate and growth conditions for transcriptional profiling. Several studies have shown that uncloned isolates of B. burgdorferi often contain subpopulations of organisms with different plasmid contents, which can result in phenotypic differences (18). Therefore, before undertaking a global analysis of the transcriptional profiles of B. burgdorferi at different temperatures, a clonal isolate of the B31-MI strain was generated. This was accomplished by infecting a mouse with the parental, uncloned B31-MI strain and cultivating organisms from an ear punch biopsy specimen for subsequent plating on solid BSK-II medium (18). A single, well-separated colony, designated B31-MI P1.1, was selected and screened by PCR using plasmid-specific primers to ensure it harbored all 21 plasmids previously identified (data not shown).

This B31-MI clone was cultivated in BSK-II medium at 23 and 35°C as described in Materials and Methods. Aliquots of the culture were analyzed for the characteristic induction of OspC protein after temperature shift by SDS-polyacrylamide gel electrophoresis and immunoblot analysis. As expected, no OspC was detected in organisms cultivated at 23°C, whereas prominent induction of OspC was observed in temperature-shifted organisms (Fig. 1).

Transcript analysis. RNA was isolated from four separate 100-ml cultures grown at each temperature. After DNase I treatment and evaluation of RNA quality (see Materials and Methods), the RNAs isolated from the four cultures at each temperature were pooled. In this manner potential variability in the transcript levels introduced during growth or RNA isolation of individual cultures was minimized. Radioactive probes were prepared by cDNA synthesis using 3′ ORF-specific primers as described in Materials and Methods and hybridized to the membrane arrays. Probe synthesis and array hybridization were performed eight separate times in two different laboratories (at the New York Medical College and at the University of Oklahoma Health Sciences Center). Pixel densities in each spot were determined by phosphorimager analysis and statistically analyzed using the ArrayStat software package. Thus, 16 data points were analyzed for each ORF.

A total of 146 ORFs (45 chromosomal ORFs, 99 plasmid ORFs, and 2 plasmid intergenic probes) did not give detectable signals at either temperature. These are listed in supplementary Table 3. Of these, 57 ORFs (31 chromosomal and 26 plasmid) do not appear to be obviously defective by sequence analysis and gene arrangement. These may well be functional genes that are not expressed at detectable levels under the
growth conditions used. However, also in this unexpressed class of ORFs are 44 pseudogenes and 45 unusually short plasmid ORFs (<300 bp). Such short ORFs are mostly found in regions rich in pseudogenes where ORFs are not tightly packed and were previously suspected not to encode any protein product (see references 6 and 7 and supplementary material therein). The substantial overrepresentation of unexpressed ORFs in these pseudogene and short-gene classes strongly suggests that these are not functional genes. Of eight intergenic regions that appeared to lack obvious ORFs, only two (BBH16.1 and BBDo6.1) showed moderate levels of expression at either temperature. This might be the result of transcriptional readthrough from the upstream ORFs. Thus, over 90% of the arrayed ORFs had at least minimal expression under one of the growth conditions.

In order to obtain values for transcript abundance for each ORF, the following normalizations were performed essentially as described elsewhere (62). The relative transcript level for each ORF was determined by dividing the average pixel density for that ORF by the sum of the total pixels of all ORFs on arrays (which exceeded noise by a factor of 2) hybridized with cDNA (prepared from total RNA) to yield a percent expression value for each ORF. A similar calculation was performed for genomic DNA hybridizations, generating a percent genomic value for each ORF. Finally, dividing the percent expression value by the percent genomic value yields a measure of relative transcript level for each expressed ORF. A list of the most highly expressed genes in B. burgdorferi cultivated at either 35 or 23°C is given in Tables 2 and 3 and a complete list is presented in supplemental Table 4.

