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Increased expression of *Borrelia burgdorferi vlsE* in response to human endothelial cell membranes

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Summary

RNA isolated from virulent *Borrelia burgdorferi* cells incubated with human endothelial or neurological tissue cells was subjected to subtractive hybridization using RNA from the same strain incubated in tissue culture medium alone. This RNA subtractive technique generated specific probes that hybridized to two restriction fragments (8.2 kb and 10 kb respectively) generated by *Eco*RI digestion of total plasmid DNA. The 10 kb *Eco*RI fragment localized to lp28-1 and was subsequently identified as the variable membrane protein-like sequence (*vls*) region, which includes an expression locus (*vlsE*) and 15 silent cassettes. *vlsE* encodes a 36 kDa outer surface protein that undergoes antigenic variation during animal infections. Primer extension analysis identified the 5′ end of a transcript and a putative promoter for *vlsE.* Quantitative reverse transcription–polymerase chain reaction (RT–PCR) suggested that the expression of *vlsE* increased when virulent *B. burgdorferi* cells were incubated with human tissue cells or purified cell membranes isolated from those same cell lines. A 138 bp region upstream of the *vlsE* region that was not reported in the genome sequence was sequenced using specific ^{32}P end-labelled primers in a DNA cycle sequencing system at high annealing temperatures. Analysis revealed that it contained a 51 bp inverted repeat, which could form an extremely stable cruciform structure. Southern blots probed with the *vlsE* promoter/operator region indicated that part or all of this sequence could be found on other *B. burgdorferi* plasmids.

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Introduction

Vector-borne bacterial pathogens, such as *Borrelia burgdorferi*, encounter different conditions as they are transmitted to various hosts. Sensing changes in these environments and modulating gene expression accordingly is likely to be important for adaptation and survival of *B. burgdorferi* within various hosts. For example, the levels of outer surface protein A (OspA), OspB, OspC, OspE and OspF shift as *B. burgdorferi* moves from the midgut of *Ixodes* ticks to the human host (Schwan *et al*., 1995; Stevenson *et al*., 1995). In the unfed tick vector, OspA and OspB are expressed, whereas OspC, OspE and OspF are barely detectable (Schwan and Simpson, 1991). Conversely, the levels of the latter increase dramatically after the ingestion of a blood meal, whereas those of OspA and OspB decrease (Schwan *et al*., 1995). These effects have been duplicated *in vitro* by shifting *B. burgdorferi* cells from 24°C to 35°C. Under these conditions, OspC, OspE and OspF again showed an increase in expression, whereas OspA and OspB were significantly reduced (Stevenson *et al*., 1995). Components of the blood meal also appear to be important in the process, as exposing unfed ticks to increased temperature is not sufficient to trigger some changes in gene expression. This suggests that OspA and OspB expression is important in unfed ticks where the temperature is $\leq$24°C, whereas the expression of OspC, OspE and OspF may be more important for transmission of the bacteria to a new host. Therefore, increase in temperature and the presence of blood components are signals that may enable *B. burgdorferi* to adapt to the environment that they are about to encounter in the mammalian host.

Likewise, it seems logical to assume that the levels of certain proteins and virulence factors that are important for establishing an infection increase when *B. burgdorferi* colonizes the skin and underlying tissue. As *B. burgdorferi* disseminates to various sites in the body, other cell types and conditions are encountered, which could trigger

additional changes in gene expression that are essential for maintaining an infection. Identifying the genes that are involved in establishing and maintaining a chronic infection is essential for understanding the pathogenesis of Lyme disease. To investigate changes in gene expression that occur as *B. burgdorferi* interacts with various host cells, we used the RNA subtractive hybridization technique developed by Utt and Quinn (1994) and Utt *et al.* (1995). This *in vitro* technique helps to identify genes whose levels rise with exposure of bacteria to human tissue culture cells. We incubated a virulent *B. burgdorferi* strain with human endothelial or neurological cells to increase the expression of genes potentially involved in establishing or maintaining an infection at those sites. Using the subtractive hybridization technique, we identified three genes that were differentially expressed when virulent *B. burgdorferi* cells were exposed to these human cells. One of these genes was *vlsE*, which encodes an outer surface protein that undergoes antigenic variation during animal infections (Zhang *et al.*, 1997). Further analysis of the promoter region of *vlsE* revealed a previously unidentified 51 bp inverted repeat that overlapped the −35 site and could form an extremely stable cruciform structure during plasmid replication. Southern hybridization analysis with the *vlsE* promoter/operator region indicated that this sequence occurs on other *B. burgdorferi* plasmids, suggesting an important role for this DNA element in *B. burgdorferi.*

Results

RNA subtraction and Southern analysis using subtractive probes

Human tissue cultures were infected with *B. burgdorferi* cells and incubated as described. After 24 h, the cultures were examined microscopically to assess any gross changes in the appearance of the tissue culture cells. The morphology of the human cells appeared normal, and they remained organized in an attached monolayer similar to cells in uninfected control flasks, suggesting that the *B. burgdorferi* cells did not dramatically effect the overall condition of the tissue culture cells. The pH of the culture medium was unchanged during the incubation period, and oxygen concentration was held constant. *B. burgdorferi* cells in the culture medium observed by darkfield microscopy were motile (> 90%), and some of the total bacterial cells were tightly associated with the monolayer of human tissue culture cells. The attachment and penetration of tissue culture cell monolayers by spirochaetes has been described (Thomas and Comstock, 1989; Haake and Lovett, 1994), but factors mediating this interaction have not been identified.

To identify the *B. burgdorferi* genes whose expression increased with exposure to tissue culture cells, RNA was extracted from *B. burgdorferi* cells recovered from flasks containing HUV-EC-C cells (some subtractions were performed using HCN-2 cells), converted to cDNA and subtracted using excess cDNA generated from RNA isolated bacterial cells exposed to tissue culture media only (described below). The resulting subtracted cDNA was labelled with digoxigenin and used as probes in Southern hybridizations of chromosomal and plasmid DNA isolated from *B. burgdorferi* strain B31-CH3. When chromosomal DNA digested with *Eco*RI, *Eco*RV, *Hin*cII or *Pst*I was probed with these subtractive products, bands totalling < 26 kb were detected (data not shown). As rRNA is difficult to remove during RNA subtractive hybridizations (Utt and Quinn, 1994; Utt *et al.*, 1995), contaminating rRNA was identified based upon known restriction digest patterns of *B. burgdorferi* DNA (Schwartz *et al.*, 1992). Of the 26 kb of chromosomal DNA identified by this procedure, at least 16 kb did not contain rRNA sequences. Probes derived from infected HUV-EC-C or HCN-2 cultures yielded the same DNA patterns, suggesting that *B. burgdorferi* responded very similarly to these very different cell types. The chromosomal DNA identified by RNA subtractive hybridization was not analysed further.

