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Combined Effects of Blood and Temperature Shift on *Borrelia burgdorferi* Gene Expression as Determined by Whole Genome DNA Array

Rafal Tokarz, Julie M. Anderton, Laura I. Katona, and Jorge L. Benach*

Department of Molecular Genetics and Microbiology, Center for Infectious Diseases, Stony Brook University, Stony Brook, New York

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***Borrelia burgdorferi* undergoes differential gene expression during transmission from its tick vector to a vertebrate host. The addition of blood to a spirochete culture at 35°C for 48 h had a dramatic effect on gene expression of this organism. Utilizing *B. burgdorferi* whole genome DNA arrays, we compared the transcriptomes of the spirochetes following a 2-day temperature shift with blood and without blood. Using combined data from three independent RNA isolations we demonstrated that the addition of blood led to a differential expression of 154 genes. Of these, 75 genes were upregulated, with 49 (65%) of them encoded on plasmids. Blood supplementation of cultures also resulted in the downregulation of 79 genes, where 56 (70%) were plasmid encoded. We verified our results by reverse transcriptase PCR of several genes in both flat and feeding ticks. In the 2-day experiment we observed the effect that exposure to increased temperature and blood combined had on *B. burgdorferi* gene expression at this crucial time when the spirochetes begin to move from the vector to a new vertebrate host. These changes, among others, coincide with the upregulation of the chemotaxis and sensing regulons, of the lp38-encoded ABC transporter, of proteases capable of remodeling the outer surface of the spirochetes, and of the recombination genes of cp32 as a transient or initial part of the stress response of the phage. These are all functions that could cause or facilitate the changes that spirochetes undergo following a blood meal in the tick.**

Lyme disease is an illness caused by an infection of the spirochete *Borrelia burgdorferi* (5, 9). The spirochete persists in nature in a zoonotic cycle between its arthropod vector, the *Ixodes scapularis* tick, and vertebrates and is responsible for over 10,000 cases of Lyme disease in the United States annually (40).

The ability of the spirochete to invade and colonize two different kinds of hosts has been an area of active research. Differential gene expression of spirochetes during tick feeding is thought to facilitate the passage of the spirochete from the tick to its new vertebrate host. In the unfed tick, the spirochetes are in the lumen of the midgut, present in low numbers, and express unique antigens, including the outer surface lipoprotein A (OspA) (10, 17, 48). As the tick begins to feed, the temperature in the midgut rises, pH drops, and in response to this radically changing environment, the spirochetes undergo drastic changes in physiology. They begin to multiply dramatically and change their gene expression profile. One well-known example of changes in gene expression is the downregulation of OspA and upregulation of OspC (15, 37, 57, 58), and this switch in expression has been used to propose pathogen-vector interactions with these lipoproteins. OspA has been implicated in adherence to the midgut of the flat tick (42, 68). Likewise, there may be a role for OspC in directing the spirochetes to the salivary glands, which may in part explain its initial upregulation (23, 43). However, this molecule may be more important for vertebrate infection than for traffic within the tick (26). Many other proteins in the spirochetes undergo differential expression during this critical time (3, 25). The spirochetes are able to penetrate the midgut and enter the salivary glands of the ticks. Once in the salivary glands, the spirochetes are deposited into the skin of the vertebrate host (17, 45, 49, 65). The entire process of the spirochete migration from the midgut through the salivary glands to the new host takes approximately 2 days (47).

The genome of *B. burgdorferi* strain B31, containing a linear chromosome and 12 linear and 9 circular plasmids, has been completely sequenced (13, 24). The genome sequence has allowed the opportunity to study the gene expression profile of the spirochete in response to experimental changes with DNA array technology. Several recent publications described such studies utilizing differential gene expression in *Borrelia* grown at temperatures mimicking unfed (flat) ticks and feeding tick-mammal conditions (39, 54, 71). Another study identified genes differentially expressed when *B. burgdorferi* spirochetes were grown in dialysis membrane chambers transplanted into peritoneal cavities of rats, i.e., in a "host-adapted" state (8). In addition, several differentially expressed genes in flat and feeding ticks were identified utilizing decal technology (34). These studies have presented valuable data on changes in gene expression and how *B. burgdorferi* gene expression oscillates throughout its life cycle due to the spirochete circulating between new environments. The studies have also shown that various results can occur not just because of the technology used but also due to differences in experimental conditions.

Several factors affect differential gene expression of *B. burgdorferi* in vitro. Changes in temperature, pH, and growth phase

* Corresponding author: Mailing address: Center for Infectious Diseases, Stony Brook University, 248 Centers for Molecular Medicine, Stony Brook, NY 11794-5120. Phone: (631) 632-4286. Fax: (631) 632-4294. E-mail: jbenach@notes.cc.sunysb.edu.

can result in differential expression of several genes (11, 12, 51, 52, 66). However, the changes during tick feeding do not occur in isolation from each other. For example, increasing temperature and pH changes in the midgut occur simultaneously as a result of the incoming blood meal and its large influx of nutrients. In fact, this interdependence of environmental factors having a role in gene expression has already been documented (66). The goal of the present study was to determine the combined effect of blood influx and temperature shift on *B. burgdorferi* gene expression, in similarity to the changes the spirochetes encounter in feeding ticks. Using *B. burgdorferi* DNA whole genome arrays, we identified genes differentially expressed when *B. burgdorferi* encounters human blood following a 48-h temperature shift from 23 to 35°C. These changes in gene expression may be essential for the spirochete in transmission and adaptation to the vertebrate host. In addition, reverse transcriptase PCR (RT-PCR) in flat and feeding ticks was used to validate array results and to show that the timing and extent of the differential expression of many of these genes in the natural tick-vertebrate cycle are the result of the combined effects of the blood and of the increase in temperature.

MATERIALS AND METHODS

Culture conditions. The *B. burgdorferi* low-passage B31 MI strain was used in all experiments. The strain contained all 21 plasmids, as confirmed by PCR using plasmid-specific primers (20). Frozen stock vials of the strain were inoculated directly into 250 ml of BSK-H medium supplemented with 6% rabbit serum (Sigma, St. Louis, Mo.) and incubated at 23°C until the cell density reached approximately 1.5×10^6 spirochetes/ml. The culture was then divided into 50-ml Falcon tubes, with 47 ml of culture placed into each tube. For blood studies, 3 ml of human blood from which the buffy coat was removed and which contained 10% 0.1 M sodium citrate was added. Control cultures received 3 ml of BSK-H medium with 10% 0.1 M sodium citrate. All cultures were incubated at 35°C for 48 h, with periodic mixing to prevent the settling of the blood. These experiments were repeated three times. Each of the three experiments yielded separate RNA preparations, which were used for three separate cDNA probe syntheses and three separate array hybridizations (see below). The pH of the cultures was measured with a 300 series pH meter (Beckman Instrument Inc., Fullerton, Ca.) prior to addition of blood, as well as at 24 and 48 h afterwards. All spirochete enumeration was performed utilizing dark-field microscopy.

RNA extraction. Following the 48-h incubation, the cultures were centrifuged at $400 \times g$ for 4 min to sediment the red blood cells. Spirochetes in the supernatant were then centrifuged in a new 50-ml tube for 10 min at $7,000 \times g$, and the pellet was resuspended in 0.4 ml of BSK-H; the remaining medium was decanted. RNA extraction was performed with Tri-Reagent LS (Molecular Research Center Inc.) according to the manufacturer's instructions. The RNA was then passed through an RNeasy spin column (QIAGEN Inc., Valencia, Ca). An aliquot of the RNA was used for A_{260}-A_{280} spectrophotometry readings as well as for agarose gel electrophoresis. Three separate 35°C and 35°C-blood RNA preparations were isolated as described above and used in the array experiment.

cDNA probe synthesis. A cDNA probe was created for hybridization to the DNA array membranes as described previously, with some modifications (2, 38). A 15-μl reaction mixture was set up which contained 5 μg of RNA and genome-directed primers (GDP) (54). Amplifications were carried out in a PTC-1152 MiniCycler (MJ Research, Waltham, Mass.) with the parameters set at 90°C for 2 min followed by a ramp to 42°C over 20 min. A final volume of 30 μl containing 1× reaction buffer (50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, pH 8.5), 50 U of avian myeloblastosis virus RT, 333 μM dCTP, 333 μM dGTP, 333 μM dTTP (Roche, Indianapolis, Ind.), and 20 μCi of [α-^{33}P]dATP (ICN, Costa Mesa, Calif.) was added to the reaction mixture. This reaction was incubated at 42°C for 2.5 h in a MiniCycler. Unincorporated radiolabeled nucleotide was removed from the labeled cDNA by use of a Panorama Sephadex G-25 column on the basis of the protocol of the manufacturer (Sigma-Genosys).

