JOURNAL OF BACTERIOLOGY, June 2002, p. 3122–3125 Vol. 184, No. 11
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.11.3122–3125.2002

NOTES

Examination of the *Borrelia burgdorferi* Transcriptome in *Ixodes scapularis* during Feeding

Sukanya Narasimhan,[1] Felix Santiago,[1] Raymond A. Koski,[2] Brandon Brei,[3] John F. Anderson,[4] Durland Fish,[3] and Erol Fikrig[1]*

Section of Rheumatology, Department of Internal Medicine,[1] and Department of Epidemiology and Public Health,[3] Yale University School of Medicine, and L[2] Diagnostics,[2] New Haven, Connecticut 06520, and Department of Entomology, Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504[4]

Received 2 November 2001/Accepted 14 February 2002

***Borrelia burgdorferi* gene expression within the guts of engorging *Ixodes scapularis* ticks was examined by use of differential immunoscreening and differential expression with a customized amplified library. Fourteen chromosomal genes involved in energy metabolism, substrate transport, and signal transduction and 10 (4 chromosomal and 6 plasmid) genes encoding putative lipoproteins and periplasmic proteins were preferentially expressed in engorging ticks. These data demonstrate a new approach to the global analysis of *B. burgdorferi* genes that are preferentially expressed within the vector during feeding.**

Borrelia burgdorferi, the agent of Lyme disease, is transmitted when *Ixodes* ticks feed on mammals (5, 17). While the tick is engorging, the antigenic composition of *B. burgdorferi* changes dramatically (1, 8, 9, 20, 21). Analysis of differentially expressed genes will provide insights into the molecular changes that occur in spirochetes during tick feeding. Recent studies have focused on the expression profiles of only a few genes, such as *ospA* and *ospC* (12, 15, 20), *erpT* (10), *bbk32* and *bbk50* (11), and more recently *rev*, *mlpA*, *erpa*/*i*/*n*, and *erpb*/*j*/*o* (14), that are expressed in the tick vector. In order to systematically identify *B. burgdorferi* antigens that are differentially expressed during tick feeding, we have used an antibody-based approach, differential immunoscreening (24), and differential expression with a customized amplification library (DECAL), a new technique to examine the transcriptomes of prokaryotic genomes. DECAL was first developed and utilized for the global analysis of gene expression in *Mycobacterium tuberculosis* grown in vitro (2). The rationale for exploiting DECAL is based on the following factors: (i) the analysis can be performed with as little as 10 ng of total bacterial RNA and can detect differences in levels of gene expression that are as small as fourfold and (ii) the technique is ideally suited for investigations where contaminating host material is present.

Differential immunoscreening of *B. burgdorferi*. *Ixodes scapularis* nymphs infected with *B. burgdorferi* strain N40 and either fed to repletion on C3H/HeN mice (9) or left unfed were dissected. The isolated guts were suspended in phosphate-buffered saline (10 μl/tick gut) and homogenized. The number of spirochetes in each preparation was measured by direct immunofluorescence (9). Equivalent doses of *B. burgdorferi* (10^6 organisms) in complete Freund's adjuvant were used to immunize C3H/HeN mice after heat inactivating the spirochetes (1 h at 60°C). Control mice were immunized with gut extracts from uninfected *I. scapularis* nymphs. Ten days after the final booster injection, antisera against spirochetes from unfed and fed ticks were collected. A λ ZAP II-*B. burgdorferi* N40 expression library (10,000 plaques) was screened with antisera from unfed and fed ticks. Hybridizing plaques were visualized with a picoBlue immunoscreening kit (Stratagene, La Jolla, Calif.). About 100 clones hybridized to sera from both unfed and fed ticks. Seven positive clones that were preferentially recognized by the antisera from fed ticks were isolated, and the insert DNAs were sequenced. Homology searches performed against sequences in the *B. burgdorferi* genome database (www.tigr.org) revealed that four clones contained *bba14* and three contained *bb0129*. To obtain a more global view of the differential expression profile of *B. burgdorferi* in engorging ticks, we exploited DECAL (2).