Probing plasmid DNA was less problematic than probing chromosomal DNA because of the absence of rRNA genes. Plasmid DNA isolated from *B. burgdorferi* strains B31-CH3 and B31-CH100 was probed with the HUV-EC-C subtractive probes (Fig. 1B) using Southern hybridizations. These probes hybridized to two plasmids isolated from virulent strain B31-CH3 (Fig. 1B, lane 1) and one plasmid from avirulent strain B31-CH100 (Fig. 1B, lane 3). Southern blots probed with HCN-2 subtractive probes yielded an identical pattern, suggesting that neurological cells had a similar effect on gene expression in *B. burgdorferi* (data not shown). The relative mobility of the two reactive bands detected from strain B31-CH3 (Fig. 1B, lane 1) suggested that they corresponded to the 28 kb and 54 kb linear plasmids lp28-1 and lp54 respectively (Barbour, 1991; Xu *et al.*, 1996; Fraser *et al.*, 1997). Only one plasmid, lp54, was detected in strain B31-CH100 (Fig. 2B, lane 3), suggesting that lp28-1 was lost during *in vitro* passage of this strain. This was consistent with previous reports describing patterns of plasmid loss and corresponding loss of infectivity in *B. burgdorferi* (Schwan and Burgdorfer, 1987; Schwan *et al.*, 1988; Xu *et al.*, 1996; Purser and Norris, 2000; Labandeira-Rey and Skare, 2001).

When *Eco*RI restriction digests of plasmid DNA from strain B31-CH3 were probed with HUV-EC-C subtractive probes, fragments of 8.2 kb (weak reaction, designated by the asterisk) and 10 kb (strong reaction, designated by the arrow) were detected (Fig. 1B, lane 2). The strength of the hybridization signal from the 10 kb fragment was very

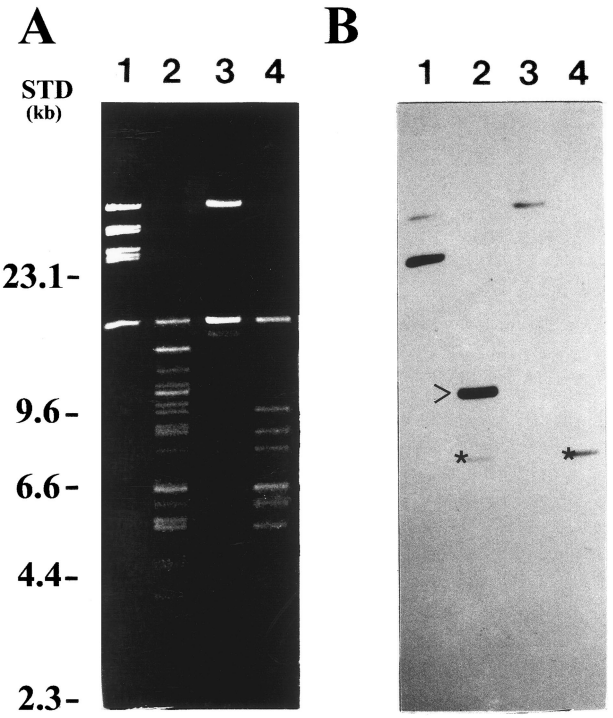

Fig. 1. Southern hybridization analysis of the RNA subtractive products.
A. Plasmid DNA from B31-CH3 (lanes 1 and 2) or B31-CH100 (lanes 3 and 4) were separated by electrophoresis on a 0.4% agarose gel. Samples in lanes 1 and 3 are uncut, whereas those in lanes 2 and 4 were treated with *Eco*RI.
B. Southern hybridization of the gel shown in (A) probed with subtractive probes generated from B31-CH3 cells exposed to HUV-EC-C cells. The arrowhead indicates the position of the 10 kb fragment (from lp28-1), and the asterisks indicate the position of the 8.2 kb fragments (from lp54). DNA size standards (in kb) are shown on the left of (A).

similar to that observed for lp28-1, whereas the weak hybridization signal from the 8.2 kb fragment was comparable with the reaction intensity observed for lp54 (Fig. 1B, lanes 1 and 2). In addition, lp28-1 and the 10 kb fragment were not detectable in strain B31-CH100 plasmid DNA, whereas lp54 and the 8.2 kb fragment were detected (Fig. 1B, lanes 3 and 4). Based upon the relative strengths of the hybridization signals and the fact that lp28-1 was not present in strain B31-CH3 plasmid preparations, it seemed likely that the 8.2 kb fragment was derived from lp54 and the 10 kb fragment originated from lp28-1.

Identification of genes detected using the subtractive probe

Because plasmid loss has been associated with loss of virulence in *B. burgdorferi*, the plasmid-encoded fragments were chosen for further investigation. To determine which genes within the 8.2 kb and 10 kb *Eco*RI restriction fragments were affected by exposing *B. burgdorferi* to tissue culture cells, we performed the following: (i) analysed the restriction patterns of lp54 and lp28-1 based upon their published nucleotide sequence (Fraser *et al*., 1997); (ii) generated polymerase chain reaction (PCR) products from genes within those fragments; and (iii) probed the PCR products using the subtractive probes. The published nucleotide sequence of lp54 indicated that the plasmid contained seven *Eco*RI restriction fragments, two of which (8.84 kb and 8.17 kb) were similar in size to the 8.2 kb fragment detected using the subtractive probes. Analysis of the sequence of the 8.17 kb fragment showed that the fragment contained several previously identified open reading frames (ORFs) including the 3′ end of the gene encoding OspA and the genes encoding OspB and the decorin-binding proteins (DbpA and DbpB). The remainder of the fragment contained additional ORFs with no prescribed function. Three PCR products were generated, one of which contained *ospAB*, a second included *dbpAB* and a third encompassing the remaining ORFs in the 8.17 fragment (*orfs1*). Southern hybridizations indicated that the subtractive probes reacted with the 2.26 kb *ospAB* product (Fig. 2B, lane 3), but not with the 1.82 kb *dpbAB* or 3.77 kb PCR products (Fig. 2B, lanes 2 and 4). These data are consistent with serological data that antibodies directed against *ospA* and *ospB* occur in some late Lyme disease patients, suggesting expression of these genes *in vivo* (Barbour *et al*., 1983; Magnarelli and Anderson, 1988; Wilske *et al*., 1990; Zoller *et al*., 1991).

