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Transcriptional Regulation of the *Borrelia burgdorferi* Antigenically Variable VlsE Surface Protein

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The Lyme disease agent *Borrelia burgdorferi* can persistently infect humans and other animals despite host active immune responses. This is facilitated, in part, by the *vls* locus, a complex system consisting of the *vlsE* expression site and an adjacent set of 11 to 15 silent *vls* cassettes. Segments of nonexpressed cassettes recombine with the *vlsE* region during infection of mammalian hosts, resulting in combinatorial antigenic variation of the VlsE outer surface protein. We now demonstrate that synthesis of VlsE is regulated during the natural mammal-tick infectious cycle, being activated in mammals but repressed during tick colonization. Examination of cultured *B. burgdorferi* cells indicated that the spirochete controls *vlsE* transcription levels in response to environmental cues. Analysis of P*vlsE*::*gfp* fusions in *B. burgdorferi* indicated that VlsE production is controlled at the level of transcriptional initiation, and regions of 5′ DNA involved in the regulation were identified. Electrophoretic mobility shift assays detected qualitative and quantitative changes in patterns of protein-DNA complexes formed between the *vlsE* promoter and cytoplasmic proteins, suggesting the involvement of DNA-binding proteins in the regulation of *vlsE*, with at least one protein acting as a transcriptional activator.

VlsE is a surface-exposed outer membrane lipoprotein of the Lyme disease spirochete *Borrelia burgdorferi*. During mammalian infection, but not during tick colonization or laboratory cultivation, segments of silent *vls* cassettes randomly recombine into the *vlsE* expression locus, continually creating novel *vlsE* gene sequences (23, 40, 58, 59). The resulting variations in the VlsE protein sequence alter its antigenic properties, which apparently allows the bacterium to evade antibody-mediated clearance by the host. Underscoring the apparently essential nature of *vlsE* are observations that mutant bacteria lacking the plasmid that carries *vls* are incapable of long-term mammalian infection (27, 28, 43, 57) and that *vls* loci are present in all examined Lyme disease spirochetes (24, 25, 55). Analogous antigenic variation systems are also involved in persistent infection by relapsing fever spirochetes (3, 47), protozoons such as *Plasmodium* spp. and *Trypanosoma* spp., and other significant pathogens (6, 15).

Several in vivo and in vitro studies indicated that *B. burgdorferi* also regulates the production of the VlsE protein. The spirochete synthesizes little or no VlsE when colonizing the midgut of a vector tick nymph (23, 40) but does make that protein during mammalian infection (14, 20, 21, 29, 31–33, 35). However, prior to this study, the complete pattern of VlsE synthesis in vivo had not been examined. In culture, bacteria increase the expression of VlsE in response to increased levels of autoinducer-2 (4,5-dihydroxy-2,3-pentanedione) (2). VlsE levels of cultured bacteria are also affected by less-well-defined signals stemming from changes in oxygen tension (49) or coincubation with mammalian tissue culture cells (22). Together, these data indicate that *B. burgdorferi* regulates the synthesis of VlsE during the natural infectious cycle in response to exogenous and endogenous signals associated with mammalian and tick infection states.

To more fully understand the mechanisms by which *B. burgdorferi* controls VlsE production, we characterized the expression pattern of VlsE during the mammal-tick infectious cycle, defined culture conditions under which VlsE is differentially synthesized, and determined that VlsE protein levels are controlled through transcriptional regulation of the *vlsE* gene. DNA elements involved in the transcriptional control of *vlsE* were identified, and data indicating that *B. burgdorferi* cytoplasmic proteins specifically bind *vlsE* regulatory DNA sequences were obtained.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strain B31-MI-16 is a wild-type infectious clone of the B31 culture sequenced by TIGR (38). Strain B31-e2 is a derivative of strain B31 that contains only plasmids cp26, cp32-1, cp32-3, cp32-4, lp17, lp38, and lp54 (1). Strain KS10 was derived from B31-e2 by the introduction of a promoterless *gfp* on plasmid pBLS590 (1). Strains BB1, BB2, BB3, BB4, and BB5 were each derived from strain B31-e2 by the introduction of plasmids pTB3, pTB11, PTB12, pTB13, and pTB14, respectively (see below). All *B. burgdorferi* isolates were grown in modified Barbour-Stoenner-Kelly II (BSK-II) medium (4) supplemented with kanamycin at a concentration of 200 μg/ml when needed. Media used to study the effect of environmental pH or isolation of proteins for electrophoretic mobility shift assays (EMSAs) (see below) were supplemented with 25 mM HEPES and buffered to pH values of 6.5, 7.0, or 8.0. Bacterial cultures were grown at either 23°C or 34°C, as required.

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Recombinant protein and production of antibodies. *Escherichia coli* strain SURE2, bearing plasmid pVlsE1-His3 (29), was cultured with aeration at 37°C to an optical density at 600 nm of 0.6. Expression of the VlsE-His fusion protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubating the cultures for an additional 3 h. Bacteria were harvested by centrifugation, and pellets were washed twice with phosphate-buffered saline (PBS) and frozen at −80°C. Aliquots of thawed cells were lysed for 15 min in 40 ml of B-PERII bacterial protein extraction reagent (Pierce Biotechnology, Inc., Rockford, IL) with the addition of 100 μl of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Crude cell lysates were mixed gently for 2 min at room temperature with 3 ml of MagneHis Ni-Particles (Promega, Madison, WI), and the beads were separated by placing them on a magnetic stand for 5 min. Beads were washed three times with 15 ml each of MagneHis Binding/Wash buffer containing 10 mM imidazole, and the target protein was then recovered by elution with 10 ml of MagneHis elution buffer containing 0.5 M imidazole. Recombinant protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie brilliant blue. Eight milliliters of 0.2 mg/ml recombinant VlsE was used to immunize two rabbits and produce polyclonal antibodies (Proteintech Group Inc., Chicago, IL). The 102-day-long antibody production procedure involved an initial injection and four additional antigen boosts before the final bleed was collected. The specificity of anti-VlsE antibodies was tested by Western blots of recombinant protein and *B. burgdorferi* B31-MI-16 total cell lysates.

Temporal analysis of VlsE expression in ticks and tick bite sites. Female BALB/c mice were infected by subcutaneous injection of 10^4 bacteria of strain B31-MI-16 that had been cultured at 34°C. Seven days later, the infection status of mice was assessed by inoculation of 1-mm^2 ear biopsies in BSK-II medium containing antibiotics (fosfomycin and rifampin) and antifungal agents (amphotericin B) (Sigma Chemical Co., St. Louis, MO). Cultured biopsies were examined 10 days later by dark-field microscopy.

Egg masses of pathogen-free *Ixodes scapularis* ticks were obtained from Jerry Bowman (Oklahoma State University, Stillwater, Okla.) and kept in separate conical tubes in a humidified chamber at 21°C until larvae hatched. For *B. burgdorferi* acquisition studies, 200 naïve larvae were placed on each *B. burgdorferi*-infected mouse (see above). The infection rate of completely engorged larval ticks was assessed by indirect immunofluorescence analysis (IFA) (see below) utilizing a monospecific antibody recognizing *B. burgdorferi* FlaB (flagellin) protein (5) and Alexa Fluor 488-labeled goat anti-mouse immunoglobulin G (IgG) (Molecular Probes, Eugene, Oreg.). Larvae that had just completed feeding after 72 h as well as larvae 96, 168, 192, and 264 h postattachment were analyzed. At least three different ticks were examined at each time point.

The remaining engorged larvae were kept in a humidified chamber at 21°C until they molted to the nymphal stage. Two weeks after emergence, flat (unfed) nymphs were analyzed by IFA (see below).

For *B. burgdorferi* transmission studies, 15 to 20 infected flat nymphal ticks each were placed onto 4- to 6-week-old naïve female BALB/c mice. Feeding nymphal ticks were forcibly removed with fine forceps after 72 h on the mice. During tick removal, a piece of mouse skin often remained attached to the hypostome of the feeding tick. These tick bite site skin samples were carefully dissected away from the tick for a separate analysis. All ticks and skin pieces were examined immediately after tick removal.