Several points are worthy of note. As expected, a substantial number of the most highly expressed genes at either temperature encode ribosomal proteins or other proteins involved in translation. The genes encoding the abundant outer surface proteins OspA, OspB, and OspC are highly expressed, indicating that the cellular levels of these proteins are regulated largely at the transcription step. Genes encoding products of no known function (i.e., annotated as hypothetical or conserved hypothetical in the sequence database) represent 40% of the 50 most highly expressed genes. This underscores the important roles likely to be played by such genes in B. burgdorferi cellular function. The lists of the 50 most highly expressed genes at the two temperatures largely overlap. Several ORFs, most notably ospC, bba32, bba74, bbi29, and bb0175, are highly expressed at 35°C but have significantly lower expression at 23°C (see below).

Expression of paralogous genes. As described above, the B. burgdorferi genome contains 175 paralogous gene families ranging from 2 to 41 members. This greatly complicates both the construction of a genome array and subsequent analysis of array data. One approach would involve choosing some subset of paralogous genes to represent all members of the family. This would require experimental determination of the parameters governing cross-hybridization and selection of only those family members that would not cross-hybridize. Alternatively, one could ignore this problem and simply amplify all genes regardless of sequence identity. As noted earlier, the second approach was taken for construction of the arrays used in the present studies. Thus, understanding the expression of individual members of a paralogous family will require more detailed examination.

The extent of sequence identity among members of specific paralogous families varies substantially. In some cases, sequence identity among the individual family members is quite low (e.g., family 54), whereas for others the genes are indistinguishable at the sequence level (e.g., family 112). It is instructive to consider the expression data for selected cases to gain insight into the extent of cross-hybridization encountered in these studies. Family 54 consists of 14 members, most with <60% sequence identity to any other family member. In this situation, cross-hybridization is of little concern, and, indeed, family 54 genes display vastly different levels of expression, and some members are upregulated at 35°C, whereas some have higher expression at 23°C (see below). At the other extreme is family 112; all eight members have >90% sequence identity, and some are 100% identical. In this case, it would be impossible by any hybridization-based technique (including RT-PCR) to differentiate expression from any one of these iden-
tical genes. This is discussed further below with regard to the nearly identical bbyAB genes. The intermediate situation is somewhat more problematic. This is exemplified by family 62, which includes nine members. Four of these—bbj19, bbd14, bbk23, and bbh26—all have significantly higher expression at 23°C relative to 35°C. bby19, bbk23, and bbh26 are 66 to 80% identical to each other but <30% identical to bbd14. This suggests that the expression data for the latter gene are unique, but those for the other three genes are unclear. Is there transcription of only one of these ORFs, or are the expression data a composite of transcription from all three genes? In such a case, further analysis by an alternative method, such as real-time RT-PCR with gene-specific primers, can differentiate between these possibilities.

In order to get some insight into the extent of cross-hybridization under the experimental conditions employed in the present study, several genes were amplified by PCR, labeled with [$\alpha$-32P]dATP, and hybridized individually to a membrane array. When labeled bba66 was used as a representative of paralogous family 54, the only significant hybridization on the array was to this ORF. All other members of the family gave signal intensities <6.6% that of bba66 (most were <3%). In contrast, using bbs25 as a representative of paralogous family 112 resulted in virtually equal signals for this gene as well as six

TABLE 3. Most highly expressed genes in B. burgdorferi cultivated at 23°C

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<tr>
<th>Gene name</th>
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a PTS, phosphotransferase.
**TABLE 4. Genes with increased expression at 35°C relative to 23°C**

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* PTS, phosphotransferase.
other close paralogs (sequence identity of 91 to 100%). *bbp41* and *bbp42*, more distantly related members of the family (sequence identity <32%), yielded signal intensities ~3% that of *bbp25*. These results are consistent with the above theoretical discussion and provide some parameters for interpretation of expression data for paralogous genes.