Zhang *et al*. (1997) reported previously a restriction map of the lp28-1 from *B. burgdorferi* strain B31-5A3, which indicated that *Eco*RI digestion of this plasmid generated three restriction fragments of 14, 10 and 4 kb. They reported that the 10 kb fragment includes the right telomere of the plasmid and the variable membrane

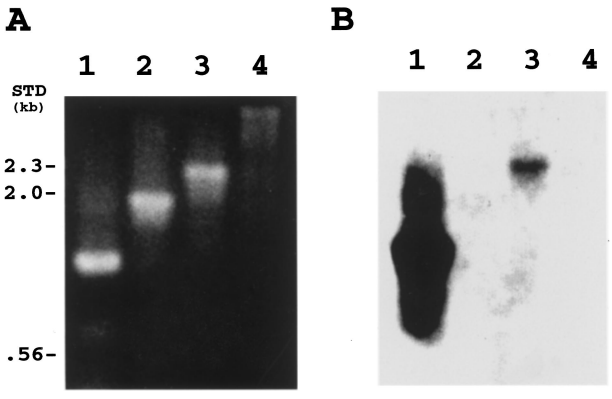

Fig. 2. Southern hybridization analysis of PCR products from lp28-1 and lp54.
A. PCR products from lp28-1 containing *vlsE* (lane 1) and those from lp54 containing *dbpAB* (lane 2), *ospAB* (lane 3) and the *orfs* (lane 4) were separated by electrophoresis on a 1.0% agarose gel. The relative positions of DNA size standards (in kb) are shown on the left.
B. Southern hybridization of the gel shown in (A) probed with the HUV-EC-C subtractive products.

protein-like sequence (*vls*) region containing an expression locus (*vlsE*) and 15 silent *vls* cassettes. *vlsE* encodes a 36 kDa outer surface protein that undergoes antigenic variation as the result of promiscuous recombination between the complete gene and the cassettes (Zhang *et al*., 1997; Zhang and Norris, 1998a,b). As the 10 kb fragment of lp28-1 contains only *vls* sequences, it seemed very likely that the RNA subtractive probes were hybridizing to *vlsE and* the cassettes contained therein. Therefore, a 1.2 kb internal fragment of *vlsE* was generated by PCR using plasmid DNA from *B. burgdorferi* strain B31-CH3 with primers *vlsE*-F and *vlsE*-R (Table 1). Southern hybridization analysis demonstrated that the subtractive probes reacted very strongly to the *vlsE* PCR product (Fig. 2B, lane1), suggesting that *vlsE* was differentially expressed during exposure of *B. burgdorferi* strain B31-CH3 to tissue culture cells.

Increased vlsE *expression*

Preliminary data from the Southern hybridizations using RNA subtractive probes suggested that expression of *vlsE* increased when *B. burgdorferi* strain B31-CH3 was exposed to human endothelial or neurological cells. However, the level of *vlsE* transcript and identification of the component(s) of the tissue culture cells responsible for the observed increase remained to be determined. It was possible that levels of certain constituents of the tissue culture extracellular fluid, either those used or secreted by the eukaryotic cells, or components of the cells (e.g. surface structures, membranes) were responsible for the changes in transcription of *vlsE.* Therefore, quantitative reverse transcription–polymerase chain reaction (RT–PCR) assays were used to detect differences in mRNA levels of *vlsE* after *B. burgdorferi* strain B31-CH3 cells were exposed to tissue culture cells, depleted medium (spent medium) or cytoplasmic membranes isolated from tissue culture cells. Quantitative RT–PCR is a very sensitive assay that has been used as an alternative to Northern hybridizations, *in situ* hybridizations and RNase protection assays to detect changes in the levels of gene expression (Gause and Adamovicz, 1995). As an internal standard for quantitative RT–PCR reactions, the levels of *flaB* mRNA were also measured (Ramamoorthy and Philipp, 1998; Stevenson *et al*., 1998), with the amount of *vlsE* mRNA expressed in arbitrary units relative to *flaB.*

Table 1. Oligonucleotide primers used for PCR, quantitative RT–PCR, sequencing and primer extension.

Primer designation	Primer sequence (5′–3′)
Primer sets for analysis of subtractive products	
Aus1[a]	GATCGGACGGTGATGTCTCGAGAGTG
Aus2[a]	GACACTCTCGAGACATCACCGTCC
orfs-F	ATTAACGAATTTACGCAAAGA
orfs-R	TATTAAACTCTTCGCCCGATA
ospAB-F	CCCACAAGAGTCAACACT
ospAB-R	AGCTGATGCCTTGTAGGG
dbpAB-F	AGTGGCTTGGTTCTAACA
dbpAB-R	AGTGGTGGACTAGCCTTA
vlsE-F	ACATCTTTTGGTGGGTTAGTA
vlsE-R	CTATCTGGTTTCCCCGTCGTA
Primer set for sequencing and Southern hybridizations of the *vlsE* promoter/operator region	
vlsE-F4	TTTGCCTACTTCCGTATCACA
vlsE-R4	GTTTGTTGGGTCGTCCTTATC
Primers sets for quantitative PCR	
vlsE-1012F	CTGAGTCTGCAGTTCGCAAAGTT
vlsE-1104R	TCACTGAATCACCGACTTTCCTTA
vlsE-1048T	FAM[b]-CTAATAGGAGACGCCGTGAGTTC-TAMRA[c]
ospB-1036F	AAAGGTGCTGAGTCAATTGGTTC
ospB-1171R	TATAACCATTAAACAAAGACACTGAGTCTTC
ospB-1111T	FAM-CTAAACAAGACCTTCCTGCGGTGA-TAMRA
flaB-586F	AATCTTTTCTCTGGTGAGGGAGCT
flaB-657R	TCCTTCCTGTTGAACACCCTCT
flaB-611T	TET[d]-AAACTGCTCAGGCTGCACCGGTTC-TAMRA
eno-229F	GCCCCAGAACTTGAAGGTATGA
eno-303R	GGTGCCATCAAGTTCAAGCA
eno-252T	TET-TGCCTTAAATCAGGTTGCAATCGAGAAA-TAMRA

a. Straus and Ausubel (1990).
b. FAM, 6-carboxyflourescein.
c. TAMRA, 6-carboxy-N,N,N′,N′-tetramethylrhodamine.
d. TET, 6-carboxy-,7,2′,7′-tetrachloroflourescein.