Membrane hybridization and array image analysis. The *B. burgdorferi* genome arrays used in these experiments consisted of 1,689 PCR amplified open reading frames (ORFs) spotted in duplicate on charged nylon membranes (8, 38, 39). All details regarding construction and handling of these membranes have been extensively documented by Ojaimi et al. (38, 39). Two membranes were utilized in the experiment. Each RNA preparation was hybridized twice, once to each membrane, to account for any slight membrane variations, and the average intensity from both experiments was calculated. Membranes were neutralized in 35- by 300-mm hybridization bottles (Robbins Scientific, Sunnyvale, Calif.) in 250 ml of 2× SSPE (0.3 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA) for 25 min; in subsequent experiments, membranes were neutralized for 5 min. Membranes were prehybridized for 60 min in 5 ml of 65°C hybridization solution (Sigma-Genosys) containing 100 μg of salmon testes DNA (Sigma-Genosys)/ml. The entire radiolabeled cDNA sample was added to 10 ml of hybridization solution containing 100 μg of salmon testes DNA/ml, denatured in a 95°C water bath for 10 min, and then added to the membranes and allowed to hybridize for 16 to 18 h at 65°C in a Model 2000 Micro hybridization incubator (Robbins Scientific). The cDNA was removed from the bottles, and 40 ml of wash solution (0.5× SSPE, 2% sodium dodecyl sulfate) was added. Bottles were inverted 10 times at room temperature, and the wash solution was decanted. This step was repeated twice. Following the third wash, 80 ml of wash solution was added and the bottles were incubated at room temperature in the hybridization oven for 20 min. Membranes were exposed to a 35- by 43-cm PhosphorImager screen (Molecular Dynamics, Sunnyvale, Calif.) for 2 to 3 days. Screens were viewed using ImageQuant 1.2 software in a Storm 860 PhosphorImager (Molecular Dynamics). Pixel densities for each spot were generated with ArrayVision software version 6.0.

Membranes were stripped in roller bottles, initially with 250 ml of 0.4 M NaOH followed by incubation with stripping solution (250 ml of 200 mM Tris-HCl, 0.1× SSPE, 0.2% sodium dodecyl sulfate, pH 7.0) for 30 min at 45°C each with rotation. This procedure was followed by incubation with 250 ml of stripping solution at room temperature in the hybridization oven for 30 min with moderate rotation. Membranes were exposed to the PhosphorImager screen (as described above) overnight to ensure that the stripping was successful so that membranes could be reprobed.

Statistical analysis and bioinformatics. ArrayVision software (Imaging Research, St. Catharines, Ontario, Canada) was used to generate raw pixel density data for every ORF from each hybridized membrane. Each B. *burgdorferi* ORF is represented by two spots. An initial gene filter created a threshold for each gene individually on the basis of the local background of each spot. In brief, the mean density of membrane areas that did not have spotted DNA was subtracted from each spot value to yield the raw data. Only genes with raw data values ≥ 2 standard deviations (SD) above the local background were analyzed. The raw data were imported into a Microsoft Excel spreadsheet (Microsoft Corp.), where each individual spot was divided by the sum of all spots to get a percent intensity value for each individual spot. All genes with expression levels above the background from individual RNA preps were plotted by calculating their log 10 percent intensity culture with blood (designated 35B culture)/culture without blood (designated 35 culture) ratio. Correlation plots between separate RNA isolations were obtained by plotting the percent intensity 35B culture/35 culture ratio for each ORF in Microsoft Excel. Transcript abundance was calculated as described previously (39). Our final analysis consisted of the mean of the combined data from all three experiments. The data was subjected to an unpaired two-tailed *t* test to determine which ORFs were differentially expressed at a *P* value of ≤ 0.01. Differentially expressed genes were further analyzed for possible functions, paralogous family members, and structural motifs, domains, and regions by the use of The Institute for Genomic Research software and protein databases (http://www.tigr.org, http://smart.embl-heidelberg.de/, http://psort.nibb.ac.jp/form.html, http://www.sanger.ac.uk/Software/Pfam/, and http://us.expasy.org/sprot/).

Quantitative real-time RT-PCR. This procedure was used for validation of whole genome DNA array experiments. One of the three RNA preparations utilized in the array study was also used for RT-PCR. For cDNA synthesis, 2 μg of total RNA was used in an RT reaction with GDP as described previously. The resulting cDNA sample was diluted 1:50 for use in the RT-PCR. Three replications were performed for each gene. LightCycler-FastStart DNA Master SYBR I Green (Roche) reaction mix was used in the experimental procedure with the LightCycler (Roche) on the basis of the manufacturer's protocol. Cycle parameters were as follows: preincubation, 1 cycle at 95°C for 5 min; amplification (quantification), 40 cycles of 95°C for 15 s, 52°C for 6 s, and 72°C for 6 s; melting curve, 95°C for 0 s, 65°C for 15 s, and 72°C for 0 s; and cooling, 1 cycle of 40°C for 30 s. 16S rRNA and *flaB* were used as internal controls for all data. Oligonucleotide primers for RT-PCR were designed using MacVector software (Kodak International Biotechnologies, Inc., New Haven, Conn.).

RT-PCR of *Borrelia*-infected ticks. Infected *I. scapularis* nymphs were placed on the neck of temporarily restrained uninfected mice. The nymphs were allowed to feed for 72 h, after which they were removed and homogenized. Total RNA was isolated using Tri-Reagent LS (MRC Inc.) according to the manufacturer's

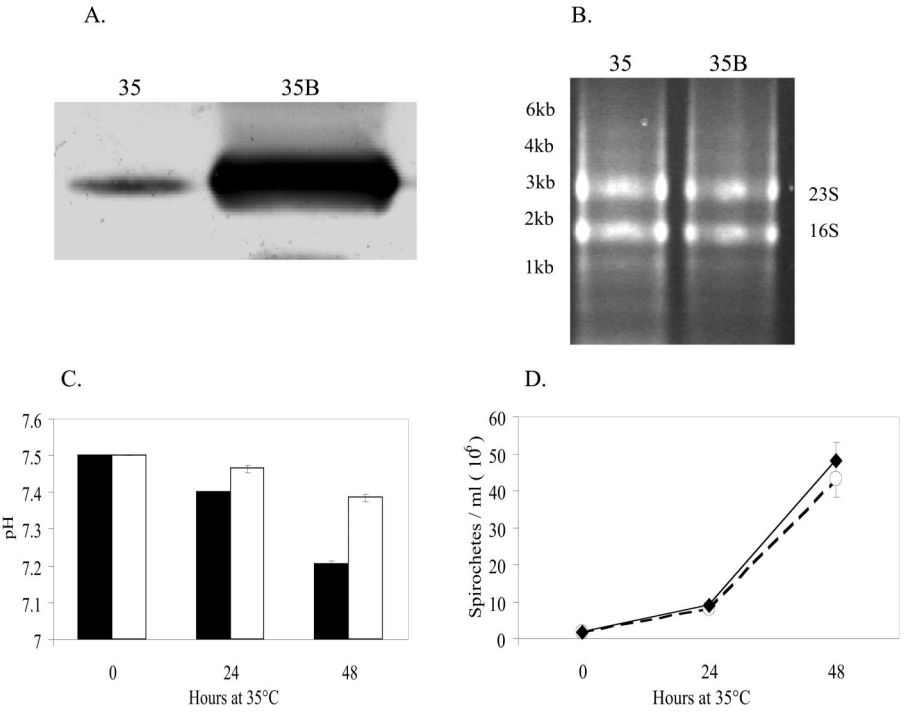

FIG. 1. Comparisons between 35B and 35 cultures. (A) Western blot of OspC from spirochetes grown in control cultures and in cultures supplemented with blood after 48 h of incubation. (B) Agarose gel electrophoresis of RNA obtained from cultures grown at 35°C and at 35°C with blood for 2 days. A total of three separate RNA isolations were performed; the figure shows a representative 16S and 23S rRNA electrophoretic pattern of one isolation. Molecular mass markers are shown on the left. (C) pH changes in blood (black bars) and control (white bars) cultures of *B. burgdorferi* at 24 and 48 h. (D) Growth curve of *B. burgdorferi* in blood (solid line) and control (dashed line) cultures of *B. burgdorferi* at 24 and 48 h.

instructions. RNA was treated with 10 U of DNase I (Roche) for 1 h at 37°C followed by purification by RNeasy kit spin columns (QIAGEN). Total RNA (5 μg) was reverse transcribed to cDNA by the use of avian myeloblastosis virus RT (Roche) and GDP. PCR consisting of 1 cycle at 94°C for 5 min and 40 cycles of 94°C for 20 s, 52°C for 30 s, and 72°C for 30 s was performed. A PCR of an RNA sample without RT was always run to ensure that no DNA contamination was present. For flat ticks, total tick RNA was isolated from adult ticks collected on Shelter Island, N.Y., and treated in an identical manner.

Western blot analysis. Minislab gels were cast with a 10-well comb, and samples (12 μl/well) were applied and run for 2 h at 20 mA/gel. Proteins were electroblotted onto nitrocellulose, blocked in 2% casein, and incubated for 1 h each in primary antibody (1/1,000 dilution of murine anti-OspC immunoglobulin G) and secondary antibody (1/1,000 dilution of a 0.2 mg/ml stock of alkaline phosphatase conjugated to goat anti-mouse immunoglobulin G [Fisher, Pittsburgh, PA]) and reacted with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Kirkegaard & Perry, Gaithersburg, Md.).