Expression of cp32 plasmid genes and a putative phage operon. The seven cp32 plasmids of strain B31 are homologous throughout most of their lengths and have several features that suggest they may be prophages (7, 8, 15). Eggers et al. (16) have found that cp32 DNA is present inside phage-like particles produced by *B. burgdorferi* strain CA-2-11A. Analysis of cp32 sequences shows that they carry homologs of two genes that may encode two proteins that are universally required for phage virion assembly, terminase and portal protein. In all known temperate phages (>100 have been sequenced), the large terminase subunit and portal protein genes are always found in a particular juxtaposition within the operon that carries the genes for construction of the phage particle (called the “late operon”). They are virtually always adjacent, the terminase gene is transcriptionally upstream of the portal gene, and the terminase gene is the second gene in the operon (S. Casjens, unpublished data). Genes *bbp42* and *bbp01* (and paralogs) have homology with the large terminase subunit and portal protein, respectively; and their juxtaposition is the same as that in the known phages. Late operon genes should not be expressed (or should be expressed at very low rates) under normal culture conditions since few phage particles are being produced. To further explore the possibility that cp32 plasmids may be temperate phages, we analyzed the relative expression of putative ORFs from several regions of the cp32 plasmids (Fig. 2).

All the genes in the cp32-1 putative late operon (*bbp42*, *bbp43*, and *bbp01* through *bbp22*) are expressed at low levels, if at all. By contrast, most genes in the putative cp32 replication-partition gene operon and the *erp* genes, both of which were previously shown to be expressed (14, 23, 53), have substantially higher average mRNA levels than genes in the putative late operon (Fig. 2). *bbp23*, *bbp24*, and *bbp25* (and paralogs), designated the *blyAB* operon, are also expressed at substantially higher levels than the late operon genes (Fig. 2). These expression data are consistent with the proposal that the cp32 plasmids are prophages and genes *bbp41* through about gene *bbp22* (and paralogs) may be the “late operon.” The genes in these regions are extremely tightly packed, as is the case in all other phages, and are about the number of genes required to build a tailed phage. There are no conflicting homologies to nonphage assembly genes in this region, and the extreme diversity of phage sequences makes it no surprise that nearly all of the genes in this region do not match any protein in the current database. Thus, global gene expression data add support to the idea that one region of the cp32 plasmids may encode phage morphogenetic genes.

Damman et al. (12) have presented evidence that *blyA* and *blyB* (*bbp23* and *bbp24* and paralogs) may encode a protein involved in lysis of the cell during phage lytic growth. Lysis genes are often found in the same operon as morphogenetic genes in other phages. However, the *blyAB* genes are expressed at levels substantially above the median of all *B. burgdorferi* expressed genes and those immediately preceding them (Fig. 2). Furthermore, a large (for this region) intergene gap precedes gene *bbp23* and paralogs that could harbor a promoter. This seems inconsistent with *bbp23-bbp24* being part of a prophage late morphogenesis-lysis operon and perhaps indicates that the role of *blyAB* should be reexamined.

Temperature-induced differential gene expression. Many studies have established that expression of a number of *B. burgdorferi* genes is regulated by changes in environmental
conditions (1, 3, 25–27, 41, 44, 49, 55, 64). The best studied of these cues is temperature (3, 26, 27, 45, 49, 55, 66). In order to determine the global changes in temperature-regulated gene expression, arrays were hybridized with cDNA generated from RNA isolated from *B. burgdorferi* cultivated at either 23 or 35°C. A total of eight independent array hybridizations were carried out for each temperature in two different laboratories. Only gene expression differences between the two temperatures that were significant at a *P* of <0.01 are considered in the following discussion. In total, 215 ORFs (15.2% of those included on the array) displayed significantly different expression at the two temperatures. A list of these ORFs is presented in Tables 4 and 5.

One-hundred thirty-three ORFs had significantly higher expression at 35°C relative to 23°C, whereas 82 genes had significantly greater expression at 23°C in comparison to 35°C. Of these, 62% (134 of 215) are annotated as unknown function. Nearly two-thirds (136 of 215) of the temperature-regulated genes are encoded on plasmids, underscoring the important roles that such genes may play in the physiology of *B. burgdorferi*, especially with regard to adaptation to environmental changes.