Mouse and tick tissues were dissected into 10 μl of PBS on glass slides and allowed to air dry overnight. Tissues were then fixed and permeabilized by immersion of slides in acetone for 15 min. Slides were air dried and then blocked overnight at 4°C in PBS containing 0.2% bovine serum albumin and 10% goat serum. After being washed in PBS–0.2% bovine serum albumin, slides were incubated for 1 h at room temperature in polyclonal rabbit antiserum specific for VlsE (see above) diluted 1:150. Slides were then washed and incubated for 1 h at room temperature in a 1:10 dilution of murine monoclonal antibody H9724, which is specific for the *B. burgdorferi* constitutively expressed FlaB subunit of the flagellum. After washing, slides were incubated in 1:1,000 dilutions of both Alexa Fluor 488-labeled goat anti-mouse IgG and Alexa Fluor 594-labeled goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) for 45 min each at room temperature. Slides were then washed, dried, and mounted with ProLong Anti-Fade mounting medium (Invitrogen, Carlsbad, CA) for viewing. Slides were viewed, and images were captured with an Axiophot epifluorescence microscope at ×100 magnification and a Spot digital camera (Zeiss, Hallbergmoos, Germany). Bacteria within 25 random fields were counted to determine the proportions of VlsE-expressing bacteria (positive for anti-VlsE staining) relative to the total number of bacteria present in a given field (as assessed by anti-FlaB staining). Slides of dissected tissues were incubated with either polyclonal anti-VlsE or monoclonal anti-FlaB antibody alone or the secondary antibodies only to serve as negative fluorescence controls.

TABLE 1. Oligonucleotides used in this work

Oligonucleotide	Sequence[a]
FLA3	5′-GGGTCTCAAGCGTCTTGG-3′
FLA4	5′-GAACCGGTGCAGCCTGAG-3′
VLSF3	5′-GTGAATTTTGCCTACTTCCGTATCAC-3′
VLSF5	5′-GAAATGAAAAATTCTCTCACCTAC-3′
VLSF13	5′-GTCATA**GGTACC**TGTGTAATGATTGTTTTGAACA TTGG-3′
VLSF15	5′-GGATTT**GGTACC**TTTGCCTACTTCCGTATCACAT AT-3′
VLSF17	5′-TGTAAAAGCCAAGTTGCTGATAAGGA-3′
VLSF21	5′-TTTTAT**GGTACC**GAATAAAAGAAATGA-3′
VLSF23	5′-CTCTCA**GGTACC**CTTGTTAAAACTCTCTTTTTG-3′
VLSF25	5′-GTTAAA**GGTACC**CTTTTTGAGTTAAGAT-3′
VLSR2	5′-GTCGTCCTTATCAGCAACTTGGC-3′
VLSR2-B	5′-/5Bio/GTCGTCCTTATCAGCAACTTGGC-3′
VLSR4	5′-TTATTCTTTATAATAAAAGAAATG-3′
VLSR6	5′-ATATTC**GGATCC**TCCTTATAATGAAAAGTATAAG TTATC-3′
VLSR10	5′-CAACAGAACCTGTACTATCTGGTT-3′

[a] Sequences of specific restriction endonuclease recognition sites are underlined. Mutations introduced into primers to generate restriction sites are indicated in boldface type.

All infection studies were performed under protocols approved by the University of Kentucky Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

Effects of temperature and pH on VlsE protein levels. B31-MI-16 spirochetes were cultured in BSK-II medium to a density of approximately 10^7 cells per ml, diluted 1:100 into fresh medium (51). Cultures were incubated at 34°C or 23°C at pH 6.5, 7.0, or 8.0, and upon reaching mid-exponential phase, cells were harvested by centrifugation. The pH values of the media were again measured following cell harvesting. Bacterial pellets were washed twice with PBS and lysed by heating in a boiling water bath for 5 min. Proteins were separated by single-dimensional SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose. Membranes were incubated with polyclonal antiserum directed against VlsE. Bound polyclonal antibodies were detected with horseradish peroxidase-linked goat anti-rabbit antibodies (Amersham) and visualized by chemiluminescence (Pierce, Rockford, IL). Membranes were then stripped and reprobed with monoclonal antibody H9724, which recognizes the constitutively expressed FlaB (flagellin) protein (5), and antibodies detected with horseradish peroxidase-conjugated anti-mouse antibodies (Amersham) were visualized by chemiluminescence.

Effects of temperature and pH on *vlsE* mRNA levels. Fifty-milliliter BSKII cultures of strain B31-MI-16 were grown to a density of approximately 10^7 cells per ml at either 34°C or 23°C and at pH 6.5, 7.0, or 8.0. Total RNA was extracted from each bacterial pellet using TRIzol (Invitrogen, Carlsbad, CA), resuspended in 50 μl of RNAsecure reagent (Ambion, Austin, TX), and treated with DNase I (RNase free) (Ambion, Austin, TX) to remove contaminating genomic DNA. Concentration and purity of RNA samples were assessed by agarose gel electrophoresis and spectrophotometric analysis at 260 nm. To test for DNA contamination, all RNA samples were subjected to PCR using 1 μg of each RNA sample, primers FLA3 and FLA4, and conditions similar to those used later for quantitative reverse transcription-PCR (Q-RT-PCR) (see below). The presence or absence of products was assessed by agarose gel electrophoresis and ethidium bromide staining. Only RNA samples that completely lacked DNA contamination (i.e., no product was obtained by PCR) were used for subsequent Q-RT-PCR analyses. Individual 0.2-μg aliquots of each DNA-free RNA preparation were then reverse transcribed into cDNA using random primers and a first-strand cDNA synthesis kit (Roche Applied Science, Indianapolis, IN).

Quantitative PCR was performed using a Light Cycler thermal cycler (Roche Applied Science) as described elsewhere previously (36). Briefly, 2 μl of cDNA or diluted genomic DNA (see below) was added to an 8-μl master mix containing Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA) (final dilution of 1:10 in enzyme diluent [Idaho Technology Inc., Salt Lake City, UT]), deoxynucleoside triphosphates (final concentrations of 0.8 mM) (Idaho Technology), 1× PCR buffer (containing $MgCl_2$ at a final concentration of 3 mM) (Idaho Technology), oligonucleotide primers (0.4 μM final concentration for each oligonucleotide) (Table 1), 1× SYBR green (final dilution of 1:10,000 in Tris-EDTA) (Molecular Probes Inc., Eugene, OR), and nuclease-free water (Promega, Madison, WI) to bring the volume of the master mix up to 8 μl. Reaction conditions for all PCRs consisted of an initial denaturation step at 95°C for 2 min to inactivate the

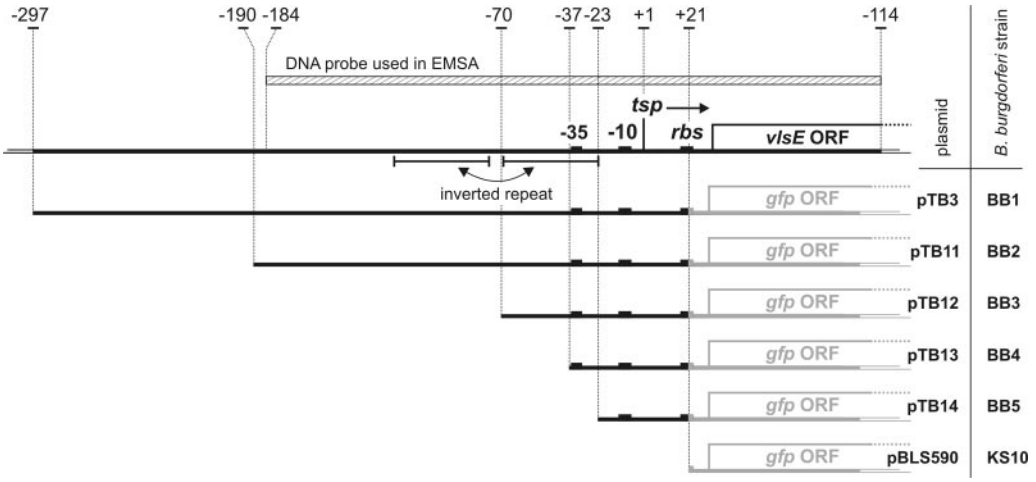

FIG. 1. Schematic map of the *vlsE* promoter/operator region and a diagram of P*vlsE*::*gfp* constructs. The −35 and −10 regions, transcription start site (*tsp*), and ribosome binding site (*rbs*) of *vlsE* are shown in boldface type. The start of the transcript synthesis is indicated by a horizontal arrow. Black lines represent the *vlsE* sequence, and gray lines represent the *gfp* gene sequence. Black and gray boxes represent open reading frames of *vlsE* and *gfp*, respectively. Sequence positions according to *tsp* are shown above the dotted lines. An inverted repeat potentially forming an extensive secondary structure is shown with bars and a double-speared arrow. The EMSA probe generated with primers VLSF3 and VLSR2 (VLSR2-B) is shown as a dashed box. P*vlsE*::*gfp* constructs present in strains BB1, BB2, BB3, BB4, BB5, and KS10 are shown below the *vlsE* sequence and marked, respectively. ORF, open reading frame.