RESULTS AND DISCUSSION

Experimental design. *B. burgdorferi* spirochetes are present in very low numbers in flat ticks, but there is a dramatic increase in spirochete numbers during tick feeding (17, 48, 49). To mimic the conditions found in tick midguts in vitro, spirochetes were grown at 23°C to a very low concentration prior to shifting them to 35°C. Once the spirochetes reached the concentration of 1.5×10^6/ml, the 23°C culture was split and then temperature shifted to 35°C either with or without blood and incubated for 48 h. The time interval was chosen because the highest number of spirochetes in feeding ticks expressing OspC was found after 48 h (57). In addition, it is around this time that passage of infectious spirochetes from ticks to animals is most likely to occur (46, 47). In our experiments, OspC protein levels vastly increased following a 2-day incubation with blood at 35°C (Fig. 1A).

Two DNA array studies have been published describing differential gene expression of *B. burgdorferi* at temperatures that would be expected in flat and feeding ticks (39, 54). In those experiments, the spirochetes were initially grown at 23°C, diluted, and shifted to either 35°C (39) or 37°C (54). The comparison was then made between the gene expression profiles at 23°C and at the elevated temperatures. These studies, while uncovering temperature-regulated genes, may have missed genes expressed transiently during the first 2 days of blood feeding. More importantly, they would not uncover genes differentially expressed due to factors in the incoming blood meal. For the array results described here, we analyzed the *B. burgdorferi* transcriptome changes that occurred after a 48-h temperature shift to 35°C of spirochetes in 35B culture compared to results for spirochetes temperature shifted in 35 culture. In a 2-day experiment we observed the effect that exposure to increased temperature and blood combined had on *B. burgdorferi* gene expression at this crucial time, when the spirochetes begin to move from the vector to a new vertebrate host.

A representative pattern of the RNA generated under these experimental conditions showed the 16S and 23S typical of prokaryotic samples and none of the 18S and 28S rRNA that could be derived from the human blood (Fig. 1B). The mean

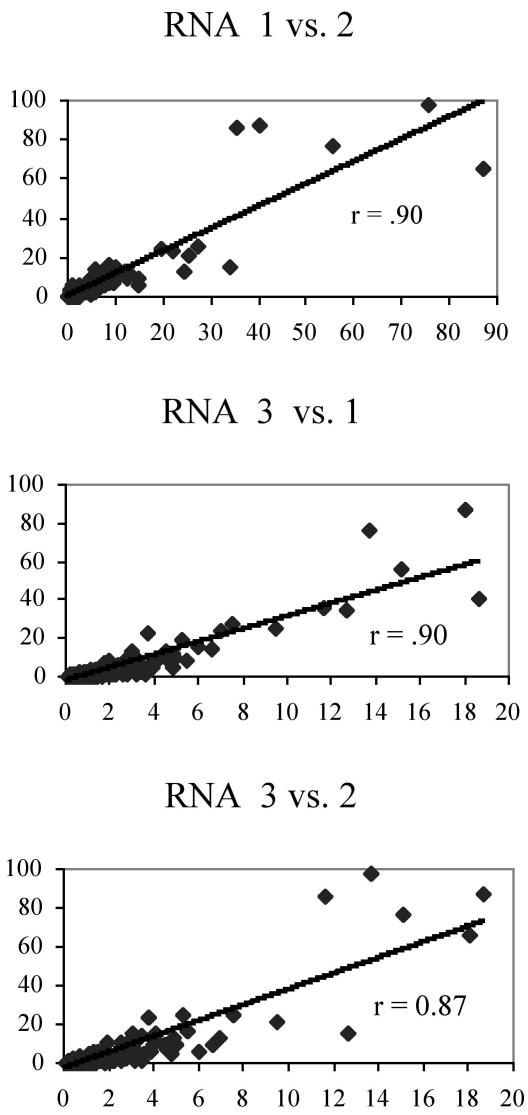

FIG. 2. Scatter plots of gene expression derived from three separate RNA isolations. Panel A compares RNA isolation 1 to isolation 3, panel B compares RNA isolation 2 to isolation 3, and panel C compares RNA isolation 1 to isolation 2. The 35B culture/35 culture percent intensity ratio from each RNA isolation was calculated and plotted against the ratios from the two other RNA isolations. The correlation coefficient *r* value is provided for each plot.

pH of the blood cultures was 7.20 compared to that of the control at 7.38 after 48 h of incubation (Fig. 1C). This drop in pH is less than the 6.8 reported for feeding ticks but may have contributed to some of the differences reported here (66). The addition of blood did not result in growth inhibition of the spirochetes in the time frame of the experiments (Fig. 1D).

Different RNA isolations produced reproducible array results. Three RNA isolations generated from three separate experiments were used to probe the arrays. To compare the RNA isolations, 35B culture/35 culture percent intensity ratios of all the ORFs were plotted, and correlation coefficients between all isolations were calculated (Fig. 2). Furthermore, the log-transformed percent intensity ratio was calculated for each experiment and plotted to identify genes differentially expressed from each RNA isolation as well as to identify genes whose expression levels differed between different RNA isolations (Fig. 3). The array results from the three RNA isolations showed a high degree of correlation to one another (Fig. 2 and 3). This allowed us to pool and analyze the data by using the mean values for all three RNA isolations. The final analysis only included genes with significant change in expression ($P \leq 0.01$) and with at least twofold differential ratios.

Incubation of *B. burgdorferi* with blood for 48 h leads to a gene expression profile that is different from the profiles obtained by temperature shifts alone. The combined data from our three separate RNA preparations yielded 154 genes that were differentially expressed, as determined by the established statistical criteria (Table 1 and Table 2). Of these, 75 genes were upregulated, with 49 (65%) of them encoded on plasmids (Table 1). Likewise, the genes with the highest fold increases were also plasmid encoded (Table 1). Blood supplementation of cultures resulted in the downregulation of 79 genes, of which 56 (70%) were plasmid encoded (Table 2). As *B. burgdorferi* contains many paralogs in its genome, it was expected that many genes sharing high sequence similarity and belonging to the same paralogous gene family would exhibit similar expression characteristics on the array, although it is unknown whether all of the paralogs are identically regulated or even expressed (8, 39).

B. burgdorferi B31 encodes approximately 150 putative lipoproteins (13). On our array, a total of 13 lipoproteins, seven belonging to the *mlp* paralogous gene family, were upregulated whereas 10 were downregulated.

Several genes described in the present study were shown to be differentially expressed in array studies by Revel et al. and Ojaimi et al. (39, 54). Some concordance would be expected, given that this study utilized a temperature shift, a variable also present in both previous studies. However, out of 154 differentially expressed ORFs in this study, only 36 (23%) were previously shown to be temperature regulated by Ojaimi et al. (39) whereas 31 (20%) were temperature and pH regulated in the experiments by Revel et al. (54). Of further note, only 14 (9%) ORFs differentially regulated in the present study were reported for both previous temperature shift array experiments. Concordance between all three studies was seen only within our top eight upregulated genes.

Blood and time in culture are important variables for gene expression. The spirochetes grown at 35°C for 2 days did not exhibit a gene expression profile that was similar to those obtained in previous temperature shift arrays in which time in culture was longer than our 48 h (39, 54). A case in point is the change in expression of *ospC*. While the spirochetes had not yet upregulated *ospC* in a 35°C culture after 48 h, the addition of blood at the same temperature had a dramatic effect on gene expression. *ospC* was highly upregulated, with the third highest fold increase of all the differentially expressed genes. In contrast, *ospC* is expressed in culture at 23°C at a very low level; after a two-day temperature shift to 35°C, its expression was not dramatically increased and it was the 107th most highly expressed gene on the array at 35°C (Table 3). However, examination of expression level data from the 35B culture demonstrated that *ospC* was the most expressed gene, containing more than double the transcript level of the second highest gene (Table 3). The vast upregulation and expression of *ospC*

