

# An unusual illegitimate recombination occurs in the linear-plasmid-encoded outer-surface protein A gene of *Borrelia afzelii*

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**In this study, we describe an unusual illegitimate recombination in the linear-plasmid-encoded outer-surface protein A gene of *Borrelia afzelii*. A 96 bp DNA segment was deleted from the *ospA* structural gene of *B. afzelii* strain R9. The nature of the rearrangement suggested that it arose by a strand slippage mechanism, which was stimulated by a 18-mer palindromic sequence and 5-mer short direct repeats at both termini of the deleted DNA. The deleted sequence could form a complex hairpin structure suggesting that it may have played important roles in pausing of replication and slippaging of the nascent strand across the replication fork. In addition, the mutant strain was isolated from a chronic Lyme disease patient, implying that the variation mechanism may have been used by the borrelial strain to avoid host immune elimination.**

Keywords: illegitimate recombination, OspA variation, *Borrelia afzelii*, Lyme disease

## INTRODUCTION

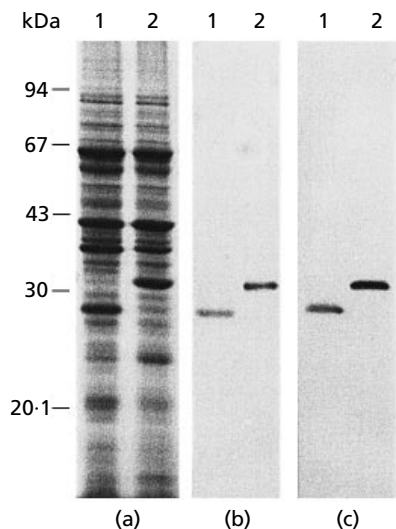
Lyme disease, a tick-borne infection causing a multi-system illness, is caused by *Borrelia burgdorferi* sensu stricto, *Borrelia afzelii* and *Borrelia garinii* (Burgdorfer *et al.*, 1982; Baranton *et al.*, 1992). All *Borrelia* spp. are distinguished by their characteristic morphology and motility, as well as a very high ( $\geq 70$  mol%) A+T content in their DNA (Barbour & Hayes, 1988). A striking peculiarity of this genus is their genome structure, consisting of linear chromosome of approximately 1000 kb and a series of circular and linear plasmids, some of which are thought to carry genes encoding factors that contribute to virulence (Saint Girons *et al.*, 1992; Simpson *et al.*, 1990). The circular and linear plasmids of the Lyme disease agents are suggested to have a common origin (Barbour *et al.*, 1996; Dunn *et al.*, 1994; Zückert & Meyer, 1996), and to replicate by a shared mechanism of rolling circle (RC) replication (Ferdows *et al.*, 1996; Marconi *et al.*, 1996). The RC mode of replication is known to lead to high

frequencies of both homologous recombination between long homologous sequences and illegitimate recombination between sequences of little or no homology (Michel & Ehrlich, 1986; Niaudet *et al.*, 1984).

Genes for the outer-surface proteins OspA and OspB are situated in a single operonic arrangement on the largest of the linear plasmids, which range in size from 48 to 60 kb (Bergström *et al.*, 1989; Jonsson *et al.*, 1992; Saint Girons *et al.*, 1992). The OspA and OspB proteins have been suggested to play important roles in host-parasite interactions during Lyme borreliosis (Coleman *et al.*, 1995; Comstock *et al.*, 1993; Fikrig *et al.*, 1992, 1994; Ma & Weis 1993; Sadziene *et al.*, 1993; Sellati *et al.*, 1996) and antigenic variation of the two Osps might enable the pathogen to survive host immune attacks and establish a chronic infection (Callister *et al.*, 1993; Fikrig *et al.*, 1995; Marconi *et al.*, 1993; Sadziene *et al.*, 1992). The *ospA* and *ospB* genes exhibit a high degree of sequence similarity, and have possibly been duplicated from an ancestral *osp* gene. Our recent study showed that the two genes from a *B. garinii* strain shared a consensus 282 bp sequence in their carboxyl termini (Wang *et al.*, 1997). Homologous recombination between the *ospA* and *ospB* genes (Rosa *et al.*, 1992) and several other variation mechanisms of OspA and OspB, including gene deletion (Marconi *et al.*, 1993), point

**Abbreviations:** Osp, outer-surface protein; RC, rolling circle.

The GenBank/EMBL/DDBJ accession numbers for the *ospA* sequence of *B. afzelii* strains R9 and XJ23 reported in this paper are U78549 and U78301.