hot-start enzyme followed by 40 cycles of amplification of 94°C for 5 s, 55°C for 5 s, and 72°C for 30 s. Programs used for melting curve analysis and cooling were previously described (16, 36). To ensure primer specificity, two additional analyses were performed. First, a melting curve analysis was included with each PCR. Second, reaction products obtained from each oligonucleotide primer pair were analyzed for the appropriate size and purity by agarose gel electrophoresis with ethidium bromide staining. All cDNA samples were analyzed in quadruplicate. A negative control that lacked the template was also included with each analysis as a check for potential DNA contamination of the reagents. Tenfold serial dilutions of B31-MI-16 genomic DNA (100 ng to 100 fg) were included in every assay for each primer set. This enabled the generation of standard curves from which the amount of *vlsE* or *flaB* transcript present in each cDNA sample could be calculated using Light Cycler software v.3.5.3 (Roche).

Three independent cDNA samples for each RNA preparation were obtained. Average expression values for *vlsE* transcript (nanograms) obtained from each cDNA sample were calculated relative to the average value (nanograms) obtained for the *B. burgdorferi* housekeeping gene *flaB*. Values reported for *vlsE* for a particular culture condition are mean expression levels (nanograms of *vlsE*/nanograms of *flaB*) of all cDNA samples. Error bars represent standard deviations of the means. Statistical analysis of the data was performed using one-way analysis of variance.

Cloning and sequencing of the *vlsE* promoter-operator element from *B. burgdorferi* B31-MI-16. All primers used in this study are listed in Table 1. The intergenic region between the *vlsE* gene and the silent *vls* cassettes contains a long inverted repeat of 46 bp, which can form a very stable cruciform structure (22). This region of the clonal strain B31-MI-16 was amplified by PCR from genomic DNA using primers VLSF3 and VLSR2 (Table 1), based on sequences under GenBank accession numbers U76405 and U76406. Amplification conditions using this highly structured template reaction mixture consisted of 0.1 ng/μl of chromosomal DNA template, 1.5 μM oligonucleotide primers, 0.2 mM of each deoxynucleoside triphosphate, 1% dimethyl sulfoxide, 1× LA (long and accurate) PCR buffer II (with Mg^{2+}), and 0.05 units/μl LA *Taq* polymerase (Takara Bio Inc., Madison, Wis.). Reaction conditions were as follows: 25 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min followed by a final 10-min extension step at 72°C. The resulting amplicon was gel purified and cloned into pCR2.1TOPO (Invitrogen, Carlsbad, CA) to produce pTB1.

Attempts to sequence the insert in plasmid pTB1 with primers M13F and M13R were only partially successful. Sequencing reactions terminated shortly after reaching the deduced stem-loop structure formed by the inverted repeats of the template. Therefore, oligonucleotides VLSF5 and VLSR4, complementary to sequences in the loop region, were designed on the basis of GenBank accession number AF314755 (Table 1). Plasmid pTB1 was used as a template for amplification with primers VLSF3 and VLSR4 or VLSF5 and VLSR2. Products were cloned into the pCR2.1TOPO vector to produce plasmids pTB1a and pTB1b, respectively. The inserts of those plasmids were completely sequenced on both strands.

Construction of *vlsE* promoter::*gfp* transcriptional fusions in *B. burgdorferi*. The *vlsE-vls* silent-cassette intergenic region was amplified by PCR using B31-MI-16 total DNA as a template under conditions similar to those used for the cloning of the *vlsE* promoter (see above). The oligonucleotide primers used for this reaction, VLSF13 and VLSR6, contain restriction endonuclease-cut sites for KpnI and BamHI, respectively. The resulting amplicon was digested with the above-mentioned enzymes and ligated between the KpnI and BamHI sites of vector pBLS590 (1). The resulting plasmid, pTB3, contains a chimera in which the *vlsE* 5′-noncoding region directs the transcription of *gfp*, with the ribosome binding site located a similar distance away from the ATG start codon as in the native *vlsE* gene (Fig. 1). The pTB3 insert junctions were sequenced on both strands to assure that no mutations had been introduced during the cloning process. Plasmid pTB3 was later used as a template for PCR with forward primer VLSF15, VLSF21, VLSF23, or VLSF25 plus the reverse primer VLSR6 to generate four DNA fragments, which were cloned into pBLS590 between the KpnI and BamHI sites. The derived plasmids, pTB11, pTB12, pTB13, and pTB14, contained *vlsE* promoter/operator fragments obtained with primers VLSF15-VLSR6, VLSF21-VLSR6, VLSF23-VLSR6, and VLSF25-VLSR6, respectively. All plasmid inserts were completely sequenced on both strands. *B. burgdorferi* B31-e2 was then transformed with plasmids pTB3, pTB11, pTB12, pTB13, and pTB14 under standard electroporation conditions (46) to generate strains BB1, BB2, BB3, BB4, and BB5, respectively.

Analysis of GFP expression. P*vlsE::gfp*-containing strains (BB1, BB2, BB3, BB4, and BB5) and control KS10 were grown to mid-exponential phase at 34°C and 23°C in medium adjusted to pH 6.5, 7.0, or 8.0. The effects of the culture conditions on green fluorescent protein (GFP) production were assessed by fluorescence microscopy, flow cytometry, SDS-PAGE of total proteins isolated from bacterial cultures, and immunoblot analysis. For fluorescence microscopy, 10-μl aliquots of each culture were placed onto a glass slide, and images were captured using an Axiophot epifluorescence microscope at ×100 magnification and a Spot digital camera (Zeiss) with the consistent use of the same picture intensity parameters for the comparison of bacteria grown at different pHs. For flow cytometry, bacteria were collected by centrifugation, washed twice with PBS, and then resuspended in PBS at concentrations of approximately 10^6 bacteria per ml. Aliquots of each bacterial suspension were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.), with excitation and detection wavelengths of 488 and 530 nm, respectively. At least 100,000 events were measured for each flow cytometry analysis. For SDS-PAGE and immunoblot, additional aliquots of bacteria were washed two times with PBS and lysed in a boiling water bath, and equal amounts of total protein from each lysis were