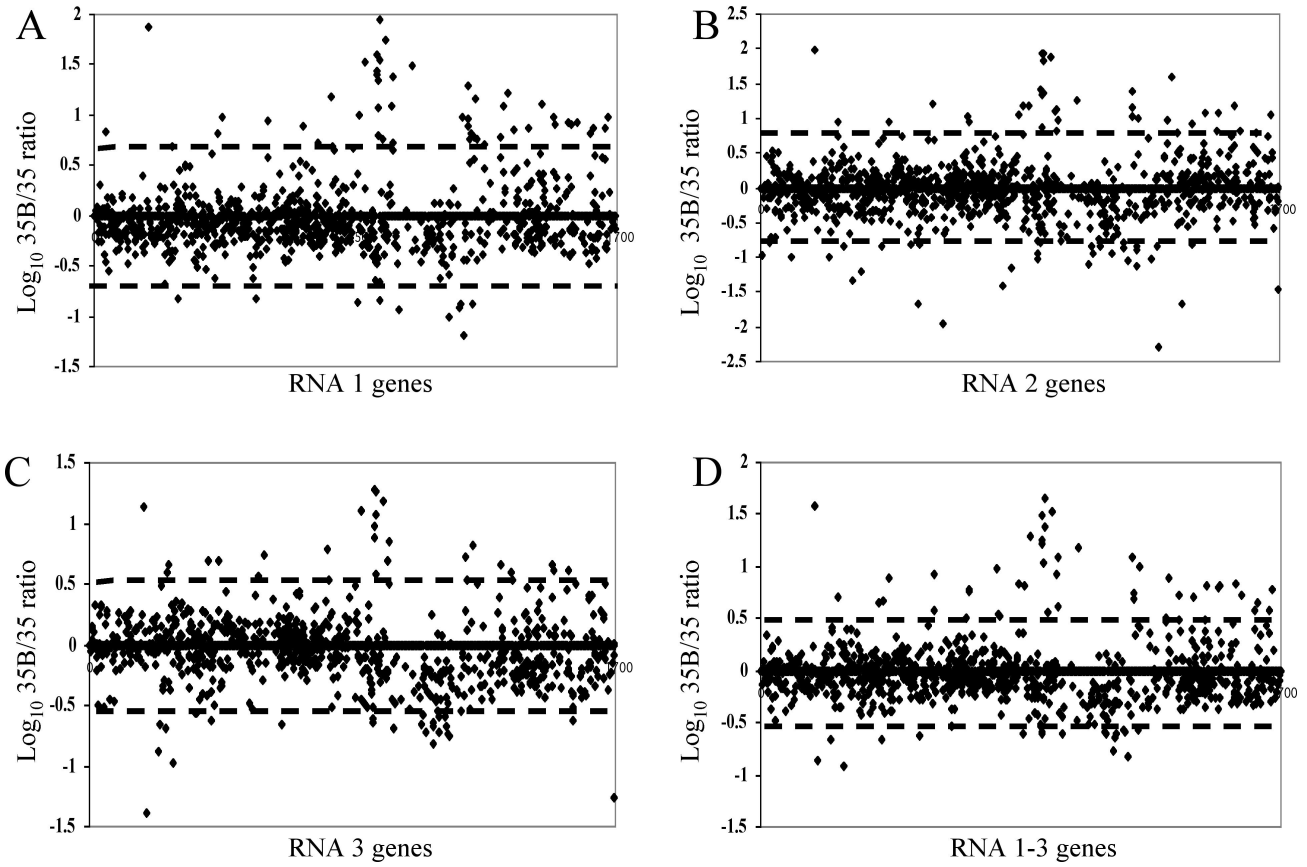

FIG. 3. Comparison of the three individual RNA isolations-hybridizations. Each individual spot represents one log 10 35B culture/35 culture percent intensity ratio of a single ORF with expression 2 SD above the background. Panels A to C represent individual cDNA results from experiments 1 to 3, while panel D represents the plot of all three combined RNA isolations. The dashed lines represent two SD above and below the means of all the plotted ORFs.

further shows that it is important for the spirochetes within the first 48 h of tick feeding.

Despite these differences, there are some important areas of agreement in all three studies. *ospC* was present as one of the top five most upregulated genes in every study. Besides *ospC*, several family 54 genes encoded on lp54 had the highest fold induction, implicating these genes as important in transmission of the spirochetes from vector to vertebrate host. *mlp* paralogs were also upregulated in all three studies. Finally, none of the genes encoded on plasmids lp5 and lp21 were differentially regulated on any arrays, including ours.

Quantitative and tick RT-PCR results validate genome array results. To validate the array results, we utilized quantitative RT-PCR analysis of a select group of genes. Primers were generated for genes that underwent significant differential expression on the array. We also randomly chose several genes which did not undergo significant changes on the array. One of the RNA isolations hybridized to the arrays was also utilized for RT-PCR, and then the results from the two techniques were compared. The quantitative data were normalized to two internal controls, *flaB* and 16S rRNA. The quantitative RT-PCR data was concordant with the array results, as all the genes showed patterns of expression similar to the array data patterns (Table 4). Array fold differences are usually underestimated; it is the inherent feature of the technology, likely as a result of cross-hybridization (21). Quantitative RT-PCR validation usually shows that fold differences are several magnitudes larger than indicated by array studies. Also, genes with very high or low levels of expression often have reduced correlation between the two methods. To further determine whether our array results of in vitro gene expression correlate with gene expression in spirochetes within ticks, we selected several genes differentially expressed on the DNA array and tested them for their expression in either the flat or feeding tick by RT-PCR. The results further confirmed our genomic array data (Table 5). The array experiment was able to identify genes upregulated during feeding or genes preferentially expressed in flat ticks. These experiments showed that our in vitro array experiments closely mimic the in vivo tick feeding environment.

lp28 genes are downregulated in blood cultures. Strain B31 MI contains four lp28 plasmids designated lp28-1, lp28-2, lp28-3, and lp28-4. A total of 24 genes from all lp28 plasmids were downregulated (Fig. 4). These results strongly suggest that the downregulated genes from lp28 plasmids are required for maintenance of spirochetes in flat ticks. That three of the plasmids, lp28-2, -3, and -4, had no effect on dissemination of *B. burgdorferi* within mice would also support a role for these genes in the flat tick (31, 50). However, lp28-1 appears to be important in vertebrate infection, as the loss of this plasmid

TABLE 1. Mean fold upregulation of genes on the cDNA array by use of three separate RNA isolations

Upregulation (fold)	Gene description	Replicon	Gene family
45.0	BBA72 hypothetical protein	lp54	
38.2	BB0175 conserved hypothetical protein	Chromosome	
33.6	BBB19 outer surface protein C (OspC)	cp26	
31.2	BBA66 antigen, P35, putative	lp54	54
24.2	BBA73 antigen, P35, putative	lp54	54
19.7	BBA24 decorin binding protein A (DbpA)	lp54	74
17.8	BBA65 hypothetical protein	lp54	54
16.7	BBA64 antigen, P35	lp54	54
15.4	BBE31 antigen, P35, putative	lp25	60
12.5	BBC12 phage exonuclease	cp9	165
12.3	BBJ25 hypothetical protein	lp38	
10.7	BBA71 hypothetical protein	lp54	54
9.9	BBJ46 hypothetical protein	lp38	
9.5	BB0771 RNA polymerase sigma factor (RpoS)	Chromosome	89
8.4	BB0565 purine-binding chemotaxis protein (CheW-2)	Chromosome	33
8.3	BBC05 phage ssb binding protein (ERF)	cp9	161
7.8	BBL36 phage exonuclease	cp32-8	165
7.6	BB0418 hypothetical protein	Chromosome	
6.9	BB0844 hypothetical protein	Chromosome	32
6.9	BBQ43 phage exonuclease	lp56	165
6.5	BBA05 antigen, S1	lp54	
6.5	BBO36 phage exonuclease	cp32-7	165
6.4	BBP35 phage exonuclease	cp32-1	165
6.3	BBP28 lipoprotein	cp32-1	113
6.1	BBS38 phage exonuclease	cp32-3	165
6.0	BB0681 methyl-accepting chemotaxis protein (Mcp-5)	Chromosome	13
5.8	BB0680 methyl-accepting chemotaxis protein (Mcp-4)	Chromosome	13
5.5	BBJ28 hypothetical protein	lp38	
5.4	BBM28 Mlp lipoprotein	cp32-6	113
5.3	BBQ74 conserved hypothetical protein, pseudogene	lp56	60
5.2	BB0253 ATP-dependent protease LA (Lon-1)	Chromosome	22
5.1	BBN35 conserved hypothetical protein	cp32-9	165
4.8	BBJ27 ABC transporter, permease protein	lp38	
4.7	BB0400 hypothetical protein	Chromosome	
4.5	BBR36 phage exonuclease	cp32-4	165
4.5	BB0385 basic membrane protein D (BmpD)	Chromosome	36
4.2	BBC11 phage integrase	cp9	96
3.8	BBR28 Mlp lipoprotein	cp32-4	113
3.7	BB0567 chemotaxis histidine kinase (CheA-1)	Chromosome	134
3.7	BBS30 Mlp lipoprotein	cp32-3	113
3.6	BBB09 hypothetical protein	cp26	
3.5	BB0776 hypothetical protein	Chromosome	
3.3	BB0782 nicotinate-nucleotide adenylyltransferase (NADD)	Chromosome	
3.2	BBL28 Mlp lipoprotein	cp32-8	113
3.2	BB0671 chemotaxis operon protein (CheX)	Chromosome	
3.1	BBM29 phage Ssb binding protein (ERF)	cp32-6	161
3.0	BBO28 Mlp lipoprotein	cp32-7	113
2.9	BBL29 phage Ssb binding protein (ERF)	cp32-8	161
2.8	BBQ37 phage Ssb binding protein (ERF)	lp56	161
2.7	BBJ43 hypothetical protein	lp38	90
2.7	BB0548 DNA polymerase I (PolA)	Chromosome	
2.7	BBS22 conserved hypothetical protein	cp32-3	142
2.6	BB0232 HbbU protein	Chromosome	
2.6	BBJ45 hypothetical protein	lp38	59
2.5	BB0279 flagellar protein (FliL)	Chromosome	
2.5	BBR29 phage Ssb binding protein (ERF)	cp32-4	161
2.5	BBN28 Mlp lipoprotein	cp32-9	113
2.4	BB0297 Smg protein	Chromosome	
2.4	BB0231 conserved hypothetical protein	Chromosome	125
2.4	BBP29 phage Ssb binding protein (ERF)	cp32-1	161
2.4	BBO29 phage Ssb binding protein (ERF)	cp32-7	161
2.3	BBM35 phage exonuclease	cp32-6	165
2.3	BB0843 C4-dicarboxylate transporter protein	Chromosome	
2.3	BBS31 phage Ssb binding protein (ERF)	cp32-3	161
2.3	BBK32 immunogenic protein P35	lp36	
2.3	BBA04 antigen, S2	lp54	44
2.2	BB0313 rRNA methylase (FtsJ)	Chromosome	
2.2	BBR44 DNA binding protein, putative	cp32-4	115
2.2	BB0689 hypothetical protein	Chromosome	
2.2	BB0017 ABC transporter, permease	Chromosome	
2.2	BBJ31 hypothetical protein	lp38	59
2.1	BB0443 SpoIIIJ-associated protein (Jag)	Chromosome	
2.1	BB0728 NADH oxidase, water-forming (Nox)	Chromosome	
2.1	BB0647 ferric uptake regulation protein (Fur)	Chromosome	
2.1	BBL38 phage integrase	cp32-8	96