Construction of a Bb-CAL. A *B. burgdorferi* customized amplified library (Bb-CAL) was constructed according to the protocol described by Alland et al. (2). Two thousand *B. burgdorferi*-ZAP II genomic clones were cored and suspended in phage dilution buffer in 96-well microtiter plates (18). *B. burgdorferi* rRNA genes were identified and removed from this library of 2,000 clones as described previously (2). The rest of the phage clones were pooled in groups of 50, the phagemids were excised, and the rescued phagemid DNA was digested with *Eco*RV and *Sma*I to release the inserts and electrophoresed on a 2% low-melting-point agarose gel. The gel region corresponding to 200 to 2,000 bp (a size range that can be efficiently amplified by PCR) was excised, and DNA was eluted from the gel, ligated with 2 pmol of Uniamp *Xho*I adapters, and PCR amplified with Uniamp primers (2). The amplified

* Corresponding author. Mailing address: 608 Laboratory of Clinical Investigation, Section of Rheumatology, Department of Internal Medicine, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520-8031. Phone: (203) 785-2453. Fax: (203) 785-7053. E-mail: erol.fikrig@yale.edu.

TABLE 1. Primary DECAL screening results

Tick group	Clone	Gene(s) present in the clones[a]	Predicted function(s) and/or gene product(s)
Fed	A-7-11[b]	*bb0237–bb0241*[c]	Central intermediary metabolism; hypothetical protein
	K-4-5	*bb0329–bb0335*[c]	Oligopeptide ABC transporters
	A-7-17	*bb0787–bb790*[c]	Translation, cellular process, unknown functions
	C-16-9	*bbg24–bbg27*[c]	Conserved hypothetical proteins
	P-15-2	*bb0420*	Response regulator/sensory transduction histidine kinase
	O-16-14[b]	*bb0435–bb0436*	DNA gyrase subunits A and B
	L-19-14	*bb0835–bb0838*	Intermediary metabolism, cellular process, unknown function
	C-10–16	*bba11–bba15*[c]	Conserved hypothetical proteins, OspA
	A-4–4	*bb0600–bb0603*[c]	Unknown function, cellular process; integrin-binding protein
	J-17-4	*bb0154*	Preprotein translocase
	A-11-9	*bb0374–bb0378*	Regulatory and unknown functions
Unfed	O-19-2	*bb0193–bb0181*[c]	Energy metabolism, regulatory function, replication, unknown function
	F-15-2	*bbl17 and bbo17*	Unknown function
	I-18-24	*bb0009–bb0013*	Energy metabolism
	I-21-24	*bb0146–bb0151*[c]	Energy metabolism, central intermediary metabolism; transport proteins
	E-11-18	*bb0420–bb0421*[c]	Sensory transduction histidine kinase, hydrolase
	F-3-2	*bb0434*[c]	Stage O sporulation protein J (chromosome segregation)
	L-9-22	*bbg10*	Hypothetical protein
	O-6-15	*bbg12–bbg14*[c]	Conserved hypothetical proteins
	E-8-14	*bb0635*	Conserved hypothetical protein

[a] Spirochete genes upregulated in both fed and unfed ticks.
[b] Identified five times. All other clones were identified more than once.
[c] Southern analysis was performed to identify the hybridization of the gene(s) to Bb-DECAL probes.

products represented the Bb-CAL encoding the proteome of *B. burgdorferi.*

Positive selection and amplification. The guts dissected from *B. burgdorferi*-infected unfed and fed (72 h) nymphal ticks (9) were placed in RNAWIZ RNA isolation reagent (Ambion, Austin, Tex.), and RNA was isolated according to the manufacturer's protocol. Contaminating DNA was removed by use of a DNA-free kit (Ambion). The total RNA (~1 μg) isolated from unfed and fed nymphs was reverse transcribed in the presence of biotin-labeled random hexamers and biotin dATP by use of the Superscript first-strand synthesis system for reverse transcription-PCR (Life Technologies, Inc., Gaithersburg, Md.) at 50°C for 1 h. Aliquots of the resultant cDNA pools were tested by PCR with *B. burgdorferi flaB* primers (25). This ensured the integrity of the cDNA and normalized the amounts of cDNA from unfed and fed ticks for use in the DECAL positive-selection step. Biotinylated cDNA was hybridized to the Bb-CAL, and the biotinylated *B. burgdorferi* cDNA–Bb-CAL hybrids were bound to streptavidin-coated magnetic beads (Dynal, Lake Success, N.Y.). The Bb-CAL DNA bound to the streptavidin-coated magnetic beads was eluted by boiling and PCR amplified by use of Uniamp primers and 20 μl of the eluted sample as described previously (2). The positively selected and amplified *B. burgdorferi* PCR probes prepared from unfed and fed *I. scapularis* ticks (Unfed Bb-DECAL and Fed Bb-DECAL, respectively) represent the B. *burgdorferi* transcriptome in the guts of unfed and fed nymphs, respectively.