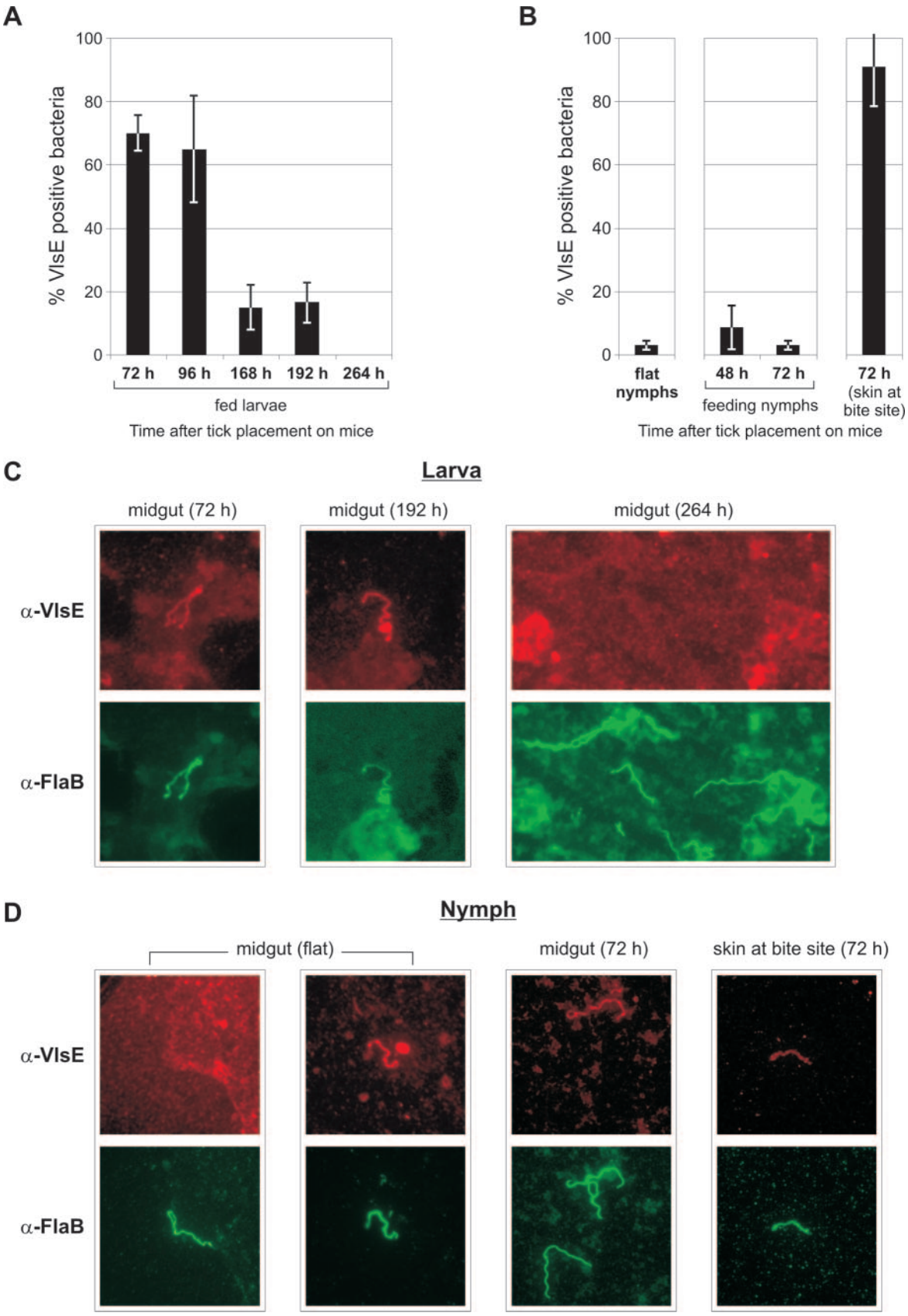

FIG. 2. Analysis of VlsE expression in ticks and tick bite sites. Results of double-labeling IFA of proportions of *B. burgdorferi* expressing VlsE. Bars are mean percentages of VlsE-positive bacteria counted in three slides (25 random fields each); error bars represent 1 standard deviation of

separated by SDS-PAGE. One of two identical gels was stained with Coomassie brilliant blue and analyzed for the presence of a band corresponding to the size of GFP (27 kDa). The other gel was analyzed by immunoblotting. Proteins were transferred onto nitrocellulose membranes and incubated with rabbit anti-GFP polyclonal antisera (Oncogene, San Diego, CA) and monoclonal antibody raised against FlaB (flagellin) to assess equal loading of the proteins. The membrane was subsequently incubated with anti-mouse and anti-rabbit infrared-labeled goat anti-rabbit IRDye 700DX and goat anti-mouse IRDye 800CW secondary antibodies (Rockland, Gilbertsville, PA) and visualized with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

EMSAs. A labeled DNA probe was produced by PCR using biotinylated 5′ primer VLSR2-B and unlabeled 3′ primer VLSF3 (Table 1), and unlabeled competitor DNA was produced using primers VLSR2 and VLSF3. Plasmid pTB1 served as a template. These DNA fragments contained a segment of the *vlsE* gene from positions −184 to +114 relative to the start of transcription. Both PCR products used for EMSAs were purified from template DNA by agarose gel electrophoresis followed by extraction using Wizard SV Gel and PCR Clean-Up systems (Promega, Madison, WI).

Whole-cell lysates were prepared from *B. burgdorferi* B31-MI-16 harvested by centrifugation. Pellets were washed twice with PBS, resuspended in 50 mM Tris (pH 8.0)–10 mM EDTA–10% (wt/vol) sucrose, and frozen in a dry ice-ethanol bath. The bacteria were thawed in a 37°C heat block, and NaCl, dithiothreitol, protease inhibitor cocktail and phosphatase inhibitor cocktail II (Sigma-Aldrich, St. Louis, MO), and lysozyme were added to final concentrations of 140 mM, 1 mM, 8 μl/ml, 2 μl/ml, and 0.4 mg/ml, respectively. Cells were then incubated on ice for 45 min with gentle mixing and then subjected to four freeze-thaw cycles using dry ice-ethanol and the 37°C heat block. Cellular debris was removed from the bacterial lysates by centrifugation in a fixed-angle rotor at 22,000 × *g* for 2 h at 4°C. The resultant protein extracts were aliquoted and frozen at −80°C.

For each EMSA, an aliquot of 0.5 to 8 μg of bacterial extract was incubated either with or without 100 ng of unlabeled specific competitor DNA in 20 μl of buffer containing 2 μg poly(dI-dC) (nonspecific competitor), 10 mM HEPES buffer (pH 7.5), 1.5 mM $MgCl_2$, 100 mM KCl, 20% (vol/vol) glycerol, 8 μl/ml protease inhibitor cocktail, and 2 μl/ml phosphatase inhibitor cocktail II. Samples were then incubated for 15 min at room temperature to allow protein binding to poly(dI-dC) and/or the competitors. Next, a 0.5-ng aliquot of labeled DNA probe was added to each sample. Samples were then subjected to electrophoresis through a 7% polyacrylamide gel (37.5:1 acrylamide-bisacrylamide) at room temperature. After electrophoresis, DNAs were transferred onto nylon membranes and cross-linked by UV irradiation. A LightShift chemiluminescent EMSA kit (Pierce Biotechnology) and exposure to X-ray film were utilized to visualize the biotinylated DNA probe.

Nucleotide sequence accession number. The sequence of the B31-MI-16 *vlsE* promoter/operator region has been deposited in GenBank under accession number DQ275473.

RESULTS

***B. burgdorferi* modulates VlsE synthesis during the mammal-tick cycle.** We first sought to develop a more comprehensive picture of VlsE expression patterns during the natural infectious cycle, as well as to gain insight about environmental cues triggering regulation, by examining bacteria sampled from many steps of infection. Naïve larval *I. scapularis* ticks were fed on chronically infected mice, with approximately 80% of tested ticks acquiring infection. These ticks were allowed to molt to nymphs, which were than fed to repletion on naïve mice. At various time points, tick and mouse tissues were examined using IFA to determine the proportions of bacteria that produced VlsE. Control analyses lacking any one labeling reagent did not yield signals, confirming that the results of all IFA studies were specific (data not shown). Each tick tissue sample examined contained >100 bacteria. Four to 14 bacteria were observed in each skin sample, with five skin samples examined.

Bacteria acquired by larval ticks feeding on infected mice exhibited high levels of VlsE expression (Fig. 2A and C). Approximately 70% of *B. burgdorferi* isolates produced detectable quantities of VlsE after 3 to 4 days of larval feeding. After completion of blood feeding, the proportions of *B. burgdorferi* isolates that produced VlsE within larvae declined significantly. By 264 h (11 days) after attachment (7 days after drop off), no bacteria that produced VlsE could be detected (Fig. 2A and C). Simultaneous use of a control antibody specific for the spirochete's flagellum indicated high levels of tick infection.

Following the molt to the nymphal stage, *B. burgdorferi* continued to repress the synthesis of VlsE (Fig. 2B and D). Similarly, very few bacteria within the midguts of nymphs feeding on mice produced detectable levels of VlsE. However, almost all bacteria found in mouse skin at tick bite sites synthesized VlsE, indicating that *B. burgdorferi* dramatically increases VlsE production upon leaving the tick midgut.