**Fig. 1.** SDS-PAGE and Western blotting of *B. afzelii* strains R9 (lane 1) and XJ23 (lane 2). Whole-cell proteins were subjected to electrophoresis on 12.5% (w/v) polyacrylamide gel. The gels were stained with Coomassie brilliant blue (a). Separated proteins were transferred onto PVDF membrane and allowed to react with mAbs P31c (b) and O31a (c). Molecular masses are indicated on the left.

mutations (Eiffert *et al.*, 1992; Rosa *et al.*, 1992) and loss of the 49 kb linear plasmid carrying the *ospA* and *ospB* gene (Hughes *et al.*, 1993), have been explored *in vitro*. These findings suggest the possibility of antigenic variation during human infection. Thus, analysis of isolates from patients with long-term infection would be useful.

In this work, a mutant *B. afzelii* strain, R9, whose OspA was changed from 32 to 28 kDa, was isolated from a patient with chronic meningitis. Sequence analysis revealed that a 96 bp segment was deleted from the *ospA* structural gene, and suggested that the deletion had arisen by an unusual illegitimate recombination event, which was stimulated by an 18 bp palindromic sequence and 5 bp short direct repeats at the termini of the deleted DNA.

## METHODS

**Bacterial strains, plasmid, and cultivation conditions.** Lyme borreliac strains used were R9 and XJ23. Strain R9 was isolated from blood of a patient with chronic meningitis (at least 4.5 years) in Mudanjiang, China, and strain XJ23 was isolated from *Ixodes persulcatus* tick in Xinjiang, China. The two strains were identified as *B. afzelii* by RFLP analysis on 5S–23S rRNA intergenic spacer amplicons (Postic *et al.*, 1994) (unpublished data). The borreliac strains were cultivated in BSKII medium at 33 °C as described by Barbour (1984). *Escherichia coli* DH5 $\alpha$ -[*supE44*  $\Delta$ *lacU169* ( $\phi$ 80 *lacZ* $\Delta$ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was grown in L broth at 37 °C. The plasmid vector used was pUC118 (Sambrook *et al.*, 1989). Plasmid-containing *E. coli* DH5 $\alpha$

strains were grown in media supplemented with ampicillin at a final concentration of 100  $\mu$ g ml<sup>-1</sup>.

**SDS-PAGE and Western blotting analysis.** The methods employed in SDS-PAGE and Western blotting analysis were as previously described (Masuzawa *et al.*, 1991). Ten to fifteen micrograms of bacterial protein (dry weight) was subjected to electrophoresis. The gels were stained with Coomassie brilliant blue. Separated proteins were transferred onto PVDF membranes (Millipore) by electroblotting. The mAbs used were OspA-specific mAbs P31c and O31a (Masuzawa *et al.*, 1991).

**DNA methodology.** Preparation of the plasmid enrichment DNA from borreliac strains and plasmid profile analysis were performed as previously described by Barbour (1988) and Li *et al.* (1994). Plasmid DNA from strain R9 was partially digested with *Sau3AI* to generate DNA fragments, mostly in the range 2–8 kb. These DNA fragments were ligated into the *Bam*HI site of pUC118 with a DNA-ligating kit (Takara) and then transformed into competent *E. coli* DH5 $\alpha$  cells using a cold CaCl<sub>2</sub> method (Sambrook *et al.*, 1989). Using colony blotting, described below, we screened the genomic library with mAb P31c and selected one *E. coli* recombinant containing the R9 *ospA* gene. It was found to express a 28 kDa OspA protein identical to the parental strain R9. The DNA fragment cloned in pUC118 was approximately 1.8 kb. To sequence *ospA*, subcloning, based on convenient restriction sites, was conducted. The subclones obtained were used for DNA sequencing. Restriction endonuclease digestions and other common DNA manipulations were performed by standard procedures (Sambrook *et al.*, 1989).