Total RNA was isolated from strain B31-CH3 ($6-8 \times 10^7$ cells ml^{-1}) incubated with fresh tissue culture medium, human endothelial cells, cell-free spent medium or tissue culture cell cytoplasmic membranes as described below. The amount of RNA in each sample was measured spectrophotometrically, and 0.1 μg of RNA was used in each RT–PCR. Primers and probes specific for *vlsE* (*vlsE*-1012F, *vlsE*-1104R and *vlsE*-1048T), *ospB* (*ospB*-1036F, *ospB*1171R and *ospB*-1111T) and the internal control *flaB* (*flaB*-586F, *flaB*-657R and *flaB*-611T) (Table 1) were used to amplify and measure relative levels of *vlsE and flaB* mRNAs. As a negative control, samples without reverse transcriptase were subjected to RT–PCR to confirm the absence of contaminating DNA (data not shown). The results of these experiments are shown in Fig. 3. The relative levels *vlsE* mRNA increased 1.5-fold when cells were exposed to HUV-EC-C tissue culture cells (Fig. 3, designated HUV) compared with cells incubated in tissue culture alone (Fig. 3, designated F12K). Levels increased fourfold when cells were exposed to purified HUV-EC-C cell membranes (Fig. 3, designated membranes) compared with spent F12K media (Fig. 3, designated spent media). Very similar results were obtained when primers for the gene encoding enolase (*eno*) (Table 1) were used as an internal control (data not shown). These data suggest that cell membranes from human endothelial cells were contributing to increased levels of *vlsE* in *B. burgdorferi* cells. Specific components of the membranes that were responsible for the effect on *vlsE* expression have not been determined. Under the conditions used in these experiments, no increase in *ospAB* mRNA was observed (data not shown) when strain B31 cells were exposed to HUV-EC-C cells or membranes. This result was surprising, as *ospAB* had been detected by the subtractive probes. It is possible that there were significant levels of *ospAB* mRNA in the cells, and the cycles of subtraction may not have eliminated these subtractive products completely. Certainly this was the case for rRNA, which was not eliminated via subtractive hybridization.

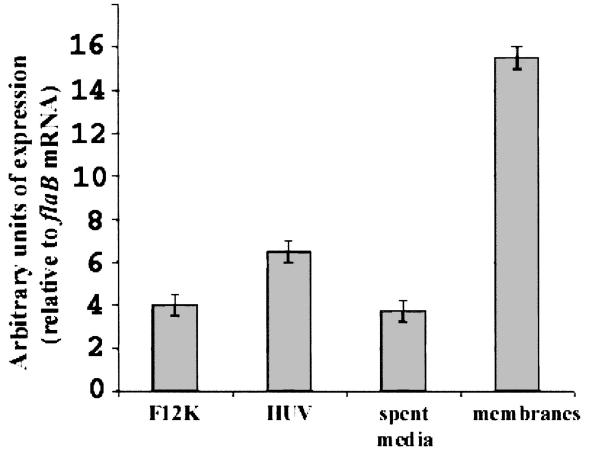

Fig. 3. Quantitative RT–PCR analysis of *vlsE* expression. Template mRNA for the quantitative RT–PCR was isolated from *B. burgdorferi* strain B31-CH3 cells incubated with tissue culture media (F12K), human endothelial cells (HUV-EC-C), spent F12K media (spent media) or purified human endothelial cell membranes (membranes). Expression of *vlsE* is expressed in arbitrary units relative to *flaB* as indicated by the grey bars. Error bars indicate the standard deviation.

Sequence analysis of the vlsE *promoter region*

To understand the regulation of *vlsE*, it was necessary to determine the sequence of the ≈ 160 bp between *vlsE and* the silent cassette *vlsE2-16.*

Previous attempts by Casjen *et al*. (2000) and Zhang *et al*. (1997) to sequence this region were unsuccessful. Our attempts to clone or sequence a 262 bp PCR product containing this region were also unproductive. After numerous attempts, the sequence of this region was finally determined using specific ^{32}P end-labelled primers in a DNA cycle sequencing system at high annealing temperatures (Fig. 4). Analysis of this sequence revealed that it contained a 51 bp inverted repeat that could form a very stable cruciform structure with a ΔG° of -46.2 kcal mol^{-1} at 37°C and a melting point of 83.2°C (Fig. 4) (Zuker, 1996–2000). Clearly, such a stable structure upstream of *vlsE* would make cloning and sequencing of this region very difficult. The *B. burgdorferi* genome database contains no complete matches to this sequence, indicating that this sequence had been missed during the sequencing of the *B. burgdorferi* genome and plasmids.

Chromosomal and plasmid DNA were probed under high stringency with the 262 bp PCR containing the inverted repeat (Fig. 5C). Surprisingly, several *Eco*RI fragments were detected, suggesting that multiple copies of this fragment were harboured in *B. burgdorferi* strain B31-CH3 (Fig. 5C, lane 2). The hybridizations against *B. burgdorferi* strain B31-5A3 also detected multiple copies of this fragment (Fig. 5C, lane 4). However, variations in the hybridization patterns detected subtle differences between these low-passage, virulent strains. Interestingly, the probe detected few *Eco*RI fragments in high-passage strain B31-CH100 (Fig. 5C, lane 6). As this high-passage strain B31 only contains plasmids lp54, cp26 and lp17 (confirmed by PCR; data not shown), it seemed likely that this fragment localized mainly to plasmid DNA.