Environmental signals affect VlsE expression. *B. burgdorferi* encounters a variety of temperatures during tick colonization or mammalian infection. Also, the physiological pH values throughout the natural life cycle can vary significantly, ranging from the neutral or slightly acidic pH of 6.5 or 7.0 within a feeding nymphal tick up to a very alkaline pH of 9.0 or 10.0 within tick saliva (7, 56). Prior studies by our laboratory and others demonstrated that *B. burgdorferi* controls expression of a number of virulence-associated proteins in response to changes in temperature and/or pH (9–12, 26, 44, 48, 50, 51, 56).

To gain further insight into external cues that influence VlsE synthesis, bacteria were cultured to mid-exponential phase at either 23°C or 34°C in medium buffered at pH 7.6. Significantly higher levels of VlsE were produced by bacteria grown at 23°C compared to those grown at 34°C (Fig. 3A). The results obtained were strikingly different from those observed for any previously examined *B. burgdorferi* protein. Bacteria were then similarly cultured in medium buffered to remain at pH 6.5, 7.0, or 8.0. All media were reexamined after collection of bacteria, and none exhibited a detectable change in pH. Bacteria cultured at pH 6.5 or 7.0 expressed higher levels of VlsE at 23°C than did bacteria cultured at 34°C at the same pH values (Fig. 3B). Bacteria cultured at either 23°C or 34°C in pH 8.0 medium expressed far higher levels of VlsE protein than bacteria cultured at either pH 6.5 or 7.0.

We next sought to determine the mechanism(s) responsible for the observed changes in VlsE protein levels. Q-RT-PCR analysis of cultured bacteria revealed a clear correlation be-

the means. (A) *B. burgdorferi* in midguts of naïve larval *I. scapularis* ticks feeding on infected mice. Ticks were examined upon completion of feeding (72 h) and at several time points after the conclusion of the blood meal. (B) Fully engorged larval ticks were allowed to molt into nymphs and were placed on naïve mice. Bacteria from midguts of flat (unfed) nymphs and nymphs pulled off during feeding (48 and 72 h) were analyzed. Bite site skin samples attached to the hypostome of the forcibly removed ticks (72 h) were thoroughly dissected away and also examined. (C and D) Representative IFA images of *B. burgdorferi* during infection of larval ticks (C) or colonization of nymphs and subsequent infection of mammalian skin (D). VlsE-directed antibody (α-VlsE) labeled only those spirochetes expressing VlsE (top rows), while anti-FlaB (α-FlaB) monoclonal antibody labeled all spirochetes in the same field (bottom rows).

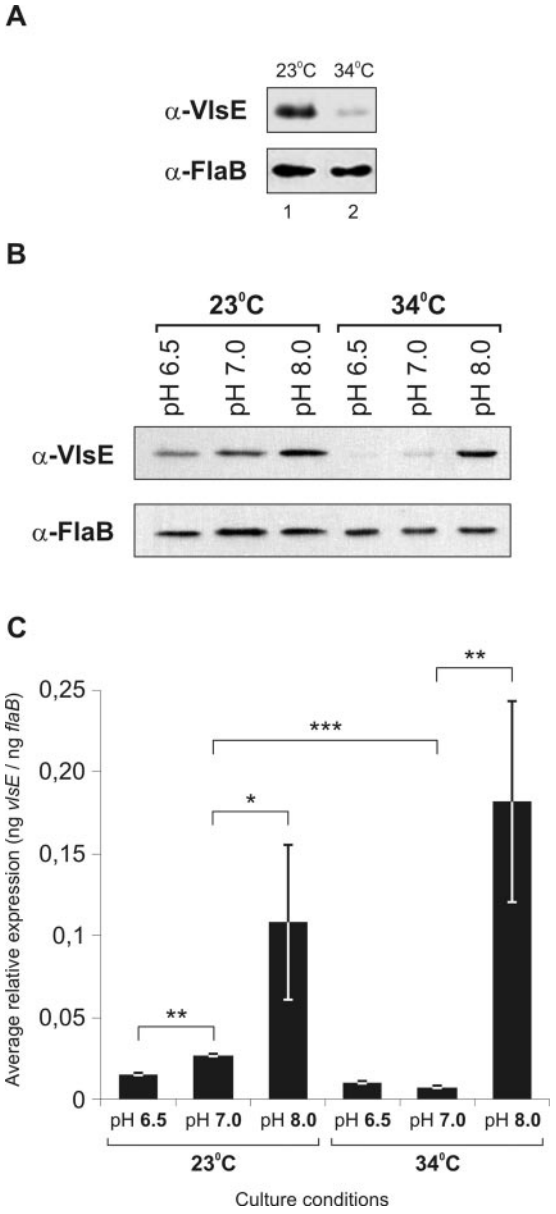

FIG. 3. VlsE protein and *vlsE* transcript levels in cultured *B. burgdorferi*. Bacteria were grown at 23°C and 34°C in media adjusted to various pH values (as indicated). (A and B) Cell lysates examined by Western blot analysis utilizing antibodies directed against VlsE (α-VlsE). Blots were stripped and rehybridized with a murine monoclonal antibody specific for the constitutively expressed FlaB protein (α-FlaB) to ensure that equal quantities of total protein were loaded in all lanes. (C) Q-RT-PCR analysis of *vlsE* mRNA levels relative to the housekeeping gene *flaB*. Graphs represent the means of three independent experiments ± standard errors. Statistically significant differences are indicated as follows: *, $P < 0.5$; **, $P < 0.01$; ***, $P < 0.001$. Other differences are not statistically significant.

tween *vlsE* mRNA levels (Fig. 3C) and the VlsE protein expression pattern (Fig. 3B). Again, alkaline culture medium stimulated *vlsE* expression. We observed a 27-fold increase in *vlsE* transcript in bacteria cultured at pH 8.0 compared with that in bacteria cultured at pH 7.0 and 34°C, and we observed a fourfold increase at 23°C. Growth at ambient temperatures yielded significantly higher levels of *vlsE* mRNA at pH 7.0 than did cultures at 34°C. These data indicate that pH and temperature each have a significant effect on *vlsE* transcript formation, with the highest amounts being produced in response to basic conditions at both temperatures, and that VlsE protein levels are determined by the level of *vlsE* mRNA levels.

Analyses of P*vlsE*::*gfp* transcriptional fusions. To begin to define the molecular mechanisms responsible for *vlsE* transcriptional regulation, plasmid pTB3 was produced to contain a transcriptional fusion between *vlsE* 5′-noncoding DNA and the gene encoding GFP (Fig. 1). This construct contains the entire DNA region between the *vlsE* open reading frame and the upstream silent cassettes. pTB3 was introduced into *B. burgdorferi* strain B31-e2 to produce strain BB1. Strain KS10, which contains a promoterless *gfp* on plasmid pBLS590 in a B31-e2 background (1), was used as a negative control. Both BB1 and KS10 were cultured at 34°C to mid-exponential phase at pH 6.5, 7.0, or 8.0, and GFP levels were then measured by several methods.

Bacteria from all three cultures of strain BB1 were visualized by epifluorescence microscopy using a GFP filter (Fig. 4A). BB1 spirochetes grown at pH 6.5 or 7.0 had uniform typical "corkscrew" appearances. However, the morphology and intensity of the fluorescence of *B. burgdorferi* from the culture grown at pH 8.0 were very different: cells were consistently brighter and thicker, and their helices were more relaxed, often resembling long rods, perhaps due to toxic effects of the abundant GFP produced by these bacteria at alkaline pHs. Strain BB1 bacteria cultured at pH 8.0 were also noticeably less motile than when grown in neutral or acidic conditions or when either strain KS10 or wild-type cells were grown at pH 8.0. As previously noted (1), green fluorescence could not be detected in cultures of the control strain KS10, regardless of growth conditions.

Lysates of bacteria used in the above-described experiment were then separated by SDS-PAGE. A protein band with a size corresponding to GFP (27 kDa) was clearly visible in all lysates of BB1 by Coomassie brilliant blue staining (Fig. 4B, lanes 2, 4, and 6). Its position and intensity were confirmed by immunoblotting with a monospecific polyclonal antibody directed against GFP (Fig. 4C). More GFP was produced by BB1 grown at pH 7.0 than at pH 6.5, and significantly higher levels of GFP were produced when BB1 was grown at pH 8.0. Transcription driven by P*vlsE* was so vigorous at pH 8.0 that the GFP band was the most intense band in the whole protein profile of strain BB1 (Fig. 4B, lane 6). No GFP was detected in lysates of strain KS10, regardless of growth conditions. Equal loading of protein lysates on the gel was confirmed by immunodetection of the constitutively expressed flagellin protein (Fig. 4C, bottom panel).