The PCR amplification was performed with a Thermo Processor model TR-100 thermal cycler (TAITEC Co.) programmed as follows: an initial denaturation step at 92 °C for 2 min, followed by 30 cycles consisting of 92 °C for 1 min, 41 °C for 30 s, and 72 °C for 1.5 min, with a final extension step at 72 °C for 10 min to allow all extension products to be completed. A frozen *Borrelia* cell suspension was lysed in a boiling water bath for 10 min and placed immediately on ice, and then centrifuged at 2000 r.p.m. for 1 min. The supernatant was used as PCR template. Primer A8 (5'-CAATTTTC-TATTTGTTATTTGTTAATC-3') designed based on the *ospA* sequence of R9) annealed with 59 bp upstream from the beginning of the R9 *ospA* ORF and primer B9 annealed to the 3' end of *ospB* (Kawabata *et al.*, 1994) were used to amplify the strain XJ23 *ospA* gene.

**Screening of *E. coli* recombinants by immunoblotting.** *E. coli* recombinants were transferred from a master plate onto nitrocellulose filters and allowed to grow on the filters at 37 °C for 15–18 h. The recombinant colonies were lysed in a solution of 0.5 M HCl for 30 min and then washed with distilled water. Immunoreactivity was then examined by blotting with mAb P31c.

**DNA sequencing.** Recombinant plasmids carrying the desired inserts and freshly generated PCR products were used for DNA sequencing. The PCR products were purified with the Wizard system (Promega) and directly sequenced by the dideoxy chain-termination method with an Applied Biosystems automated sequencer (model 373A) according to the manufacturer's protocol. Primers used in sequence analysis were as follows: M13 forward primer (5'-TGTAACGACGG-CCAGT-3') and M13 reverse primer (5'-CAGGAAACAG-CTATGAC-3') purchased from Takara, and the *ospA*-specific primers AR1 (5'-AGCTTTTCCGGTTCATCGC-3', nt 486–467), AF1 (5'-GAGAAAATGGAACCAACTTG-3', nt

416–436), AR2 (5'-ATTTTCGACTGCTGTGCCTTC-3', nt 766–785) and A8. The nt positions refer to the strain XJ23 *ospA* sequence.

## RESULTS AND DISCUSSION

### OspA variation during human infection

*B. afzelii* strains R9 and XJ23 were subjected to SDS-PAGE and Western blotting analysis (Fig. 1). Strain XJ23 had a 32 kDa OspA as *B. afzelii* usually expresses, but strain R9 expressed a 28 kDa protein instead. The 28 kDa protein was determined as OspA by OspA-specific mAb P31c and O31a. The OspA protein had been shown to be homogeneous including the molecular mass and mAb reactivities among *B. afzelii* strains isolated from various sources around the world (Masuzawa *et al.*, 1996; Will *et al.*, 1995). The different OspA expression of strain R9 indicated a protein variation from 32 to 28 kDa. Although strain R9 had a different OspA expression, it also had a plasmid content identical to that of strain XJ23 and several other strains isolated from various geographical and biological sources in China, e.g. M7 and H20 from *I. persulcatus* in Mudanjiang, L8 from *I. persulcatus* in Liaoning, SR1 from *Rattus coxingi* in Sichuan, and FP1 from patient in Sichuan (Li *et al.*, 1994). Plasmid analysis is one of the most sensitive tools for distinguishing borrelial strains (Barbour, 1988). The identical plasmid content indicated a very close relationship between the strains. The XJ23 type strain might be the prototype form of strain R9, or R9 might have been derived from the XJ23 type strain by OspA variation. Strain R9 was isolated from a chronic Lyme disease patient but not found in the environment, which may indicate that the OspA variation occurred during human infection.