Differential-expression analysis with a Bb-CAL. Unfed Bb-DECAL and Fed Bb-DECAL were randomly labeled with [α-^{32}P]dATP, and the amounts were normalized based on hybridization intensity to the *flaB* PCR product prior to the probing of duplicate *B. burgdorferi* genomic arrays. A *B. burgdorferi* N40 genomic library constructed in the plasmid vector pHYB/LEX-ZEO (Invitrogen, Carlsbad, Calif.) was used to print *Borrelia* genomic arrays at Incyte Genomics (St. Louis, Mo.). The arrays were probed with Unfed Bb-DECAL and Fed Bb-DECAL. Hybridization was scored visually, and intensity values ranging from 0 to 3, with 0 being the weakest and 3 being the strongest hybridization signal observed by autoradiography, were assigned. Differentially hybridizing clones with spot intensity scores of 2 and 3 on each array were selected for the initial analysis. Sixteen clones were preferentially expressed in unfed ticks, and 32 were preferentially expressed in fed ticks. The *B. burgdorferi* genes in these clones were identified by DNA sequencing and homology searches as described above (Table 1). The list of genes identified by DECAL appears skewed towards chromosome-borne (*bb0*) genes. *B. burgdorferi* replicates rapidly in the guts of ticks during feeding (9). It is therefore reasonable to observe the recruitment of functions involved in cellular processes such as (i) DNA metabolism, (ii) energy metabolism, and (iii) the uptake of essential substrates, the majority of which are indeed chromosome encoded (13). Virulence genes predominantly carried by the extrachromosomal elements of *B. burgdorferi* (16, 22) are perhaps more selectively expressed than genes involved in cellular processes. When we extended our efforts to the sequencing of differentially hybridizing signals in a low intensity range (intensity 1), we observed a preponderance of differentially expressed plasmid-borne genes (data not shown).

Southern analysis. The DECAL hybridization profile of each of the genes carried by the clones with more than one gene (Table 1) was investigated by Southern analysis. PCR products corresponding to the individual genes present in these clones were electrophoresed and transferred to nylon membranes. The membranes were probed with the [α-^{32}P]dATP-labeled Unfed Bb-DECAL or Fed Bb-DECAL probe and exposed to autoradiography. The Southern hybridizations identified individual genes contributing to DECAL