These studies of strain BB1 demonstrated transcriptional regulation of the *vlsE* promoter. Additional P*vlsE*::*gfp* transcriptional fusions were designed to narrow down DNA sequences responsible for the control of *vlsE* transcription. Plasmid pTB11 lacks only 107 bp distal to the *vlsE* promoter, pTB12 is missing half of the inverted repeat, pTB13 contains the *vlsE* promoter through position −37, and pTB14 contains only P*vlsE* DNA proximal to −23 bp (i.e., it lacks the −35 sequence) (Fig. 1). Plasmids pTB11, pTB12, pTB13, and

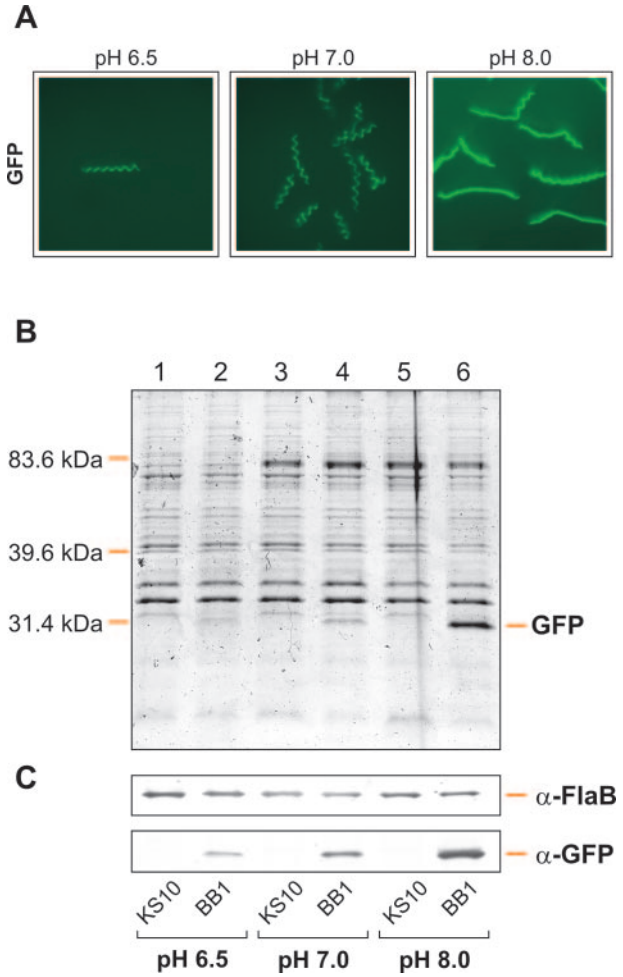

FIG. 4. Influence of various pH conditions on GFP production by strain BB1. (A) Fluorescence microscopy of *B. burgdorferi* BB1 cultured to mid-logarithmic phase in media adjusted to the indicated pHs. All images were captured using the same intensity parameters and the same original magnification of ×100. (B) Total protein lysates prepared from mid-exponential-phase cultures of KS10 or BB1 grown under the indicated pH conditions. Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. The indicated band corresponds to the size of GFP (27 kDa). (C) Proteins were transferred onto a nitrocellulose membrane and probed with anti-GFP (α-GFP) and anti-FlaB (α-FlaB) antibodies to confirm equal loading of protein lysates in all lanes.

pTB14 were introduced into *B. burgdorferi* strain B31-e2 to produce strains BB2, BB3, BB4, and BB5, respectively.

All transformed *B. burgdorferi* strains (including BB1 and the negative control, KS10) were then cultured at 34°C in medium buffered to pH 7.0 or 8.0. Neither strain BB5 (missing the consensus −35 sequence of the promoter) nor KS10 expressed detectable levels of GFP (Fig. 5A and B). Strains BB1, BB2, BB3, and BB4 all produced GFP, and expression of all four promoter fusion constructs was positively affected by alkaline conditions (Fig. 5A and B). Interestingly, even though the promoter fusion carried by strain BB4, pTB13, contains only first 37 bp of P*vlsE*, the culture medium pH still had a significant effect on GFP levels.

B. burgdorferi strains with reporter constructs were also diluted into fresh medium buffered to remain at a pH of either 7.0 or 8.0 and then incubated at 23°C. Strains KS10 and BB5 did not produce GFP (Fig. 6A and C). GFP expression by strain BB4 was still regulated by pH, although there was no significant effect of temperature (Fig. 6B and C). Those data indicate that the effects of culture temperature and pH on *vlsE* transcription are independent and that the 37 bp of P*vlsE* in pTB13 contains the minimal operator for pH-dependent transcriptional control.

Despite several efforts, BB1, BB2, and BB3 were not able to grow at 23°C. We noted increasing amounts of dead cells and debris in cultures as incubation proceeded. These effects may have been due to a combination of toxicity from elevated levels of GFP plus the inhibition of bacterial metabolism arising from cultivation at cool temperatures (51).

Specific protein-DNA interactions between the *vlsE* promoter/operator DNAs and cytoplasmic proteins. Bacteria frequently use DNA-binding proteins to affect transcription initiation. Therefore, we tested lysates from wild-type *B. burgdorferi* grown under different pH and temperature conditions for the presence of proteins that bound *vlsE* 5′ DNA. For these studies, a 5′-biotinylated 298-bp DNA fragment extending from positions −184 to +114, relative to the *vlsE* transcription start site (22), was generated by PCR (Fig. 1). Soluble proteins were isolated from cultured *B. burgdorferi* and used in EMSAs.

Lysates obtained from bacteria incubated at 23°C formed two main complexes (C1 and C2) with the *vlsE* promoter/operator DNA (Fig. 7). A more slowly migrating complex (C3) was also observed following long film exposures. Proteins from cultures grown at 34°C also formed these same complexes but only at high total protein concentrations. Relative proportions of these complexes also differed between the lysates at 23°C and the lysates at 34°C. All three complexes could be competed by the unlabeled *vlsE* probe, indicating specificity of protein-DNA interactions (Fig. 7A).

To further characterize the differences in the levels of transcription from the *vlsE* promoter in response to pH, cytoplasmic proteins obtained from cultures grown at pH 6.5 or 8.0 and at 23°C or 34°C were used for additional binding studies. Bacterial culture pH did not exert any qualitative effects on gel retardation patterns of the *vlsE* promoter/operator DNA probe (Fig. 7B). However, for both culture temperature conditions, complexes formed with lysates from pH 8.0 medium were markedly more intense than those from pH 6.5 medium. These phenomena can be explained by either an increase in the concentration or increased DNA affinity of a *vlsE* operator-binding protein(s) at a more basic pH.

DISCUSSION

B. burgdorferi controls synthesis of the VlsE surface antigen during the mammal-tick infectious cycle, producing the protein during mammalian infection and repressing its synthesis during tick colonization. Data from this report are consistent with previous studies that examined small parts of the infectious cycle. Ohnishi et al. also noticed that the protein was expressed by very few (<1%) bacteria in midguts of unfed or partially fed nymphs (40). Indest et al. observed some VlsE expression in flat nymph midguts but not after feeding commenced (23). In