Primer extension analysis of RNA isolated from strain B31-CH3 identified the 5′ end of the *vlsE* mRNA (Figs 4 and 6, denoted by the +1). Based upon these data, a putative promoter was identified (Figs 4 and 6, denoted by −10 and −35), which corresponded to the promoter proposed by Zhang *et al*. (1997). The promoter sequence was similar to σ^{70}-dependent promoters identified in

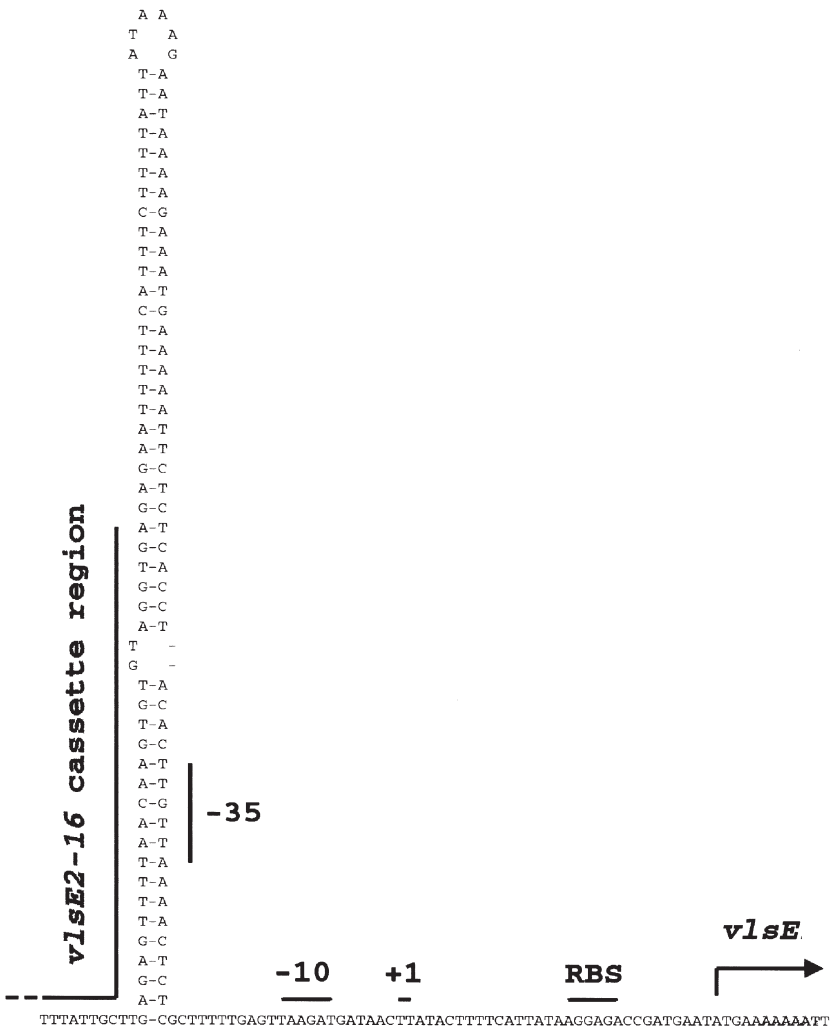

Fig. 4. Analysis of the region of DNA between *vlsE* and the silent upstream cassettes. Putative stem–loop structure predicted by the MFOLD DNA folding program (Washington University School of Medicine, St Louis, MO, USA). The region showing the 3′ end of the *vls* cassettes (*vlsE2*-16), putative promoter (−35 and −10), transcriptional start site (+1), ribosome binding site (RBS) and the translational start site of *vlsE* on lp28-1 are labelled with solid lines.

Escherichia coli. Interestingly, the −35 region of the promoter lies within the inverted repeat (Fig. 4). It is not known whether the inverted repeat influences the expression of *vlsE*, but it seems likely that the region between *vlsE and vlsE2-16* may contain sequences (i.e. enhancer sequences) that contributed to the increased expression observed by quantitative RT–PCR. However, no such sequences have been identified experimentally, and the role this region plays in the expression of *vlsE* remains to be determined.

Discussion

As *B. burgdorferi* is shuttled from a reservoir animal to a human host by *Ixodes* ticks, it encounters different environmental conditions and must alter gene expression to adapt, survive and cause disease. Preliminary data from several laboratories suggest that *B. burgdorferi* alters gene expression in response to increases in temperature (Schwan *et al*., 1995; Stevenson *et al*., 1995), oxidative stress (O^{2-} and peroxide) (Whitehouse *et al*., 1997; Smith and Austin, 1998) and pH (Carrol *et al*., 1999; Yang *et al*., 2000). Characterizing the regulatory mechanisms responsible for differential expression, as well as identifying the genes they regulate, will lead to greater understanding of the pathogenesis of Lyme disease. To understand these processes in *B. burgdorferi*, we have used an mRNA subtractive hybridization technique to detect differences in gene expression in the virulent *B. burgdorferi* strain B31 upon incubation with human tissue culture cells. This technique has been used to detect differences in gene expression in virulent strains of *Mycobacterium bovis* (Mahairas *et al*., 1996), *Listeria monocytogenes* (Utt and Quinn, 1994) *and Haemophilus influenzae* biogroup *aegyptius* (Utt *et al*., 1995).

Using the RNA subtractive technique, we identified an 8.2 kb *Eco*RI fragment derived from the lp54 and a 10 kb *Eco*RI fragment that originated from lp28-1. Zhang *et al*. (1997) have shown previously that the 10 kb *Eco*RI fragment of lp28-1 contains a gene locus that encodes a