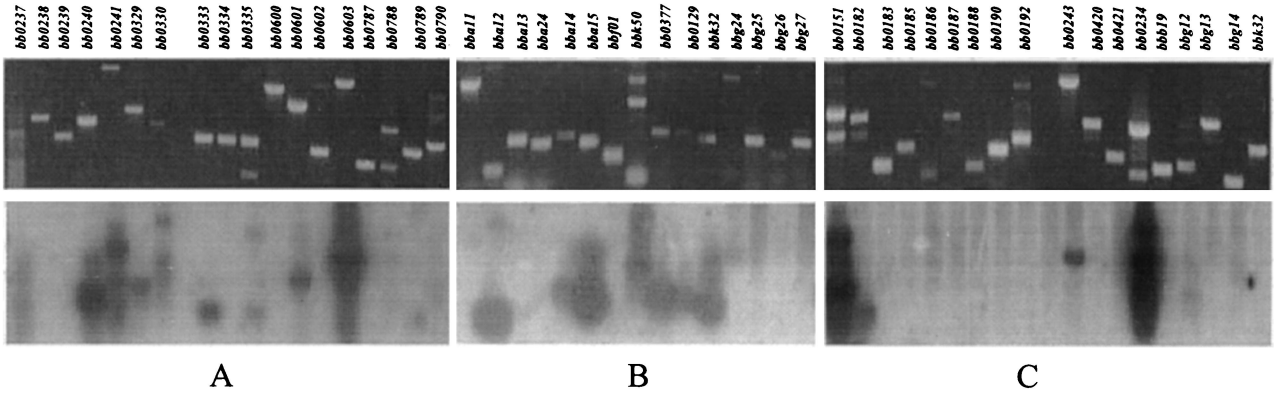

FIG. 1. Southern analysis of the individual genes carried by the differentially hybridizing *B. burgdorferi* clones. The genes in selected differentially hybridizing clones were PCR amplified by use of gene-specific primers. The PCR products were Southern blotted to nitrocellulose membranes. Shown are Southern blots hybridized to the radioactively labeled Fed Bb-DECAL probe (A and B) and to the radioactively labeled Unfed Bb-DECAL probe (C).

differential hybridization (Fig. 1). Of physiological significance is the hybridization of Fed Bb-DECAL to amplicons corresponding to *bb0240* (glycerol uptake facilitator) and *bb0241* (glycerol kinase). It is likely that *bb0240* and *bb0241* are upregulated to enable the uptake of glycerol as an energy source (23). The Southern analysis (Fig. 1A) also suggests that only a subset of the oligopeptide ABC transporter genes contained in the *opp* operon corresponding to *bb0329*, *bb0330*, *bb0333*, and *bb0335* are upregulated on spirochetes during the vector's feeding. The hybridization of amplicons corresponding to *bbk32* and *bbk50*, but not to *bba24* and *bbf01* (Fig. 1B), to Fed Bb-DECAL and the lack of hybridization of Unfed Bb-DECAL to *ospC* are consistent with earlier observations on the expression profiles of these genes (6, 11, 21). The hybridization of Fed Bb-DECAL to the PCR product corresponding to *bba14* and *bb0129* (Fig. 1B) is also consistent with the results obtained by differential immunoscreening. These data emphasize the utility and validity of DECAL.

Temporal analysis of expression by RT-PCR. The results obtained by DECAL and differential immunoscreening were confirmed by RT-PCR analysis. RNA isolated from the guts of unfed and fed *B. burgdorferi*-infected *I. scapularis* ticks was reverse transcribed. The resultant cDNA was used as a template to amplify PCR products corresponding to several *B. burgdorferi* genes identified by DECAL and differential immunoscreening. The expression profiles observed (Fig. 2) confirmed the results obtained by DECAL (Table 1) and differential immunoscreening. The presence of spirochetes in the guts of engorging ticks increased the expression of *bb0420* and *bb0377* (Fig. 2). The *bb0420* gene encodes a sensory transduction histidine kinase, and *bb0377* encodes a homolog of LuxS (Table 2). LuxS has been shown to be a key enzyme in the biosynthesis of a quorum-sensing signal molecule in several gram-negative bacteria (19). We speculate that BB0377 and BB0420 may be components of signaling circuits operating in *B. burgdorferi* that presumably mediate intracellular responses to environmental stimuli. Gene *bb0603* encodes an integrin receptor that facilitates binding to mammalian host tissue (4, 7). Its upregulation in spirochetes upon tick feeding (Fig. 2) perhaps signifies a preparation for entry into the mammalian host. The observation that *bb0151* (*nagA*, *N*-acetylglucosamine-6-phosphate deacetylase) is upregulated on spirochetes in unfed ticks (Fig. 2) supports the hypothesis that *N*-acetylglucosamine (a constituent of chitin in tick midguts) may serve the spirochete as a substrate for energy and cell wall biosynthesis (13,