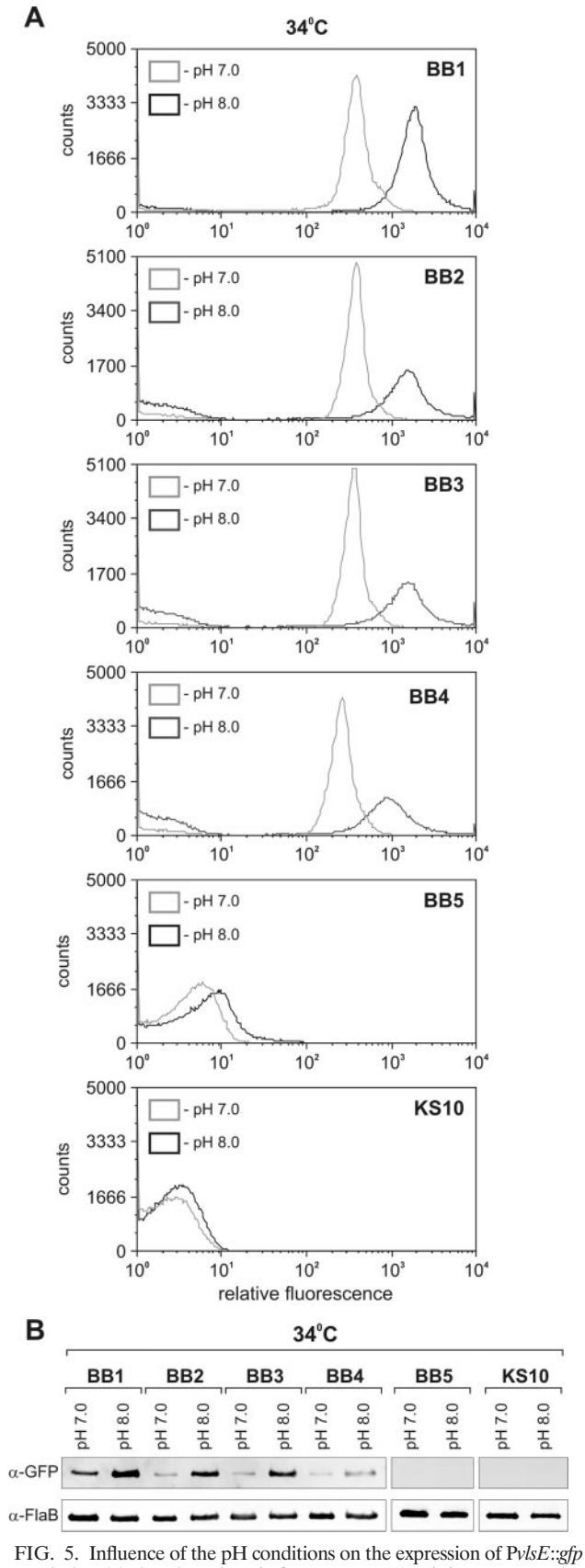

FIG. 5. Influence of the pH conditions on the expression of P*vlsE*::*gfp* transcriptional fusions in *B. burgdorferi* grown at 34°C. (A) Representative GFP expression by *B. burgdorferi* carrying P*vlsE*::*gfp* fusions (strains BB1, BB2, BB3, BB4, and BB5) and a promoterless *gfp* (KS10). Bacteria were grown at 34°C to mid-exponential phase at a constant pH of either 7.0 (gray) or 8.0 (black) and analyzed by flow cytometry. (B) Total protein lysates from cultures were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-GFP (α-GFP) and anti-FlaB (α-FlaB) antibodies to confirm equal loading of protein lysates in all lanes.

both of those studies, ticks were artificially infected with cultured bacteria using capillary feeding tubes, and the results of immunofluorescence staining were not specifically quantified. The lack of VlsE during tick colonization is consistent with observations that the VlsE-encoding plasmid is not required for *B. burgdorferi* persistence within the arthropod vector (18, 53) and may even occasionally be lost by bacteria surviving artificial tick infections (23). The expression of protein during stages of mammalian infection is congruous with its proposed role as a varying surface antigen that enables *B. burgdorferi* to avoid clearance by host antibodies. Since *vlsE* recombination occurs only during mammalian infection, times when *vlsE* is actually transcribed, the process of transcription and recombination may be controlled by some of the same mechanisms.

Many steps in the pathway between a gene and its functional product may be exploited to maintain control of this process. For reasons of economy, bacteria frequently regulate the initiation of RNA transcript formation (8). In our studies, analyses of *vlsE* mRNA levels by Q-RT-PCR and GFP protein synthesis by P*vlsE*::*gfp* operon fusions correlated with the fluctuations observed in VlsE protein levels, indicating that transcriptional initiation is an important level of VlsE control. Similar results were obtained from analyses of the infectious strain B31-MI-16, which contains a large complement of natural plasmids, and derivatives of B31-e2, which contains only seven small natural DNAs (cp26, cp32-1, cp32-3, cp32-4, lp17, lp38, and lp54). Therefore, it is evident that all the proteins necessary to sense changes in the environment, transfer signals to the *vlsE* 5′-regulatory region, and modify transcription initiation from P*vlsE* are encoded by either the main chromosome or those seven small DNAs. Significantly, genes encoding transcriptional regulators of *vlsE* must be encoded on DNA(s) other than the linear plasmid that carries the *vls* locus. Studies are ongoing to identify those regulatory factors, a search made simpler by the ability to screen the less complex strain B31-e2.

Transcriptional regulation in bacteria frequently involves DNA-binding proteins with a selected affinity for the promoter regions, interacting with transcriptional machinery to modulate the local concentration of RNA polymerase at the sites of transcriptional initiation (34, 41). We observed both quantitative and qualitative changes in the complexes formed by DNA-binding proteins and the *vlsE* 5′-noncoding region depending upon growth conditions. Complex C2 was very faint or absent in EMSAs of bacteria grown at 34°C, but the stimulation of VlsE synthesis at 23°C correlated with the formation of the strong C2 complex between proteins from those bacteria and *vlsE* promoter/operator DNA. Generally, concentrations of proteins that formed the characteristic complexes C1, C2, and C3 were higher in the cells grown under conditions stimulating VlsE expression: they were higher at 23°C compared to 34°C

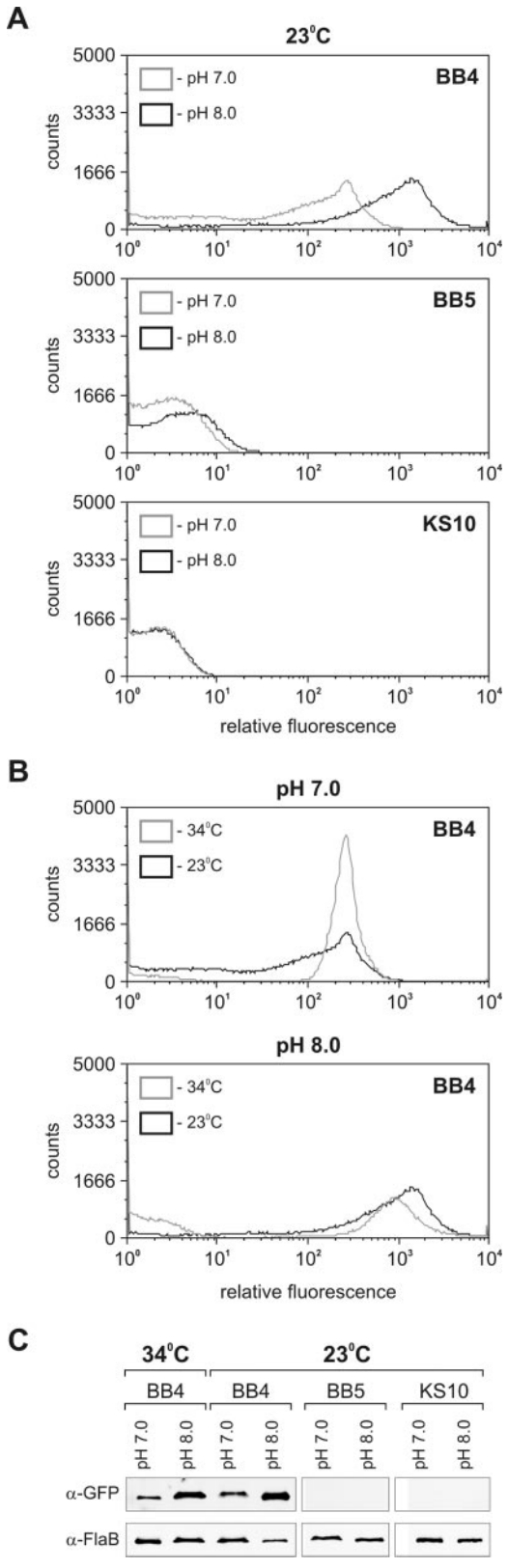

FIG. 6. Effects of different pH and temperature conditions on the expression of P*vlsE*::*gfp* transcriptional fusions. (A) Representative flow cytometry analyses of *B. burgdorferi* carrying P*vlsE* from position −37 to the *rbs* fused to *gfp* (BB4), only the segment at the −10 position of P*vlsE* fused to *gfp* (BB5), and a promoterless *gfp* (KS10) grown at 23°C. (B) Flow cytometry analysis of temperature-dependent GFP expression by strain BB4 grown at pH 7.0 and 8.0. (C) Western blot analysis of lysates of cultures shown in panels A and B probed with anti-GFP (α-GFP) antibodies. Reprobing of the membrane with anti-FlaB (α-FlaB) antibodies confirmed equal loading of protein lysates in all lanes.