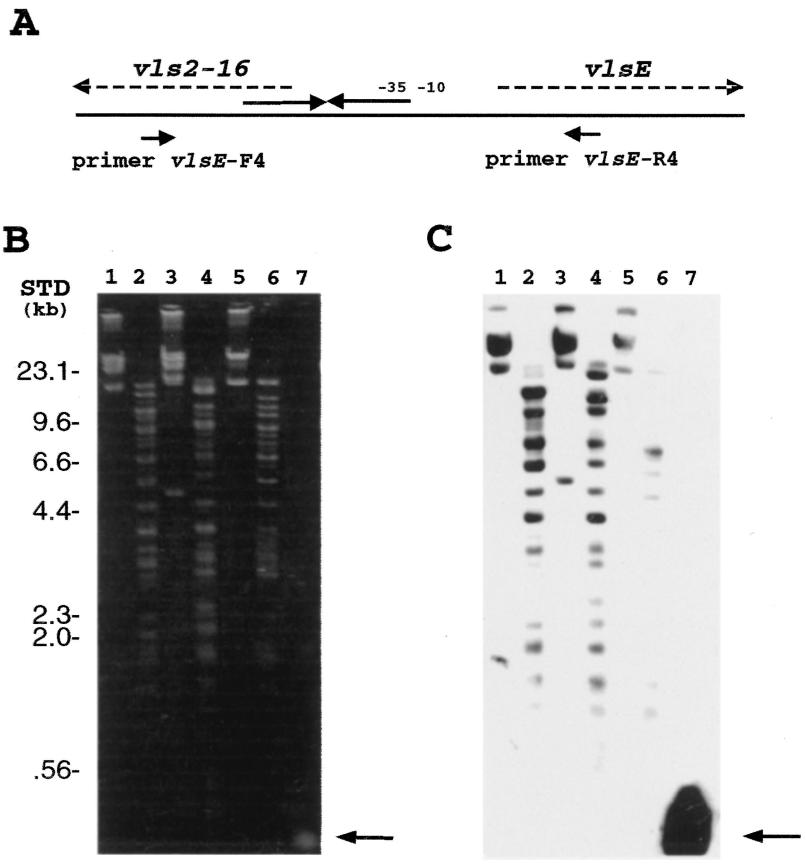

Fig. 5. A. Schematic diagram of the *vlsE* promoter/operator region including the positions of primers *vlsE*-F4 and *vlsE*-R4.
B. Agarose gel electrophoresis (0.5%) of undigested chromosomal and plasmid DNA from low-passage, virulent *B. burgdorferi* stains B31-CH3 and B31–5A3 (lanes 1 and 3 respectively) and high-passage, avirulent B31-CH100 (lane 5). Corresponding DNA samples treated with *Eco*RI are shown in lanes 2, 4 and 6. A 262 bp DNA fragment including the *vlsE* promoter/operator region generated by PCR is shown in lane 6 (denoted by the arrow). The relative positions of DNA size standards (in kb) are shown on the left.
C. Southern hybridization of the gel shown in (A) probed with labelled PCR product generated using primers *vlsE*-F4 and *vlsE*-R4.

36 kDa outer membrane protein designated the variable membrane protein-like sequence (*vls*) on account of similarities to the variable membrane protein (VMP) of *Borrelia hermsii* (Barbour *et al*., 1982; 1991; Kitten and Barbour, 1990; Barbour, 1991; 1993). This region includes an expression locus (*vlsE*) and 15 silent *vlsE* cassettes. Promiscuous recombination between the cassette regions and the *vlsE* locus in *B. burgdorferi* strain B31-5A3 during mouse infections demonstrated that *vlsE* undergoes antigenic variation to escape the host immune system. In addition, convalescent human sera of a Lyme disease patient was shown to react strongly with *vlsE*, suggesting that the gene is expressed during human infections (Zhang *et al*., 1997; Zhang and Norris, 1998b). The DNA sequence of the allele isolated from *B. burgdorferi* strain B31-CH3 compared with that from strain B31-5A3 suggested that recombination events had occurred in the strain B31-CH3 allele. However, the DNA sequences of PCR products generated using *vlsE*-specific primers and DNA isolated from *B. burgdorferi* strain B31-CH3 before and after exposure to tissue culture cells were identical. Taken together, these data suggest that *vlsE* expression increased when *B. burgdorferi* cells were incubated with human endothelial or neurological cells, but that eukaryotic cells alone do not affect recombination or antigenic variation.

Quantitative RT–PCR indicated that the expression of *vlsE* increased 1.5-fold in response to incubation of *B. burgdorferi* with human tissue culture cells, whereas sixfold increases in *ospAB* mRNA were dependent on cell density not cell–cell interactions. These data also indicated that *vlsE* was expressed constitutively in low-passage virulent strain B31-CH3. This low level of expression may be required to promote the initial interaction of *B. burgdorferi* with human tissue culture cells. RT–PCR using RNA isolated from *B. burgdorferi* incubated with tissue culture cell membranes or spent medium indicated that *vlsE* expression increased in response to cell membranes. This supports the hypothesis that *vlsE* is required for interaction with the host cell surface (i.e. attachment, nutrient acquisition, etc.). The specific components of the tissue cell membranes (e.g. carbohydrate, protein or lipid) responsible for the increased levels of *vlsE* transcript have not been determined.

The nucleotide sequence of the putative regulatory region between *vlsE* and the upstream cassettes revealed a unique inverted repeat. Analysis of potential secondary structure in this region indicated that it could form a very stable cruciform structure. Such stable cruciform structures are generally associated with eukaryotic or viral (both prokaryotic and eukaryotic) genomes (Shlyakhtenko *et al*., 1998). These structures are poorly maintained in

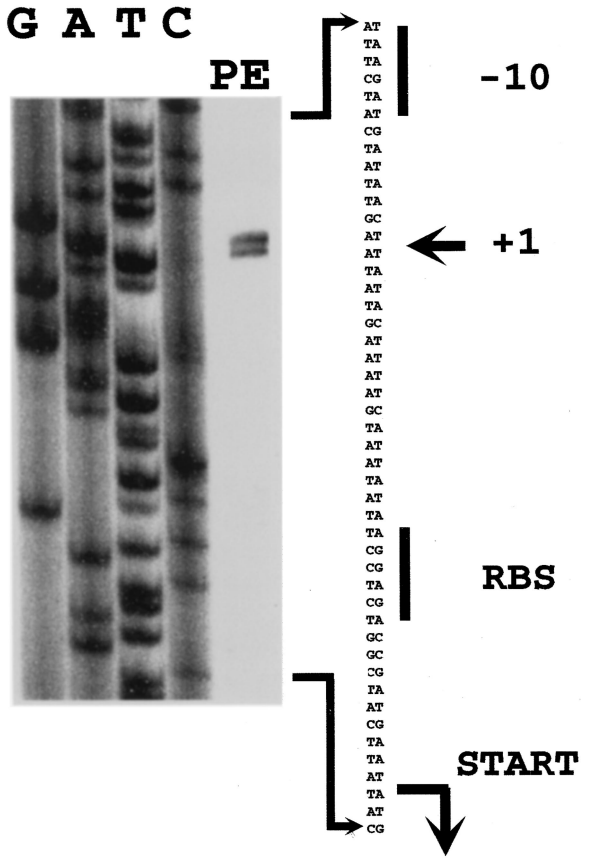

Fig. 6. Primer extension and sequence analysis of the region 5′ of *vlsE*. Sequencing gel showing the primer extension product (lane designated PE) and sequencing reactions (lanes designated G, A, T and C). The putative −10, 5′ end of *vlsE* mRNA (designated +1), ribosome binding site (RBS) and translation start site for *vlsE* are indicated on the sequence.