**Genomic location of temperature-regulated genes.** The chromosome was found to contain 79 temperature-regulated genes, while 136 were located on the various plasmids. Of the 21 sequenced B31-M1 PI.1 plasmids, only 3 (lp5, lp21, and cp9) did not encode any ORFs differentially regulated by temperature (Fig. 3A). Among the other 18 plasmids, lp54, lp38, and the circular plasmids cp26, cp32-3, and cp32-6 had more than 20% of their ORFs regulated by temperature (42, 22, 24, 24, and 21%, respectively) (Fig. 3A). For lp54, lp38, and cp26, this number is likely to be accurate, since none of the temperature-regulated genes are expected to cross-hybridize with paralogs. As noted above, the true number of regulated genes on cp32 plasmids cannot be determined with certainty, since many of these genes are members of paralogous families with substantial sequence identity. For example, for cp32-3 and cp32-6, five of the temperature-regulated genes are members of such paralogous families.

Linear plasmid lp54 is of particular interest. Over 80% (26 of 32) of its temperature-regulated genes are clustered in the right half of the plasmid (Fig. 3B). Moreover, all the lp54 genes that are more significantly expressed at 23°C are clustered in a single region between *bba38* and *bba56*, and these are all transcribed in the same direction. Casjens et al. (7) have speculated an ancient insertion of a linearized cp32 plasmid into lp54, with *bbp01* to *bbp18* roughly corresponding to *bba38* to *bba56*. Interestingly, none of the genes in the equivalent regions of any cp32 plasmids are upregulated at 23°C. Thus, if the paralogous regions in lp54 are indeed the result of insertion of a cp32, subsequent changes in lp54 must have included acquisition or evolution of regulatory sequences which respond to temperature. Curiously, all the remaining temperature-responsive genes on lp54 (all of which are upregulated at 35°C) lie outside the putative cp32 insertion. It is reasonable to suggest that lp54 has evolved to play an important role in the adaptation of the spirochete to growth at different temperatures.

**Functional categories of differentially expressed *B. burgdorferi* genes.** All *B. burgdorferi* ORFs have been grouped into 23 different functional categories based on sequence similarities to known genes and proteins from other organisms (22). Of these, 16 contain ORFs that responded differentially to a change in temperature (Fig. 4). As noted above, the most-striking observation was that of the 215 genes that are temperature regulated, 134 encode proteins of unknown function (categories HX and U). Of genes encoding proteins of known function, the greatest number was found to be putative cell envelope constituents, with 26 being differentially expressed at the two temperatures. This is consistent with the proposal that *B. burgdorferi* remodels its surface in response to changes in environmental conditions (3, 26, 41, 45, 49, 55, 64). Interestingly, almost all of the differentially expressed cell envelope genes (21 of 26) were found to be upregulated at 35°C. Additionally, almost 20% (11 of 59) of the genes encoding putative transport proteins were differentially expressed—five more highly at 35°C and six more highly at 23°C. Eight genes annotated as being involved in intermediary metabolism (IM) were differentially expressed; interestingly, seven were more highly expressed at 23°C. The possible role of these genes in adaptation to the lower temperature is discussed below. The only gene in this category upregulated at 35°C is *bb0376*, a *metK* (*S*-adenosylmethionine synthetase) homolog, which was recently shown to be essential for growth in *Escherichia coli* (63). Interestingly, the adjacent gene, *bb0377* (annotated as a conserved hypothetical), was recently shown to be a *B. burgdorferi* luxS ortholog (52), and the expression of this gene is also significantly higher at 35°C. Schauder et al. have proposed that these two genes comprise an operon with *bb0375* (a putative 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase) whose products would be responsible for synthesis of the quorum-sensing autoinducer, AI-2 (47). In the present study, *bb0375* transcript levels were very low and differential temperature-dependent expression was not observed.

The other functional category for which a substantial number of genes are temperature regulated is the category of putative hemolysins; 13 are significantly upregulated (1.5- to 2-fold) at 35°C. Sixteen of the 21 putative hemolysin genes are located on cp32 plasmids. The eight *blyA* sequences are 100% identical, and the eight *blyB* sequences are 93 to 100% identical. Thus, as noted above, it is not possible to determine whether one, some, or all of these genes are expressed. The three cp32-encoded putative hemolysin genes (*bbn23*, *bbn24*, and *bbq31*) that did not meet the stringent statistical criteria for differential regulation (i.e., *P* < 0.01) were all upregulated at 35°C, with *P* values of 0.02, 0.015, and 0.013, respectively. It therefore appears that these genes are more significantly expressed at 35°C, and it is tempting to speculate that this may have an in vivo correlation to the temperature increase in the tick gut on acquisition of the blood meal in that expression of hemolysin may be required for its digestion.