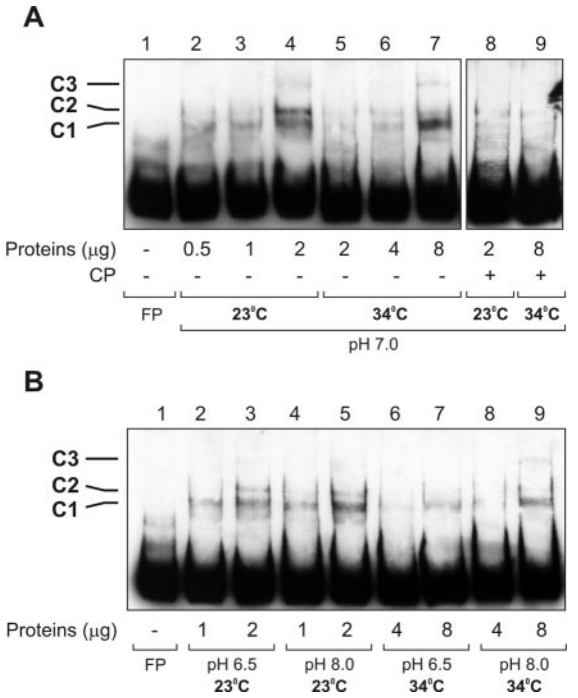

FIG. 7. EMSAs identifying three complexes formed by the *vlsE* promoter/operator DNA and cytoplasmic proteins from *B. burgdorferi*. A biotin-labeled 305-bp promoter/operator DNA fragment extending from positions −190 to +114 relative to the *vlsE* transcription start site was incubated with soluble proteins from wild-type *B. burgdorferi* cultured at 23°C or 34°C and under various pH conditions. Total soluble proteins were used as indicated. Protein-DNA complexes are labeled C1, C2, and C3. FP represents free probe, and CP represents an unlabeled *vlsE* competitor probe obtained with nonbiotinylated primers.

(Fig. 7A) and at pH 8.0 compared to pH 6.5 (Fig. 7B). These observations led us to hypothesize that protein-mediated transcriptional activation is responsible for P*vlsE* expression.

Proteins from cultures grown at pH 8.0 did not form any additional complexes with the *vlsE* promoter region compared to those grown at pH 6.5, but the existing bands were markedly more intense when lysates from alkaline-grown bacteria were used. This suggests that the transcriptional activation of *vlsE* in response to pH does not require the synthesis of any novel regulator(s) but that the concentration of DNA-binding proteins and/or their activity could be increased at more basic growth conditions. Changes in activity may arise from the interactions with small ligands, such as ppGpp in other bacteria, that facilitate a quick and efficient response to the environment (13). The number of *B. burgdorferi* genes responding to the changes in pH constitute a relatively large stimulon (9, 11), so it is possible that a common regulator directly affects the ac-

tivity of RNA polymerase at certain promoters. This hypothesis can also explain our results using truncated P*vlsE*::*gfp* fusions. Promoter/operator DNA present in strain BB4 encompasses little more than the consensus −10 and −35 sequences but is sufficient to exert pH-dependent transcriptional control over the reporter gene.

The 5′-noncoding region of *vlsE* contains a very long inverted repeat of 46 bp, which can potentially form a very stable cruciform structure (22). The stability of this stem-loop structure often impeded laboratory procedures such as PCR or DNA sequencing (see Materials and Methods). The predicted −35 region of the *vlsE* promoter sequence lies within the palindrome (Fig. 1). Although it is possible that an initial step in the transcriptional initiation from P*vlsE* is the rearrangement of the DNA secondary structure to facilitate the access of RNA polymerase to the promoter, deletion of one side of the inverted repeat in pTB12 had no detectable effect on promoter activity. These data indicate that if the *vlsE* inverted repeat performs a specific function, it is not to limit access to the promoter.

B. burgdorferi detects natural environmental signals as it passes through its infectious cycle and responds by modulating the composition of its outer membrane (16, 19, 37–39, 48). In the initial model that sought to explain the effects of culture temperature on *B. burgdorferi* gene expression, it was postulated that cultivation at 23°C represented conditions within the unfed tick, while 34°C mimicked conditions during transmission and infection of mammalian hosts (48). We observed that bacteria cultivated at 23°C produced considerably more VlsE protein than bacteria grown at 34°C (especially under neutral conditions). However, VlsE is not well expressed in the arthropod vector but is expressed during mammalian infection. Clearly, the original model is not adequate to describe all the mechanisms involved in *B. burgdorferi* gene regulation (52). The means by which *B. burgdorferi* senses temperature differences are as yet undefined, so one can only speculate at this time about why cooler culture temperatures result in increased production of VlsE. Regardless of how one models the effect of temperature on *vlsE* expression, it is significant that this culture condition affects VlsE synthesis, thereby allowing researchers to conduct detailed in vitro studies on the pathways exerting control over this gene.

B. burgdorferi encounters at least two pH changes during transmission from the feeding tick to the mammalian host: a slight acidification of the midgut during blood ingestion (56) and a profound alkalinization upon entry to the saliva, which has a pH of 9.5 to 10 (7, 30, 56). Few bacteria produced detectable levels of VlsE while within the tick midgut, while the great majority of *B. burgdorferi* isolates at the tick bite site made large quantities of VlsE. A previous study reported that *vlsE* mRNA is more abundant in the feeding tick's salivary glands than in midguts (42). Consistent with these observations, VlsE synthesis was repressed in bacteria cultured at pH 6.5 and 7.0, while the more alkaline pH of 8.0 had a strong stimulatory effect on VlsE production. We propose that the encounter with alkaline tick saliva serves as a signal to trigger the synthesis of VlsE during transmission of *B. burgdorferi* into the mammalian host. A similar mechanism may be partially responsible for regulation of the *B. burgdorferi* mammal-associated virulence factor complement regulator-acquiring surface protein 1 (54), which is also up-regulated in vitro in response to alkaline growth conditions and in vivo upon transmission into the body of mammals. It is also notable that proteins associated with antigenic variation of other vector-borne pathogens are often synthesized within the organs producing saliva. Enhanced expression of the major surface protein 2 class of proteins of the tick-borne bacterium *Anaplasma marginale* occurs in tick salivary glands during transmission feeding (45). Synthesis of the variant surface glycoprotein of African trypanosomes is reinitiated specifically in tsetse fly salivary glands (17).

In conclusion, detailed temporal analysis of VlsE expression during the *B. burgdorferi* infectious cycle indicated that the synthesis of the VlsE protein is regulated in vivo. Studies of cultured bacteria demonstrated that VlsE protein levels corresponded with both levels of *vlsE* mRNA and initiation of P*vlsE* transcription. Both environmental pH and temperature affected *vlsE* transcriptional initiation, apparently through distinct mechanisms. An effect of pH was observed on transcription from a minimal P*vlsE* extending only to bp −37, while the effect of culture temperature required additional 5′ DNAs. At least three specific protein-DNA complexes are formed between *B. burgdorferi* cytoplasmic proteins and *vlsE* 5′ DNA. Correlation between the *vlsE* promoter activity and the presence and/or intensity of these complexes suggests the involvement of DNA-binding proteins in the regulation of *vlsE* expression, with at least one acting as a transcriptional activator. Since the VlsE protein performs crucial functions in the evasion of the mammalian immune response, dissection of the molecular mechanisms underlying its regulation may provide new insights into the pathogenesis of *B. burgdorferi* as well as direct novel treatment strategies for Lyme disease. Additionally, since recombination within the *vls* locus occurs only in mammals, conditions that also favor transcription of the *vlsE* gene, the two phenomena may be linked, so characterization of *vlsE* regulation in *B. burgdorferi* may also lead to a better understanding of the control of recombination.

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