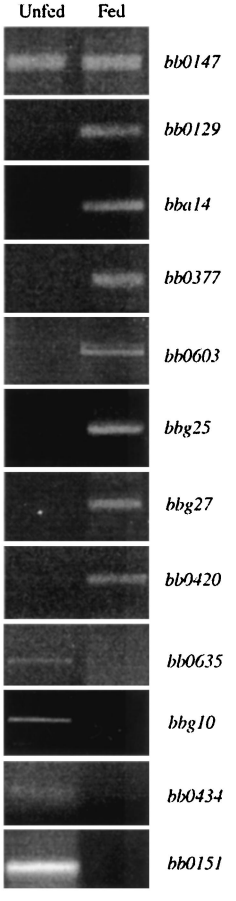

FIG. 2. Confirmation of DECAL and differential immunoscreening results by RT-PCR. cDNA prepared from *B. burgdorferi*-infected unfed and fed *I. scapularis* nymphs were used as templates in PCRs with gene-specific primers.

TABLE 2. Homologs of differentially expressed genes that were identified as hypothetical proteins in the *B. burgdorferi* genome database

Protein	NCBI best match[b]	NCBI E value[b]
BB0129[a]	Pseudouridine synthase	3.00E−58
BB0788[a]	GMP synthetase	1.00E−54
BB0377[a]	Autoinducer 2 synthase (LuxS)	1.00E−47
BBA14[a]	*B. burgdorferi* Mlp lipoprotein family (ORF D)	6.00E−14
BBA11[a]	*B. burgdorferi* ORF X upstream of *blyA* and *blyB*	1.00E−08
BBG25[a]	*B. burgdorferi* Mlp lipoprotein family (ORF D)	1.10E+00
BBG10	Putative tail protein (*Streptococcus pneumoniae* bacteriophage MM1)	2.00E−06
BB0635	Nicotinic acid phosphoribosyl transferase	1.00E−50

[a] Protein whose corresponding gene was preferentially expressed on spirochetes in fed nymphs. ORF, open reading frame.
[b] See www.ncbi.nlm.nih.gov. E value is a statistical parameter to estimate sequence similarity scores.

23). To glean information on the potential biochemical functions of some of the genes identified by DECAL that encode proteins with unknown functions, we compared their protein sequences with those in the National Center for Biotechnology Information (NCBI) nonredundant protein database by use of the BLAST-P program (3). The best matches obtained are listed in Table 2. In future experiments, analysis will be conducted on potential genes to follow up on the pathways and mechanisms implicated by the DECAL results.

We have for the first time demonstrated the utility of DECAL for the examination of the *Borrelia* transcriptome in ticks during feeding. DECAL is a powerful tool that overcomes the limitations of the differential immunoscreening technique and can potentially be exploited to examine the in vivo dynamics of *B. burgdorferi* gene expression in any tissue.

This work was supported by grants from the National Institutes of Health and the American Heart Association. E. Fikrig is the recipient of a Burroughs Wellcome Clinical Scientist Award in Translational Research.

We thank David Alland for his advice during the construction of the Bb-CAL library and Lisa Wilson at Incyte Genomics for printing the *B. burgdorferi* genomic arrays. We also thank Yvonne Pannekoek for helpful discussions.