**Differential expression of cell envelope genes.** Twenty-six genes encoding putative cell envelope constituents were differentially expressed. Among these were a number of genes known to be expressed preferentially in vivo or at 35°C, including *ospC* and *dbpAB*, the levels of expression of which were elevated at 35°C by 5.5-fold and 4.5- to 7-fold, respectively. Several members of paralogous family 54—*bbha64*, *bbha65*, *bbha66*, *bbha69*, *bbha71*, and *bbha73*—are more highly expressed at 35°C (between 1.8- and 6.26-fold). Of these, only the *bbha64* protein is known to be on the cell surface (17), but *bbha66,*
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* PTS, phosphotransferase.
bba73, bba65, and bba69 each have potential signal peptidase II sites and are predicted to be cell envelope constituents. Sequence identity between any of these genes is less than 33%, and therefore, the signals obtained for each of these genes should be specific. bba66 to bba64 and bba73 to bba71 likely constitute operons. bba64 encodes a protein designated P35, which is reported to be regulated by cell density, growth phase, or pH and is undetectable in the unfed tick (24, 29, 44, 45). This would be consistent with elevated expression at 35°C.

bbk32, which encodes a fibronectin binding protein (42) and whose expression is induced in feeding ticks and mice (21), is significantly upregulated at 35°C (1.7-fold).

Three lipoprotein genes—bbm28, bbp28, and bbs30, which are all members of paralogous gene family 113 and have recently been designated Mlps (66)—are significantly upregulated at 35°C (2.3- to 4.3-fold). The mlp family is comprised of eight genes with substantial sequence identity (72 to 88%, except for bbq35). In strains 297 and B31, some members of this family were not expressed at 23°C, were expressed somewhat at 35°C, and were expressed more substantially at 37°C (41, 66). Consistent with the findings here, prior Northern blot analyses of Mlp expression revealed substantial temperature-dependent expression of mlpA (bbp28), mlpC (bbs30), mlpF (bbm28), and mlpH (bbk28); the remaining mlp genes were poorly expressed, as was the case in the present study (41). We cannot rule out the possibility that expression of some mlp genes is regulated only by mammalian host factors, as previously shown for mlp orthologs in B. burgdorferi strain 297 (1). These putative factors would not have been present under our experimental conditions. The combined data, however, strongly suggest that only a subset of the mlp genes is temperature regulated.

Another paralogous family whose members are upregulated at 35°C is family 143. These genes have no known function, but

FIG. 3. (A) Temperature-regulated genes encoded on each genetic element. The percentage of genes on the chromosome and each plasmid differentially expressed at either 23 or 35°C. (B) Temperature-regulated genes encoded on lp54. The 76 ORFs encoded on lp54 are represented as bars linearly along the x axis. Closed bars denote ORFs expressed from the positive strand, and open bars represent those transcribed off the minus strand. Genes with greater expression at 35°C are indicated as up-regulated, and those with greater expression at 23°C are shown as down-regulated. Paralogs of genes also encoded on cp32 plasmids are indicated by shaded boxes.
all contain a signal peptidase II modification and processing site, strongly suggesting that they encode lipoproteins. In contrast to the Mlp family, all cp32-encoded family 143 genes are temperature regulated (up to two- to threefold at 35°C). However, since all the genes share 94% sequence identity, it is not clear whether the observed expression is the result of mRNA transcript from one or more of these genes. Thus, although by database annotation the levels of expression of 21 cell envelope constituents are significantly increased at 35°C, the actual number may be somewhat higher than that (31, if one considers bba65, bba69, and the family 143 genes). Regardless of the precise number, our results underscore the notion that substantial remodeling of the spirochete’s cell surface occurs as a consequence of growth at the elevated temperature (e.g., during tick feeding and transmission).