TABLE 2. Mean fold downregulation of genes on the cDNA array by use of three separate RNA isolations

Downregulation (fold)	Gene description	Replicon	Gene family
−2.0	BBS33 conserved hypothetical protein	cp32-3	57
−2.0	BBO01 hypothetical protein	cp32-7	146
−2.0	BB0562 hypothetical protein	Chromosome	35
−2.0	BBG18 hypothetical protein	lp28-2	
−2.0	BB0335 ABC transporter, ATP-binding protein (OppF)	Chromosome	4
−2.0	BBK22 conserved hypothetical protein	lp36	50
−2.0	BB116 hypothetical protein	lp28-4	60
−2.0	BBR31 conserved hypothetical protein	cp32-4	57
−2.0	BBB10 conserved hypothetical protein	cp26	62
−2.1	BBG06 conserved hypothetical protein	lp28-2	57
−2.1	BBQ51 hypothetical protein, authentic frameshift	lp56	146
−2.1	BB0367 hypothetical protein	Chromosome	
−2.1	BBF10 hypothetical protein	lp28-1	70
−2.1	BBR34 conserved hypothetical protein	cp32-4	49
−2.1	BB119 conserved hypothetical protein	lp28-4	57
−2.1	BBS01 hypothetical protein	cp32-3	146
−2.1	BBH29 conserved hypothetical protein	lp28-3	49
−2.1	BBH14 hypothetical protein	lp28-3	
−2.1	BB107 hypothetical protein	lp28-4	
−2.2	BBH12 hypothetical protein	lp28-3	
−2.2	BBJ08 hypothetical protein	lp38	12
−2.2	BBF24 plasmid partition protein, putative	lp28-1	32
−2.2	BB0242 hypothetical protein	Chromosome	
−2.2	BB0365 lipoprotein LA7	Chromosome	
−2.2	BBD18 hypothetical protein	lp17	
−2.2	BBK23 hypothetical protein	lp36	62
−2.2	BB0261 hypothetical protein	Chromosome	
−2.3	BBO30 conserved hypothetical protein	cp32-7	57
−2.3	BB0396 ribosomal protein L33 (RpmG)	Chromosome	
−2.3	BB0256 ribosomal protein S21 (RpsU)	Chromosome	
−2.3	BBK01 hypothetical protein	lp36	12
−2.3	BBM33 conserved hypothetical protein	cp32-6	49
−2.3	BBL01 hypothetical protein	cp32-8	146
−2.3	BB0034 hypothetical protein	Chromosome	48
−2.3	BB0631 hypothetical protein	Chromosome	
−2.3	BBP30 conserved hypothetical protein	cp32-1	57
−2.4	BB0324 hypothetical protein	Chromosome	
−2.4	BB0083 hypothetical protein	Chromosome	
−2.4	BB0425 hypothetical protein	Chromosome	
−2.4	BBH37 hypothetical protein	lp28-3	12
−2.4	BB0428 hypothetical protein	Chromosome	
−2.5	BB0403 hypothetical protein	Chromosome	
−2.5	BBA42 conserved hypothetical protein	lp54	150
−2.6	BBD05.1 conserved hypothetical protein, pseudogene	lp17	57
−2.6	BB0356 hypothetical protein	Chromosome	
−2.6	BBJ19 conserved hypothetical protein	lp38	62
−2.6	BB0785 stage V sporulation protein G	Chromosome	
−2.6	BBH09.1 conserved hypothetical protein, pseudogene	lp28-3	95
−2.7	BBA52 outer membrane protein	lp54	
−2.8	BBG04 hypothetical protein	lp28-2	
−2.8	BBH18.1 conserved hypothetical protein, pseudogene	lp28-3	65
−2.8	BBD14 conserved hypothetical protein	lp17	62
−2.9	BBA23 conserved hypothetical protein	lp54	144
−2.9	BBA74 outer membrane porin (Oms28)	lp54	171
−2.9	BBE30 hypothetical protein	lp25	49
−2.9	BBH26 hypothetical protein	lp28-3	62
−3.0	BB0049 hypothetical protein	Chromosome	
−3.2	BBA60 surface lipoprotein P27	lp54	
−3.3	BBF32 recombination cassettes Vls2-Vls16, authentic frameshift	lp28-1	170
−3.3	BBG15 hypothetical protein	lp28-2	
−3.6	BBA59 lipoprotein	lp54	
−3.7	BBJ41 antigen, P35, putative	lp38	54
−3.8	BB136 antigen, P35, putative	lp28-4	54
−3.8	BBA62 lipoprotein	lp54	
−3.8	BBH16 hypothetical protein	lp28-3	
−3.8	BBI39 hypothetical protein	lp28-4	54
−4.0	BBH20 outer membrane porin, authentic frameshift (Oms28)	lp28-3	171
−4.0	BBI13 conserved hypothetical protein	lp28-4	
−4.0	BBA63 hypothetical protein	lp54	
−4.1	BBA03 outer membrane protein	lp54	
−4.1	BBD13 hypothetical protein	lp17	
−4.3	BB0519 grpE protein (GrpE)	Chromosome	
−4.5	BB103 hypothetical protein	lp28-4	
−4.5	BB0229 ribosomal protein L31 (RpmE)	Chromosome	
−4.6	BB0395 preprotein translocase subunit (SecE)	Chromosome	
−5.9	BB102 conserved hypothetical protein	lp28-4	84
−6.7	BBJ09 outer surface protein D (OspD)	lp38	
−7.2	BB0187 hypothetical protein	Chromosome	
−8.1	BB0273 flagellar biosynthesis protein (FliR)	Chromosome	

TABLE 3. List of the ten most highly expressed genes at 35B according to transcript abundance array data[a]

Culture 35B results		Gene description	Culture 35 results	
Rank	Expression level		Rank	Expression level
1	92.26	BBB19 outer surface protein C	106	2.74
2	43.30	BB0355 transcription factor, putative	3	42.69
3	36.83	BB0232 HbbU protein	19	14.09
4	32.94	BB0162 hypothetical protein	1	57.42
5	30.26	BB0294 flagellar basal-body rod protein (FlgB)	4	28.81
6	23.55	BB0426 hypothetical protein	12	20.78
7	20.59	BB0477 ribosomal protein S10 (RpsJ)	7	25.80
8	18.50	BB0231 conserved hypothetical protein	35	7.61
9	18.46	BB0440 ribosomal protein L34 (RpmH)	5	26.40
10	18.33	BB0425 hypothetical protein	2	44.20

[a] For comparison the expression at 35 is also provided along with the gene's rank.

leads to loss of infectivity in mice (31, 50). One suggested reason why lp28-1 may be essential to infectivity is that it encodes the *vls*E locus, which undergoes recombination during mammalian infection and may aid in immune evasion (69, 70).

All but one of the downregulated lp28 genes are undescribed hypothetical proteins. The one exception is BBI16, designated VraA antigen, which encodes a lipoprotein with 21 consecutive 9-amino-acid repeat sequences near its amino terminus (30). Database searches did not provide any hints for possible functions for any of the downregulated lp28 genes. Along these lines, four genes from lp17 were also downregulated and none were upregulated, again suggesting a role for this plasmid in flat ticks similar to that of lp28 genes.

Differential expression of genes on lp54 plasmid. The lp54 plasmid, with nine upregulated and nine downregulated genes, had the highest number of differentially expressed genes of all the plasmids (Fig. 4). These results underscore the importance of this plasmid in the life cycle of *B. burgdorferi*. In fact, six of the eight genes with the largest fold increase on the cDNA array were encoded on lp54. Several of these highly upregulated genes belong to paralogous gene family 54. Family 54 contains 14 members, 8 encoded at one end of the lp54 plasmid. Five genes in this family, BBA64, BBA65, BBA66, BBA71, and BBA73, were upregulated more than 10-fold on our array (Fig. 5). All but BBA71 encode putative lipoproteins. We tested for the expression of BBA64 and BBA66 in ticks, and both were expressed in feeding ticks only (Table 5). Since the genes of this family represented some of the most active genes on our array, it is likely that they are functional in the passage of *Borrelia* from tick to vertebrate.