E. coli plasmids, and the difficulties experienced in cloning and sequencing of this region from lp28-1 explain why it is not represented in the published genome or plasmid sequences (Fraser *et al*., 1997; Casjens *et al*., 2000). In fact, Casjens *et al*. (2000) and Zhang *et al*. (1997) reported that several sequences were missing from regions near the telomeres of lp17 (right and left ends), lp28-1, lp56 and both ends of the chromosome. Southern hybridizations using the *vlsE* promoter region as a probe detected 8–10 low-passage associated fragments that contained sequences with homology to this region. Thus, the inverted repeat sequence reported here may represent part of the estimated 2000 bp of unsequenced DNA from the linear plasmids and close gaps in the published sequence (Casjens *et al*., 2000).

Possible functions for these inverted repeats remain unknown. Similar structures have been implicated in plasmid replication and segregation, recombination or regulation (Glucksmann *et al*., 1992; Kornberg and Baker, 1992). This region has limited homology with single-strand origins of replication found on broad-host-range rolling-circle plasmids (Kramer *et al*., 1999). However, preliminary analyses of *B. burgdorferi* linear plasmids using GC skew suggest that their origins localize near the centre rather than the telomeres of these replicons (Casjens *et al*., 2000; Picardeau *et al*., 2000). It seems more likely that these sequences provide regions of homology for recombination between various linear plasmids (Casjens *et al*., 2000). This role for the *vlsE* inverted repeat is supported by the reported sequence redundancy among plasmids (Casjens *et al*., 2000), indicating that recombination had occurred between various replicons during evolution. Finally, the increased levels of *vlsE* transcript detected in the presence of tissue culture cells or membranes also suggests a possible regulatory role for the promoter region of *vlsE*. As yet, the function of this region in *B. burgdorferi* or its potential role in the pathogenesis of Lyme disease remains to be determined.

Experimental procedures

Bacterial strains, tissue cells and growth conditions

Virulent, low-passage *B. burgdorferi* strain B31-CH3 (passage 3, this laboratory), strain B31-5A3 (passage 3, provided by S. J. Norris, University of Texas at Houston, Health Sciences Center, Houston, TX, USA) and avirulent high-passage strain B31-CH100 were grown in BSKII medium (Barbour, 1984) at 37°C under an atmosphere of 5% O_2−5% CO_2−90% N_2. The cells were examined and enumerated using darkfield microscopy until the desired cell density was obtained. *E. coli* strain XL1-Blue (Stratagene) was grown in Luria broth (LB) or LB supplemented with 100 μg ml^{-1} ampicillin (Am) (Sambrook *et al*., 1989). Human umbilical cord endothelial cells (HUV-EC-C) and human cerebral cortical neuron cells (HCN-2) were obtained from American Type Culture Collection (ATCC), Rockville, MD, USA. HUV-EC-C cells were grown in Ham Kaighn's modification mixture (F12K; Sigma) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 100 μg ml^{-1} heparin (Sigma) and 40 μg ml^{-1} endothelial cell growth supplement (ECGS; Sigma). HCN-2 cells were grown in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals). HUV-EC-C or HCN-2 cells were grown for 7–10 days in 15 ml of the appropriate medium in T-75 cm^2 tissue culture flasks (Corning) at 37°C under a 5% CO_2–air atmosphere until a monolayer of cells (2×10^6 cells ml^{-1}) was observed using a Diaphot-TMD inverted microscopy (Nikon). Bacterial cells were grown in BSKII medium as described above, harvested by centrifugation (8000 *g* for 15 min at 26°C), resuspended in 15 ml of the tissue culture medium (DME or modified F12K) and transferred to a tissue culture flask. The cells were incubated for 48 h at 37°C in a CO_2 incubator (Fischer Scientific) and enumerated as described above.

Isolation of RNA

Virulent *B. burgdorferi* strain B31-CH3 was grown to a density

of $6-8 \times 10^7$ cells ml^{-1} in 125 ml of BSKII medium. Cells were collected by centrifugation (8000 *g* for 15 min at 26°C) and suspended in 125 ml of F12K (HUV-EC-C) or DME (HCN-2). Spent medium was removed aseptically from four tissue culture flasks containing either HUV-EC-C or HCN-2 cells, and 15 ml of the *B. burgdorferi* cells was added to each flask. Four tissue culture flasks containing tissue culture medium were inoculated with 15 ml of bacterial cells to provide subtractive RNA. All flasks were incubated at 37°C under 5% CO_2 for 24 h, and the pH of the culture was measured using a Corning model 320 pH meter. Isolation and purification of mRNA was performed according to the procedure described by Utt and Quinn (1994). First, unattached bacterial cells from all flasks were harvested by collecting the spent medium. Attached bacterial cells were harvested by successive washing of the tissue culture cells with 25 mM NaCl in 20 mM HEPES, pH 7.4 (HEPES buffer) and pooled. Total bacterial cells were then harvested by centrifugation (8000 *g* for 25 min at 4°C) and suspended in 10 ml of ice-cold phosphate-buffered saline (PBS). An equal volume of Tris-saturated phenol (pH 8.0) was added immediately, the solution was mixed vigorously, and organic and aqueous phases were separated by centrifugation (10 000 *g* for 30 min at 26°C). The aqueous phase was collected, extracted twice with phenol–chloroform–isoamyl alcohol (25:24:1), and nucleic acids were isolated as described by Sambrook *et al*. (1989). DNA was removed by treatment with RNase-free DNase I (Sigma) (Utt and Quinn, 1994), and RNA concentration was determined as described by Sambrook *et al*. (1989). Eukaryotic mRNA was selectively removed using the PolyATtract system (Promega).