Several cell envelope genes are expressed at significantly higher levels in organisms grown at 23°C. Most noteworthy is the differential expression of plasmid lp38-encoded ospD (bbj09), which was 13-fold elevated at 23°C relative to 35°C. OspD was first characterized as a protein that was unique to low-passage-number isolates of B. burgdorferi (37). No information regarding its regulation by temperature has been reported, and its function is not known. Plasmid lp38 is missing from a subtype of B. burgdorferi human patient and animal isolates (30, 40), suggesting that it is not required for mammalian infection. By analogy to several other proteins that are known to be upregulated at 23°C and expressed in the tick midgut (e.g., OspA), this suggests that OspD may play a role during tick infection.

It previously has been shown that the various cp32s harbor genes encoding a large family of immunogenic lipoproteins, termed Erps (for OspEF-related proteins) (17, 53, 56). However, a closer examination of the Erps has revealed that they can be divided into three different and evolutionarily distinct paralogous gene families (families 162, 163, and 164). This separation is supported by the combined annotation of the complete strain B31 genome and the detailed analyses of these gene families in strains 297 and B31 (2, 5, 7, 54). It has been proposed that paralogous families 162, 163, and 164 be termed OspE-related proteins, Elps, and OspF-related proteins, respectively (2, 5). Interestingly, several studies in multiple borrelial strains have shown that some members of the 162, 163, and 164 protein families are regulated by temperature as well as other mammal-specific factors (1, 3, 26, 53, 55, 57, 60). In the present study, only expression of bbn39 (family 163) was found to be significantly altered by temperature, and surprisingly, its expression was found to be significantly greater (1.6-fold) at 23°C than at 35°C. It is worth noting in this regard that expression of bbo39 (erpL) and bbr42 (ospF) was undetectable at 23°C but was observable at 35°C (these are not listed in Table 4 because the differences in expression did not meet the required stringent statistical criteria). Prior studies have shown that the genes comprising families 162, 163, and 164 are typically upregulated at 35°C (53, 55), but the reasons for the
discrepancy between the array results presented here and in earlier studies are not clear. They may be related to differences in the growth medium employed here compared to that used in prior studies (BSK-II versus BSK-H medium), which has been shown to be important in the overall regulation of these genes (65). Alternatively, it may be that expression of these various gene families is more complex than previously recognized and that factors other than temperature also influence their expression. In this regard it should be noted that prior studies of several borrelial strains, including strains N40 and 297, have shown that mammalian host factors are important in regulating transcription and translation for several family: 162, 163, and 164 orthologs (1, 3, 13, 27, 57, 60). Future studies using real-time PCR and specific primers will likely be needed to fully elucidate the complex regulation of the genes which comprise these three lipoprotein gene-encoding families.

**Differential expression of putative transporter genes.** A number of genes with possible transporter functions are differentially expressed by temperature. Two operons are of particular interest. *bb0216 to bb0218* encode components of a putative phosphate ABC transporter, and *bb0216 and bb0218* are significantly upregulated 1.5- to 1.75-fold at 35°C. Expression of *bb0217* was also greater at 35°C (1.4-fold) but with a *P* value of 0.019, which did not reach significance. Therefore, it is tempting to speculate that phosphate transport may be enhanced at the higher temperature.

A cellubiose/chitobiose transporter operon is encoded on circular plasmid cp26. This operon has an unusual organization; *celC* and *celA* (*bb005* and *bb006*) are transcribed off the plus strand, whereas *celB* (*bb004*) is transcribed divergently. (The *cel* genes were renamed *chb* in keeping with their role in chitobiose utilization and nomenclature in *E. coli* [59], but the original *cel* designation is used here.) Interestingly, *celC* and *celA* were found to have increased expression at 35°C (2.14- and 2.0-fold, respectively), whereas *celB* had significantly higher expression at 23°C. A previous study demonstrated that *celB* expression was higher in organisms grown at 23°C, but that of *celCA* was not affected by temperature (59). The *celB* gene product is required for utilization of chitobiose, a dimer of N-acetylgalactosamine, the subunit of chitin and a component of the tick cuticle. This led to the hypothesis that chitobiose may serve as a nutrient source for the spirochete within ticks (59). This would be consistent with elevated expression of chitobiose-related genes at the lower temperature. Interestingly, *bb0002*, which encodes a β-N-acetylhexosaminidase, is also significantly elevated in spirochetes cultivated at 23°C (Table 5). This enzyme could presumably be involved in the metabolism of chitobiose that is taken up via the transporter.