Five other genes in this family—BBA68, BBA69, BBI36, BBI39, and BBJ41—were downregulated (Fig. 5). BBI36, BBI39, and BBJ41 were all downregulated >3.5-fold, while BBA68 and BBA69 were both downregulated 1.8-fold. Since genes in family 54 undergo both up- and downregulation, our data agrees with the hypothesis that despite some sequence homology, the genes in this family are under the control of different regulatory mechanisms (39). Analysis of upstream and downstream regions of the downregulated family 54 genes revealed the presence of a 24-bp inverted repeat sequence,

TABLE 4. Comparison of cDNA array and quantitative RT-PCR results for selected genes[a]

Gene	Forward and reverse primer (5′–3′)	Several induction (severalfold)		
		cDNA array	16S RNA	*flaB*
BBA72	GCATAAGGAGAGTGTTTTGAC and AACTTCTTGGTAGGTGCTTC	18.2	15.5	18.6
BBB19	GAGGTTGAAGCGTTGCTGTC and TCCATTGTGATTATTTTCGGTATC	15.11	51	61.2
BBA24	AGATAAAAAAACGGGTAGTGGG and GCTTCCTCTTCTATTGCTATTACG	12.66	13.7	16.44
BB0175	CGTATGAGGATTCTGATGATGC and CCTTTTTGTTTACCTTGTCTCCC	13.7	1.79	2.15
BBJ46	TCTTTACTATTGGGGCTTTGTG and TTGTAGACTGTTGTTTATGGATTTG	6.62	6.33	7.59
BBJ25	CCAAAATGGGTCAAGACAGTC and GCGGACAATACTAAAAATAAATCAG	5.25	34.97	41.96
BB0565	ATAATAGGGGCAAAATCGTTCC and CCAATCTTAGGAGCATCATCAATAC	5.47	1.79	2.15
BBC05	GAAAACCCTAAGGATGAACTTGC and TAGAACTTGTTGCTCCCCCAC	4.86	16.53	19.84
BBM35	CTGGAGTTGAGTTTGATTGG and GGCTTTTCTCTTTTTTTATTTTCG	1.58	1.11	1.33
BB0647	AACTTGGAGAAAACTGGGAAAC and TGGGACATTATTGTTATTATGGG	1.42	−1.26	1
BB0147	GCTTCTGATGATGCTGCTGG and ATGTGCCGTTACCTGATTGAACTGCC	1.29	1.2	1
BB0377	CATACAAAACTCAACCCTGGC and CGGTCCAAACTTCATTATTTCTAAG	1.17	1.46	1.75
BB0690	TTGCCCTCAATGGAAAGC and ATCGTGACAAAAACAATCGC	−1.17	−2.26	−1.88
BBA15	AAGAGCAGACGGAACCAGACTTG and AACCACCAATGTTGTTTTTTCAGC	−1.29	−1.53	−1.27
BBA74	GCTGTTTCTGTTGCTGGTGAAG and AACTTTTTGAGCCTCTTGAACTG	−1.44	−2.43	−2.02
BBD18	GGGGGCATAAAAGGAAC and CACCGGTTTGCATATTAAC	−1.28	−12.68	−10.56
BBH37	CGTAGAATCTGCGGTGTCTTTAG and CCTCAGTAGAAGGGAAAAAATCCTC	−1.65	−6.43	−5.35
BBI39	TGAAAAAGAAGCCGAAAAGTTG and GTAAGCGTCAATGGTTGCG	−2.52	−6.74	−5.61
BBA62	TATTATTTGTTGCTTGCGAAACTAC and GCTTCATTGATTTGGTATTTTTGTC	−3.31	−10	−8.33
BBJ09	GGCAAATAAAGTTGTAGAAGCG and TTTCAGCAGAATCAGAATAGTCAG	−3.55	−18.58	−15.48

[a] Forward and reverse primers for each of the selected genes are provided, each with its respective fold changes. The quantitative RT-PCR assay results were normalized to 16S rRNA and flaB, utilized as internal controls.

TABLE 5. RT-PCR results of expression assays of select *B. burgdorferi* genes from flat and feeding ticks

Gene	RT-PCR[a]		cDNA array induction (severalfold)
	Flat tricks	Feeding ticks	
BB0647	−	+	2.08
BB0690	+	−	−1.57
BBA15	+	+	−1.73
BBA64	−	+	16.68
BBA66	−	+	31.22
BBA72	−	+	45.06
BBA74	+	+	−2.93
BBA62	+	−	−3.80
BBD18	+	−	−2.22
BBB19	−	+	33.60
BBJ25	−	+	12.33
BBH37	+	+	−2.41

[a] A plus sign indicates an amplified transcripts, while a minus sign indicates lack of amplified transcript. Each gene's severalfold up- or downregulation on the cDNA array is shown on the right for comparison. Note that a downregulated gene on the array may be expressed in both flat and feeding ticks. It's likely that such a gene is preferentially expressed in the flat tricks, but the transcript can still be detected in feeding ticks, as spirochete populations within feeding ticks are heterogeneous.

AAAGAGAAGAATTAACTTCTCTTT, which may act as a transcriptional terminator for this group of genes. This sequence is located exactly 40 bases downstream from the end of BBI36, BBI39, and BBJ41 as well as BBI38, a paralog of BBI36 that was not expressed on our array. The sequence is also located 41 bp downstream of BBA68. The repeat sequence is found only in only one other region of the genome, approximately 1 kb upstream of BBA69. It has been proposed that these five genes, along with BBI38, encode lipoproteins despite a variation in the consensus signal peptidase II sequence (13). These genes all contain an isoleucine at −1 and a threonine at −3 relative to the cysteine residue, while the rest of the family 54 genes have different residues at these positions or lack the cysteine altogether (www.tigr.org). Our findings show that the downregulated genes of family 54 all share several common characteristics which may lead to their similar regulatory patterns.

BBA74, which encodes a porin designated Oms28, was downregulated more than threefold (59). This ORF was also downregulated in both vertebrate host-adapted arrays but was shown to be upregulated in both temperature shift experiments (8, 39, 54). We found that BBA74 was expressed in flat ticks and undergoes downregulation during feeding. The downregulation of this gene in our array and in the experiments of Brooks et al. (8) suggests that some mammalian factors present in the incoming blood meal may drive the repression of BBA74. Without these factors, temperature alone seems to increase its transcription. This is the first direct evidence of a protein described as a porin in *B. burgdorferi* that is preferentially expressed in flat ticks and that may be instrumental in acquisition of small molecules while the spirochetes are in the nutrient-poor environment of the tick midgut.

Three lp54-encoded lipoproteins, BBA59, BBA60, and BBA62, were downregulated (at least threefold), implicating these genes as being preferentially expressed in the flat tick and downregulated during feeding. BBA62 encodes lp6.6, which has previously been shown to be downregulated during mammalian infection (32, 66). Finally, *ospA* and *ospB* were downregulated in our experiments but the decreases in expression were −1.73 and −1.75, respectively.

Upregulation of lp38-encoded ABC transporter system. A total of 11 genes on lp38 were differentially expressed in our array (Fig. 4). Of particular interest was a cluster of three upregulated genes on lp38, BBJ25, BBJ27, and BBJ28 (12.3-, 4.8-, and 5.5-fold upregulation, respectively) (Table 1). BBJ27 is predicted to be in an operon with BBJ26. BBJ26, which was also upregulated in our array but did not pass our criteria for filtering genes, has been identified as an ATP-binding ABC transporter, the substrate of which has not yet been identified. ABC (ATP binding cassette) transporters are active transport systems. The transporters have a common global organization with three types of components: two integral membrane permease proteins, two peripheral membrane proteins that bind and hydrolyze ATP, and a periplasmic substrate binding protein. The genes for all three components frequently form an operon (64). BBJ27 contains four predicted transmembrane

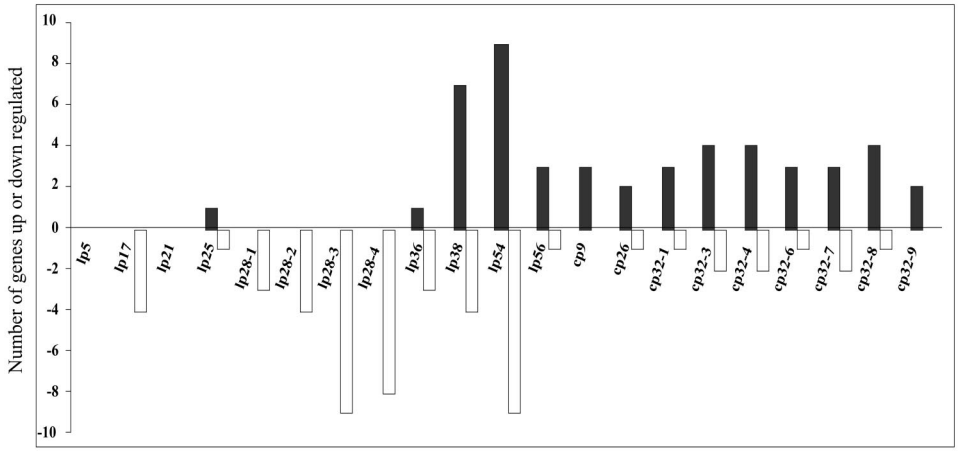

FIG. 4. Differential (more than twofold) expression from all 21 circular (cp) and linear (lp) plasmids of *B. burgdorferi* on the cDNA array. The *y* axis represents the number of genes up- or downregulated in spirochetes cultured with blood compared to the results for spirochetes cultured without blood. Black bars indicate the numbers of genes that were upregulated; white bars indicate the numbers of genes that were downregulated.