Subtractive hybridization

Subtractive hybridization was performed according to the method of Utt *et al*. (1995) with the following modifications. Total RNA from infected and uninfected flasks was used as templates to synthesize cDNA using the random-primed Riboclone cDNA synthesis system (Promega) according to the manufacturer's instructions. The resulting cDNA was purified using agarose gel electrophoresis, completely digested with *Sau*3A (Promega), and the resulting fragments were ligated to AUS1 and AUS2 primers/linkers (Table 1), which were prepared as described by Straus and Ausubel (1990). Recombinant DNA fragments were then separated from excess primers/linkers by agarose gel electrophoresis and isolated from the gel using the Qiagen gel extraction kit. To obtain sufficient DNA for the subtraction, partially purified recombinant fragments were amplified by PCR using the AUS2 primer (Table 1) as described below. After PCR, cDNA (generated from flasks containing only bacterial cells) was labelled with biotin using the BioNick labelling system (Gibco BRL) according to the manufacturer's instructions. Amplified cDNAs from bacterial cells (BC) and bacterial cells incubated with tissue culture cells (BCTC) were combined at a 1:10 ratio (BCTC:BC) in 100 μl of 0.6 M NaCl, 2 mM EDTA, 0.2% SDS in 20 mM Tris, pH 7.5 (hybridization buffer), denatured (95°C for 10 min) and hybridized at 70°C for 48 h. Double-stranded and hybrid biotinylated cDNAs were removed using streptavidin-coated magnetic beads (Promega) according to the manufacturer's instructions. Remaining cDNA, representing unique sequences from the infected flasks, was amplified by PCR using the AUS2 primer and labelled with digoxigenin (DIG)-dUTP (Boehringer Mannheim) for use as subtractive probes.

Incubation of B. burgdorferi *strain B31-CH3 cells with spent medium or HUV-EC-C cell membranes*

Four flasks containing HUV-EC-C cells were grown to monolayers as described above. Fresh F12K (15 ml) was added to each flask, and cultures were incubated for 24 h at 37°C. Spent medium was collected, detached cells were removed by centrifugation (1500 *g* for 15 min at 4°C), and supernatant was filter sterilized by passing the spent medium through a 0.22 μm filter (Millipore). The HUV-EC-C monolayer was detached from the flask by scraping, and cells were harvested by centrifugation (1500 *g* for 15 min at 4°C). Cells were washed twice, suspended in 5 ml of sterile water and lysed by two 30 s pulses at 50% power with a Sonic dismembrator (Fisher Scientific). Membranes were harvested by centrifugation (100 000 *g* for 1.5 h at 4°C), suspended in 15 ml of fresh F12K medium and filter sterilized. *B. burgdorferi* strain B31-CH3 cells were grown in BSKII medium to a cell density of $6-8 \times 10^7$ cells ml^{-1} and collected by centrifugation as described previously. Half the cells were added to the 15 ml of spent medium, and the remaining cells were added to the HUV-EC-C membranes. Cultures were incubated at 37°C for 24 h, harvested by centrifugation (8000 *g* for 10 min at 4°C), washed twice, and RNA was isolated as described above.

DNA manipulations, PCR, electrophoresis and Southern hybridization

Chromosomal and plasmid DNA were isolated from *B. burgdorferi* strains B31-CH3 and B31-CH100 as described previously (Barbour, 1988; Barbour and Garon, 1987). PCR products were generated using the PCR master kit (Boehringer Mannheim) in a PTC-100 thermocycler (MJ Research) (1 min at 95°C, 45 s at 50°C and 2 min at 72°C for 35 cycles) using the primers indicated in Table 1. Products generated by PCR were separated by electrophoresis (100 V for 1 h) on 1.0% agarose gels in 1 mM EDTA, 40 mM Tris-acetate, pH 8.0 (1× TAE), and DNA was visualized using ethidium bromide and a UV Trans-illuminator (Fisher Scientific) (Sambrook *et al*., 1989). For Southern hybridizations, chromosomal and plasmid DNA was separated by electrophoresis (30 V for 16 h at 4°C) in 0.4% horizontal agarose gels in 2× TAE (Barbour, 1988), transferred to nylon membrane, positively charged (Boehringer Mannheim) using a PosiBlot pressure blotter (Stratagene) and probed with the DIG-labelled subtractive probe under high stringency (2% SDS in 0.1× SSC at 65°C) according to the manufacturer's instructions (Boehringer Mannheim). A 262 bp PCR product generated using primers *vlsE*-F4 and *vlsE*-R4 was also DIG labelled and used as a probe in Southern hybridizations to detect DNA sequences between *vls2-16 and vlsE*. Restriction endonucleases were used according to the manufacturer's instructions (Promega) (Sambrook *et al*., 1989).

Quantitative RT–PCR

Amounts of specific mRNA transcripts were determined using the *Taq*Man Gold RT–PCR kit and the ABI Prism 7700 sequence detection system (PE Applied BioSystems) as described previously by Ursla *et al*. (1996). Primers and probes specific for *vlsE* (*vlsE*-1012F, *vlsE*-1104R and *vlsE*-1048T), *ospB* (*ospB*-1036F, *ospB*1171R and *ospB*-1111T) and the internal control, *flaB* (*flaB*-586F, *flaB*-657R and *flab*-611T) were designed using the PRIMER EXPRESS 1.0 program (PE Applied BioSystems) and are listed in Table 1. Primers were added to a final concentration of 900 nM, labelled probes were added to a final concentration of 250 nM, and assays were performed according to the manufacturer's instructions. Duplicate samples were assayed for each experiment, and absolute quantities of each transcript were determined by comparison with a standard curve of known amounts. The amount of each transcript was normalized by comparison with the internal control, *flaB* (Stevenson *et al*., 1995).

Sequence analysis of the region between vlsE *and* vlsE2-16

The region between the N-terminal coding region of *vlsE* and the beginning of the *vls* cassettes was amplified by PCR using primers *vlsE*-F4 and *vlsE*-R4 (Table 1) as described above, except that the annealing temperature was 60°C. The resulting 262 bp PCR product was sequenced with the *fmol* cycle sequencing system (Promega) using ^{32}P end-labelled primers (*vlsE*-F4 and *vlsE*-R4) according to the manufacturer's directions, again with an annealing temperature of 60°C. The sequencing reaction products were separated on a Castaway gel (7% acrylamide, 8 M urea) electrophoresis system (Stratagene) and visualized by autoradiography. The MFOLD computer algorithm (http://mfold2.wustl.edu/~mfold/dna/form1.cgi; Washington University School of Medicine, St Louis, MO, USA) was used to predict possible stem–loop structures in this region. The sequence of the *vlsE* promoter/operator region has been submitted to GenBank (accession number AF314755).

Primer extension analysis

Total RNA from *B. burgdorferi* cells and ^{32}P end-labelled primer *vlsE*-R4 were used with the primer extension system AMV (Promega) to generate a primer extension product. A sequence ladder was also generated with the *fmol* cycle sequencing system (Promega), and these reaction products were separated and visualized as described above.

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