REFERENCES

1. **Akins, D. R., K. W. Bourell, M. J. Caimano, M. V. Norgard, and J. D. Radolf.** 1998. A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state. J. Clin. Investig. **101:**2240–2250.
2. **Alland, D., I. Kramnik, T. R. Weisbrod, L. Otsubo, R. Cerny, L. P. Miller, W. R. Jacobs, Jr., and B. R. Bloom.** 1998. Identification of differentially expressed mRNA in prokaryotic organisms by customized amplification libraries (DECAL): the effect of isoniazid on gene expression in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **95:**13227–13232.
3. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
4. **Bunikis, J., L. Noppa, and S. Bergstrom.** 1995. Molecular analysis of a 66-kDa protein associated with the outer membrane of Lyme disease *Borrelia*. FEMS Microbiol. Lett. **131:**139–145.
5. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216:** 1317–1319.
6. **Cassatt, D. R., N. K. Patel, N. D. Ulbrandt, and M. S. Hanson.** 1998. DbpA, but not OspA, is expressed by *Borrelia burgdorferi* during spirochetemia and is a target for protective antibodies. Infect. Immun. **66:**5379–5387.
7. **Coburn, J., J. M. Leong, and J. K. Erban.** 1993. Integrin alpha IIb beta 3 mediates binding of the Lyme disease agent *Borrelia burgdorferi* to human platelets. Proc. Natl. Acad. Sci. USA **90:**7059–7063.
8. **de Silva, A. M., and E. Fikrig.** 1997. Arthropod- and host-specific gene expression by *Borrelia burgdorferi*. J. Clin. Investig. **99:**377–379.
9. **De Silva, A. M., and E. Fikrig.** 1995. Growth and migration of *Borrelia burgdorferi* in *Ixodes* ticks during blood feeding. Am. J. Trop. Med. Hyg. **53:**397–404.
10. **Fikrig, E., M. Chen, S. W. Barthold, J. Anguita, W. Feng, S. R. Telford III, and R. A. Flavell.** 1999. *Borrelia burgdorferi erpT* expression in the arthropod vector and murine host. Mol. Microbiol. **31:**281–290.
11. **Fikrig, E., W. Feng, S. W. Barthold, S. R. Telford III, and R. A. Flavell.** 2000. Arthropod- and host-specific *Borrelia burgdorferi* bbk32 expression and the inhibition of spirochete transmission. J. Immunol. **164:**5344–5351.
12. **Fingerle, V., H. Laux, U. G. Munderloh, U. Schulte-Spechtel, and B. Wilske.** 2000. Differential expression of outer surface proteins A and C by individual *Borrelia burgdorferi* in different genospecies. Med. Microbiol. Immunol. **189:** 59–66.
13. **Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. C. Venter, et al.** 1997. Genomic sequence of a Lyme disease spirochaete, Borrelia burgdorferi. Nature **390:** 580–586.
14. **Gilmore, R. D., Jr., M. L. Mbow, and B. Stevenson.** 2001. Analysis of *Borrelia burgdorferi* gene expression during life cycle phases of the tick vector *Ixodes scapularis*. Microbes Infect. **3:**799–808.
15. **Gilmore, R. D., Jr., and J. Piesman.** 2000. Inhibition of *Borrelia burgdorferi* migration from the midgut to the salivary glands following feeding by ticks on OspC-immunized mice. Infect. Immun. **68:**411–414.
16. **Labandeira-Rey, M., and J. T. Skare.** 2001. Decreased infectivity in *Borrelia burgdorferi* strain B31 is associated with loss of linear plasmid 25 or 28–1. Infect. Immun. **69:**446–455.
17. **Lane, R. S., J. Piesman, and W. Burgdorfer.** 1991. Lyme *borreliosis*: relation of its causative agent to its vectors and hosts in North America and Europe. Annu. Rev. Entomol. **36:**587–609.
18. **Sambrook, J., T. Maniatis, and E. F. Fritsch.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Schauder, S., K. Shokat, M. G. Surette, and B. L. Bassler.** 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol. Microbiol. **41:**463–476.
20. **Schwan, T. G., and J. Piesman.** 2000. Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete , *Borrelia burgdorferi*, during the chain of infection in ticks and mice. J. Clin. Microbiol. **38:**382–388.
21. **Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa.** 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proc. Natl. Acad. Sci. USA **92:**2909–2913.
22. **Skare, J. T., D. M. Foley, S. R. Hernandez, D. C. Moore, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 1999. Cloning and molecular characterization of plasmid-encoded antigens of *Borrelia burgdorferi*. Infect. Immun. **67:**4407–4417.
23. **Subramanian, G., E. V. Koonin, and L. Aravind.** 2000. Comparative genome analysis of the pathogenic spirochetes *Borrelia burgdorferi* and *Treponema pallidum*. Infect. Immun. **68:**1633–1648.
24. **Suk, K., S. Das, W. Sun, B. Jwang, S. W. Barthold, R. A. Flavell, and E. Fikrig.** 1995. *Borrelia burgdorferi* genes selectively expressed in the infected host. Proc. Natl. Acad. Sci. USA **92:**4269–4273.
25. **Thomas, V., J. Anguita, S. W. Barthold, and E. Fikrig.** 2001. Coinfection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis alters murine immune responses, pathogen burden, and severity of Lyme arthritis. Infect. Immun. **69:**3359–3371.