**Glycolysis and glycerol uptake and utilization.** Among the genes annotated as being involved in intermediary metabolism, several are significantly upregulated by growth at 23°C. In particular, *bb0240 to bb0243* appear to constitute an operon encoding proteins involved in glycolysis uptake (glpF; *bb0240*) and utilization (glpK and glpA; *bb0241* and *bb0243*, respectively). These gene products would mediate glycerol uptake (glpF) and its conversion to dihydroxyacetone phosphate via a glycerol 3-phosphate intermediate (glpK and glpA). All four putative genes are significantly upregulated between 2.2- and 3.1-fold at 23°C. RT-PCR analysis indicates that all four genes are cotranscribed (data not shown). *bb0242* has no database match, and its role in this process is unknown. Interestingly, genes that provide a second route to dihydroxyacetone phosphate are also elevated significantly at 23°C. This would be mediated by a pyrophosphate-dependent phosphofructokinase (*bb0020*) and aldolase (*bb0445*), which are upregulated 1.5- to 1.8-fold in organisms grown at 23°C. Genes encoding the enzymes that catalyze the reactions downstream of dihydroxyacetone phosphate in glycolysis exist in *B. burgdorferi*. Of these, only *pgk* (encoding phosphoglycerate kinase) was moderately elevated at the lower temperature. Taken together, these data suggest that lower temperature induces *B. burgdorferi* to increase its cellular level of dihydroxyacetone phosphate, presumably to enhance the rate of energy production via glycolysis. Alternatively, these genes may be repressed at the higher temperature.

**Temperature shift and global transcriptional regulation.** It was not clear previously if *B. burgdorferi* undergoes a heat shock response due to the rapid rise in temperature it experiences during tick feeding. In the present study, none of the genes encoding typical heat shock proteins (e.g., dnaK or groEL) are up-regulated by temperature shift. Furthermore, there was no discernible down-regulation in components of the translational machinery, such as ribosomal proteins, after the temperature shift. This global gene expression profile would suggest that a shift in temperature from 23 to 35°C does not induce a heat shock or general stress response (10). These observations lead us to propose that the altered gene expression profiles observed on temperature shift from 23 to 35°C are directly related to an adaptive response for migration of *B. burgdorferi* from the tick midgut to the salivary glands and/or transmission of *B. burgdorferi* from the tick to mammalian host.

Hubner et al. (28) recently demonstrated that the enhanced expression of *ospC* and *dbpA* in response to environmental cues such as elevated temperature and decreased pH proceeds via a novel regulatory network involving the alternate sigma factors RpoN and RpoS. In addition, RpoS protein levels were elevated in cells grown at 35°C relative to those cultivated at 23°C. Consistent with this, a 1.6-fold increase in RpoS mRNA was observed in the current study in spirochetes grown at 35°C (Table 4).

**Correlation between microarrays and real-time RT-PCR.** Real-time quantitative RT-PCR was employed to validate the final microarray data. Sixteen ORFs with differential expression values ranging from approximately 3-fold-higher expression at 23°C to 5.5-fold-higher greater expression at 35°C were chosen for the real-time PCR analysis (Table 1). As shown in Fig. 5, there was a high correlation between the expression values obtained by real-time PCR analysis and those measured by array analysis (r = 0.93).