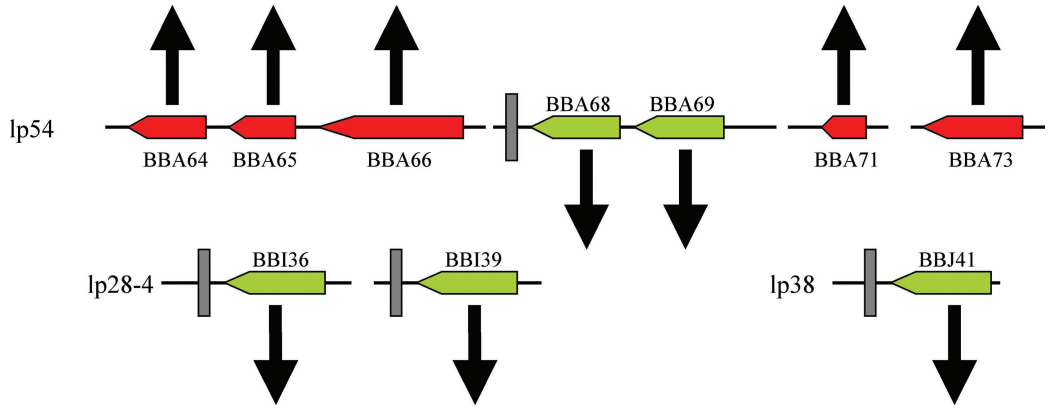

FIG. 5. Family 54 genes differentially expressed on the cDNA array. All genes except BBA71 encode putative lipoproteins. Upregulated genes are shown in red; downregulated genes are shown in green. The gray rectangle represents a 24-bp inverted repeat located 40 bp downstream from the downregulated genes.

regions and shares homology with many other ABC transporter permeases. Both BBJ25 and BBJ28 have predicted signal peptidase cleavage sites, indicating that they may be secreted to the periplasm, where one or both may function in substrate binding. Besides this lp38-encoded system, all but two of the *Borrelia* ABC transporter systems are encoded on the chromosome. Notable exceptions are two known plasmid-encoded oligopeptide binding proteins. The lp38 ABC transporter system, however, is the only known *B. burgdorferi* system in which the genes encoding the ATP binding protein and the permease are next to each other on a plasmid. Only one other gene identified as having potential ABC transporter functions was upregulated in our array. BB0017, upregulated 2.2-fold, encodes a putative permease, but unlike other ABC transporter operons, no ATP binding protein is encoded nearby.

As shown on the basis of our DNA array data, this transporter system is not expressed or is expressed at a very low level in flat ticks and is upregulated during tick feeding. Moreover, RT-PCRs showed that BBJ25 was expressed only in feeding ticks (Table 5). Once upregulated during feeding, the system likely remains expressed throughout mammalian infection, as Brooks et al. found BBJ26 and BBJ27 to be upregulated in dialysis chambers (8). Therefore, this may be the only known transporter system utilized by *Borrelia* specifically during the transmission and infection process.

Differential expression of cp32 paralogous gene families. Cp32 plasmids are members of a group of circular plasmids found in *B. burgdorferi*. Strain B31 MI may contain up to seven of these plasmids designated cp32-1, cp32-3, cp32-4, cp32-6, cp32-7, cp32-8, and cp32-9 (13). The cp32 plasmids are very similar to one another, and paralogs of cp32 genes are also found on cp9 plasmids and lp54 and lp56, which contain insertions of cp32. Cp32 plasmids have been implicated as prophages (13, 16, 18, 19). In our array experiments a total of 34 genes were upregulated on cp32 plasmids, all but 2 belonging to four paralogous gene families (Fig. 6). Seven paralogs of family 113 were upregulated over twofold. Family 113 encodes Mlps, which are lipoproteins that have previously been shown to undergo upregulation due to increase in temperature and/or decrease in pH and are expressed in feeding ticks but not flat ticks (25, 27, 67).

Nine paralogs of family 165 and eight paralogs of family 161 were upregulated. Casjens et al. indicated that an upstream region of genes encoding members of family 161 and 165 contained identical inverted repeat sequences of approximately 180 bp (13). It was hypothesized that these genes may undergo common regulation, and our results are in agreement. Genes in family 161 encode a protein similar to ERF single-stranded binding proteins encoded by bacteriophages. Furthermore, family 165 members have been implicated as putative

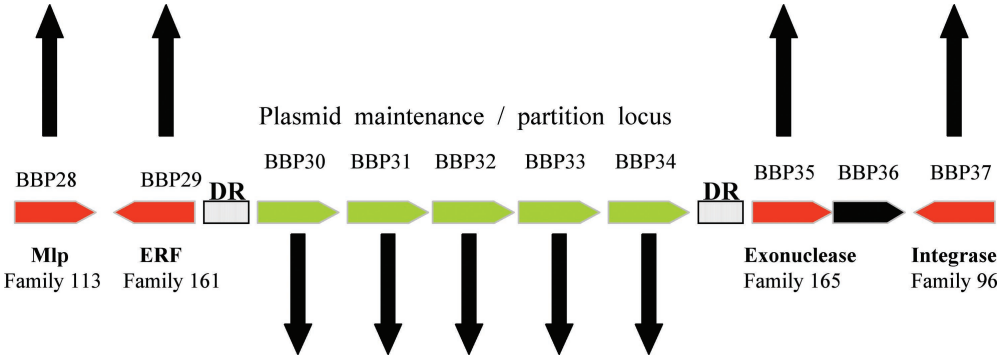

FIG. 6. Schematic of a cp32 region which underwent significant differential expression on the cDNA array. The region depicted is from cp32-1; a similar expression pattern was observed for several cp32 paralogs. Arrows indicate whether a gene underwent upregulation or downregulation. DR stands for direct repeat regions located in front of family 161 and 165 ORFs.

lambda exonuclease homologs, which are enzymes involved in recombination and repair in bacteriophages (4). Finally, two homologs of family 96 which encode a putative phage integrase were also upregulated. These results seem to indicate that an increase in temperature combined with some mammalian factors may serve as a signal to initiate a stress response leading to the upregulation of phage recombination proteins in *B. burgdorferi*. Furthermore, BB0548, a chromosome-encoded DNA polymerase I, was upregulated 2.71-fold. DNA polymerase I is involved in repair and recombination, and no other polymerases were differentially expressed in our experiments. What role the phage recombination machinery may serve during the infection process remains to be elucidated. The upregulation of these genes may be a transient part of a stress response of the phage, occurring early following exposure to blood and temperature. Alternatively, it may have more pronounced functions in the overall spirochete life cycle, perhaps generating genetic variability on cp32, other plasmids, and perhaps the chromosome itself.

Of the downregulated cp32 genes and their paralogs, nearly all were grouped to five families. Four of these are families 32, 49, 50, and 57. These families encode genes in a five-member locus which has been implicated on the basis of homology as functioning in plasmid maintenance and/or partitioning (Fig. 6). Members of family 32 specifically are homologous to the *parA* gene of *Escherichia coli* bacteriophage P1. ParA is an ATPase belonging to a large family of genes implicated in bacterial plasmid partitioning proteins (13, 61). On other *B. burgdorferi* plasmids, family 62 genes replace family 57 genes within the locus; similarly, four homologs of family 62 were downregulated more than twofold. Therefore, the downregulation of the putative maintenance locus was occurring on all the plasmids, not just cp32.

Four paralogs of family 146 were downregulated. This gene has been implicated as a bacteriophage portal protein, which allows the phage to be packaged prior to release from the cell. The downregulation of this protein would seem to indicate that the environment acts against phage release. In support of this idea, and as also noted by Ojaimi et al., very few genes found in the putative "late phage operon" (between BBP01 and BBP26 on cp32-1) were differentially expressed or even expressed at all. Ojaimi et al. (39) also noted upregulation of *blyA* and *blyB*, the putative phage holins, which we did not observe in our experiments. In fact, both failed to meet our criteria for expressed genes on our array, indicating they were either expressed at a very low level or perhaps not expressed at all.

Chromosomal genes. BB0175 was the chromosomal gene most highly upregulated under our array conditions as well as under conditions of temperature induction (39). This gene encodes a conserved protein with an undescribed function and contains a region of homology to the von Willebrand factor A (vWA) domain. This domain is associated in both prokaryotes and eukaryotes with cell-to-cell interactions, often via integrin receptors. BB0175, however, has an incomplete vWA domain, lacks two of the five conserved residues of the ligand binding Midas motif, and does not appear to be a secreted protein (www.tigr.org). One of two *B. burgdorferi lon* ATP proteases, BB0253, was upregulated over fivefold. In bacteria, the Lon protease functions in degradation of abnormal proteins and has also been shown to be responsible for the regulation of various pathways in many bacteria by its degradation of regulatory proteins. Recent reports focusing on Lon in *Salmonella* spp. showed that it can function as a regulator of genes responsible for cell invasion (62, 63). In *Pseudomonas syringae*, it functions as a negative regulator of type III protein secretion systems (7). Since the *B. burgdorferi lon* upregulation occurs at a time of vast changes in the expression of many genes, it is possible that the protease may serve similar regulatory functions in *Borrelia* and to degrade proteins such as OspA/B that disappear from the organisms in the midguts of the feeding ticks.

Two known regulatory proteins were upregulated in our experiment. BB0771 encodes an *rpoS* sigma factor. RpoS is upregulated by temperature and controls the expression of both OspC and DbpA and likely other *B. burgdorferi* antigens through sigma factor *rpoN* (28). On our array *rpoS* was upregulated 9.5-fold, while *ospC* and *dbpA* were upregulated 33.6- and 19.7-fold, respectively. Another upregulated regulator was BB0647, the *B. burgdorferi fur* homolog. Members of this family of proteins function as transcriptional regulators in response to metals. *fur* was upregulated 2.1-fold in our array and was expressed in feeding ticks (Table 5). It is possible that upregulation of *fur* occurred due to the influx of metals in the incoming blood meal. A recent report implicated *fur* in acting as a transcriptional activator of NapA (BB0690), a putative DNA binding protein functioning in oxidative stress response (6). Our data does not support this hypothesis, as we found that *napA* was downregulated on the array and was expressed in flat ticks. Our results would argue that Fur acts as a repressor of NapA.

HbbU, a *B. burgdorferi* HU/IHF protein family homolog, was upregulated 2.6-fold in our array. HbbU protein has been shown to bind DNA, induce a dramatic bend, and restrain negative supercoils upon binding (29). A recent study described how temperature and supercoiling affects the transcription of *ospA*/*B* as well as *ospC* (1). *Borrelia* DNA was found to be more negatively supercoiled at 23°C than in cultures grown at 35°C. Following relaxation of negative supercoils, *ospA*/*B* expression and promoter activity decreased while *ospC* levels increased. This implies that during tick feeding, Hbb may act in transcriptional regulation by relaxing negative supercoils, resulting in the well-described changes in *ospA*/*B* and *ospC* expression during feeding.

BB0782 encoding a nicotinate-nucleotide adenylyltransferase was upregulated 3.3-fold. This enzyme catalyzes the adenylation of nicotinate mononucleotide (NaMN) to nicotinic acid adenine dinucleotide (NaAD), which is then directly processed to NAD by NAD synthetase (Swiss-Prot). BB0728 was upregulated 2.1-fold, and it encodes an NADH oxidase. Its upregulation may be due to the increase in oxygen. During tick feeding, the spirochetes in the tick midgut are exposed to the oxygen in the incoming blood meal. Borrelia may upregulate NADH oxidase to allow it to deal with this increase in oxygen levels which may be toxic to the cells. A similar function has been shown in the spirochete *Brachyspira*, in which NADH oxidase is utilized to protect the bacteria from oxygen toxicity and therefore contributes to its pathogenicity (60).

Of the downregulated ORFs, several encode previously characterized proteins. BB0034, downregulated 2.3-fold, encodes a protein designated P13. This protein is a 13-kDa porin

which is posttranslationally processed at both ends and modified by an unknown mechanism (35, 41). BB0365, encoding LA7, a lipoprotein, was downregulated 2.2-fold. This ORF was upregulated in both previous temperature shift array studies. It has been shown, however, that in similarity to results seen with OspA/B, few patients generate antibodies to this protein in early Lyme disease, lending further evidence that this gene is likely downregulated in feeding ticks (33, 53). Three genes coding for ribosomal proteins RpmG, RpsU, and RpmE were downregulated. In *Anabaena* and *Synechocystis* cyanobacteria, as well as in the soil bacterium *Sinorhizobium meliloti*, *rpsU* is preferentially expressed at colder temperatures; this gene has been suggested to be associated with adaptation to changes in the environment (36, 55, 56).

Chemotaxis-motility genes. Several ORFs functioning in chemotaxis and motility were upregulated in our experiments. In a typical bacterial chemotaxis pathway, MCPs (methyl-accepting chemotaxis proteins) are the transmembrane chemoreceptors that regulate the autokinase activity of the histidine kinase CheA. The coupling protein CheW stabilizes the complex formed between CheA and the receptor. CheY, a response regulator, is activated by phosphorylation by CheA and then regulates flagellar activity. Adaptation enzymes CheB and CheR covalently modify specific adaptation sites on the receptor cytoplasmic domains (22, 44).

B. burgdorferi contains multiple copies of chemotaxis sensory transduction genes, encoded primarily in two operons. It has been suggested that the spirochete may preferentially express its chemotaxis genes in different habitats (14). We observed that the addition of blood had a major effect on movement, with the spirochetes exhibiting a great increase in motility. Not surprisingly, several chemotaxis genes were upregulated on our array. Two out of five MCPs encoded by *B. burgdorferi* were upregulated 6- and 5.8-fold, respectively; the two genes, designated *mcp-4* and *mcp-5*, are encoded on a probable operon. *cheA-1* was upregulated 3.7-fold, while *cheA-2* was upregulated only 1.6-fold. *cheW-2*, one of the three *B. burgdorferi* copies of *cheW*, was upregulated 8.3-fold. *cheX*, another gene associated with chemotaxis, was upregulated 3.7-fold. No induction of *cheY* or *cheB* was observed. Our results are in full agreement with the data of Revel et al. (54), as the same chemotaxis genes were upregulated in both experiments. Since no upregulation of chemotaxis genes was reported by Ojaimi et al. (39), it's likely that lower pH or mammalian blood factors may drive the upregulation of these genes in *B. burgdorferi*.

Summary. Spirochetes in the midgut of flat (unfed) ticks are metabolically active but very likely at a very low rate. Thus, genes that are expressed during this phase of the spirochete life cycle would be expected to encode proteins that are associated with the maintenance of the most elemental functions for survival in a nutrient-depleted, possibly anoxic environment with a potential wide range of temperatures. A number of genes from all four copies of lp28 are involved in maintenance of spirochetes in the midgut of flat ticks, as these genes are unanimously downregulated with the influx of blood. The same is true for some genes in lp54, including the now well-known downregulation of *ospA* and *ospB*. Without knowledge of the function of most of these plasmid genes, it is possible to speculate that genes expressed in the flat midgut may be engaged in specialized transport of simple molecules and in survival at different temperatures. In fact, in flat ticks, the lumen of the midgut is poor in residual nutrients, as nutrient storage is the function of the cells of the midgut epithelium. These cells store macromolecules (for example, undigested hemoglobin and albumin and other macromolecules) in endosomes, essentially leaving the lumen devoid of nutrients.

Although the temperature of the midgut and that of the tick itself rises upon blood feeding, this is not the only time when ticks undergo temperature shifts. Questing ticks have a constantly changing temperature that can fluctuate by several degrees within a 24-h period, at various locations within the tick microclimate, and at different seasons in their 2-year life cycle. Therefore, temperature-regulated genes would have to express differentially often and not just at the time of blood feeding. Under poor nutrient conditions, this would be unnecessarily stressful for the organisms unless accompanied, as in the blood meal, by an influx of nutrients. Some of these gene products need to change not only as a result of blood influx and its increase in temperature but also to protect the spirochete from the temperature fluctuations of the flat tick. Genes that are downregulated as the organisms come in contact with blood must be specialized enough to maintain the minimum requirements for viability.

The transformation of spirochetes as blood begins to fill the midgut and its diverticula is fueled by changes in gene expression, and these induce the marked changes in the cell cycle as the spirochetes begin to divide, the increase in motility, and in the visible changes in morphology. These changes coincide with the upregulation of the chemotaxis and sensing regulons, of the lp38-encoded ABC transporter, of proteases capable of remodeling the outer surface of the spirochetes, and of the recombination genes of cp32 as a transient or initial part of the stress response of the phage. These are all functions or occurrences that cause or facilitate the changes that spirochetes undergo following a blood meal in the tick. Most importantly, the highly active genes of unknown function that we have detected in this study may hold a lot of the answers to vector-host interactions.

In summary, we have demonstrated that the addition of blood has a dramatic effect on gene expression of *B. burgdorferi*. By verifying our results by tick RT-PCR, we also showed that our in vitro study was a close mimic of a feeding tick environment. When the results of our study are analyzed in combination with earlier array studies, we can obtain the picture of which genes the pathogen preferentially expresses in either the vertebrate host or the vector and may further our understanding of *B. burgdorferi* pathogenesis.

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