**Concordance with other microarray analyses.** Several recent reports have described the use of gene arrays to facilitate *B. burgdorferi* transcriptional analysis (33, 36, 46). Narasimhan et al. spotted clones from a strain N40 genomic library and probed the arrayed clones with a DECAL cDNA library generated from either fed or unfed tick midguts (36). In this manner they identified a number of *B. burgdorferi* genes expressed in either the fed- or unfed-tick state. Liang et al. limited their analysis to 137 putative lipoprotein genes and studied their expression during murine infection (33). All of these genes were expressed during in vitro growth, but a sub-
substantial number were downregulated during murine infection. The limited overlap between the genes identified in these studies (33, 36) and those in our data set is most likely the result of substantial differences in array design and transcript source. Revel et al. recently reported an analysis of global regulation of *B. burgdorferi* gene expression similar to that presented here (46). Of 215 genes found to be differentially regulated by temperature in the present study, only 25 (11.6%) were regulated under the fed-tick condition (that most comparable to the present study, only 25°C (11.6%) were regulated). Of 215 genes found to be differentially regulated by temperature in the present study, only 25 (11.6%) were regulated under the fed-tick condition (that most comparable to the present study, only 25°C (11.6%) were regulated). The concordance was somewhat better when the comparison was limited to those genes which were upregulated at 35°C (17.3%; 23 of 133) and still better if only those genes upregulated at 35°C by twofold or more in this study were considered (41%; 17 of 41). The limited correspondence between the two data sets may be a result of several methodological differences. The temperature shift in this study was from 23 to 35°C compared to 37°C in the Revel et al. study (46). In addition, Revel et al. also altered the pH between the two conditions, so the observed changes may be a combination of both temperature and pH regulation. Technical differences such as the cDNA labeling method and membrane versus glass slide arrays may, in part, account for the observed disparity. In addition, a cloned isolate of B31-M1 was used in the present study; nonclonal isolates of B31-M1 have been shown to consist of subpopulations that have very different genotypes (i.e., plasmid content) with various phenotypes (18). Additional *B. burgdorferi* gene array studies performed under similar experimental conditions are necessary to reconcile these overlapping data sets.

**Concluding remarks.** Microarray experiments are often regarded as unfocused since they typically have broad and encompassing hypotheses (9). However, much has been gleaned from microarray analyses, especially when designed to characterize differences in gene expression due to a single environmental stimulus. In the present study, we specifically chose to examine the response of a cloned isolate of *B. burgdorferi* to a single environmental variable (temperature) to help identify genes encoding proteins that could be important in the adaptation of *B. burgdorferi* for growth in the tick and the mammalian host.

It is apparent from the present study that plasmid-encoded ORFs are integral to the temperature shift response. Most prominent among the findings are the previously unrecognized roles for many lp54 genes in this response, a possible role for OspD in the tick environment, and the contribution of cp32-encoded genes to the enzootic life cycle of *B. burgdorferi*. The latter point is supported by the findings that (i) more than 20% of the ORFs encoded on many of the cp32s are regulated by temperature, with almost all having increased expression at 35°C; (ii) the expression of many of the putative hemolysins encoded on these plasmids was significantly up-regulated by temperature, which may be related to digestion of the blood meal; (iii) several cp32 lipoprotein genes encoding surface antigens are differentially up-regulated by temperature (e.g., *mpl* genes), suggesting they may be important at the host-pathogen interface; and (iv) the overall transcriptional profiles and gene homologies are consistent with the cp32s being the genetic elements that may encode a *Borrelia*-specific bacteriophage. This is especially noteworthy since our analysis of the cp32 transcriptomes is consistent with recent speculation that cp32-encoded bacteriophage may be involved in horizontal transfer of genetic information between spirochetes during tick feeding (i.e., at the same time the cp32 ORFs are up-regulated) (5, 14).

The combined data indicate that major alterations in the expression of borrelial outer surface antigens, cell envelope constituents, nutrient transporters, and metabolic genes take place in response to a shift in growth temperature from 23 to 35°C (as may occur during tick feeding). These changes are likely important for the parasitic strategy of *B. burgdorferi* as it adapts to growth in the tick environment and the mammalian host and for enabling the spirochete to traverse the tick environment during migration from the midgut to salivary glands. The results provide numerous new avenues for focused, hypothesis-based investigations to help delineate the role(s) of specific genes, operons, and regulons in the life cycle of this pathogen. In addition, several previously unrecognized temperature-regulated surface proteins were identified. These may provide new vaccine candidates or targets for novel therapeutics for the treatment or prevention of Lyme disease.

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C. Ojami and C. Brooks contributed equally to this work.

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**REFERENCES**


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