### DNA deletion within the *ospA* structural gene

To confirm the OspA variation, we cloned and sequenced the *ospA* gene of strain R9 and compared it to the *ospA* sequence of strain XJ23 and other published data. The XJ23 *ospA* gene was obtained by PCR amplification with primers A8 and B9, and the PCR product was directly sequenced with primers A8, AR1, AF1 and AR2. The sequence data revealed that the R9 *ospA* gene has an ORF of 726 nt encoding a putative lipoprotein of 241 aa with a calculated molecular mass of 26319 Da, and the *ospA* gene of strain XJ23 has an ORF with 822 nt encoding a putative lipoprotein of 273 aa with a calculated molecular mass of 29629 Da. The XJ23 *ospA* sequence was almost identical to all published *B. afzelii* *ospA* gene sequences except for several base changes, e.g. it had one base change at nt 687 from C to T in comparison with that of strain ACA1 (Jonsson *et al.*, 1992). Comparing the two *ospA* genes in Fig. 2, a DNA deletion from the R9 *ospA* structural gene was found. The deleted sequence was 96 bp long from nt 142 to 237, and the upstream and downstream sequences from the deleted DNA were almost identical to the XJ23 *ospA* gene except for four base changes, from T to C at

nt 441, A to G at nt 543, G to C at nt 574, and A to G at nt 691. With the deletion, 32 aa were lost and the deduced molecular mass of OspA protein changed from 29629 Da to 26319 Da.

### Molecular mechanism of the postulated DNA rearrangement

To clarify the mechanism leading to the R9 *ospA* recombination, attention was focused on the nature of the deleted DNA and the sequences flanking the recombination sites. The deletion occurred at positions nt 141/142 and nt 237/238. Beginning at position nt 136 were a pair of 6-mer direct repeats (AAAGAC) in tandem, and following and overlapping it by four bases (AGAC), was an 18-mer inverted and complementary repeat (AGACGGTAAGTACAGTCT) at nt 144–161. Furthermore, a 5-mer short direct repeat (AAAGA) was found at both termini of the deleted DNA (nt 142–146 and nt 238–242). These findings suggested a possibility of an illegitimate recombination event.

Illegitimate recombination has two different mechanisms: (i) end-joining, mediated by enzymes which cut and join DNA, such as topoisomerases, site-specific DNases and proteins which initiate RC replication, and involve sequences which include or resemble those on which such enzymes normally act; (ii) strand slippage, where, after pausing at the replication fork, the nascent strand is able to dissociate from one template and pair with another (Ehrlich *et al.*, 1993; Pinder *et al.*, 1997). About half of the reported illegitimate recombination events involve short homologous sequences, its key feature being slippage of the tip of a growing DNA chain from one repeat to the other (Ehrlich *et al.*, 1993). No special motif recognized by specific cut-and-join enzymes could be identified in the sequence, indicating that the deletion event may have arisen by a strand slippage mechanism. Using a computer program (Genetyx-MAC/ATSQ Automatic connection of sequences, version 8.0; Software Developments), we analysed the structure of the deleted DNA by searching for hairpins, palindromes and secondary structures, and propose a model to explain the rearrangement in the *ospA* gene (Fig. 3). Sequences which have the potential to form unusual secondary structures (e.g. palindromes, which can form hairpins when single-stranded, or cruciforms when double-stranded) are known to promote pausing of DNA replication and stimulate replication slippage (Michel & Ehrlich, 1986; Ehrlich *et al.*, 1993). Long DNA palindromes are unstable in both eukaryotes (Collick *et al.*, 1996) and prokaryotes (Peeters *et al.*, 1988; Weston-Hafer & Berg, 1989, 1991) and have been shown to halt the progress of the replication fork *in vitro* (LaDuca *et al.*, 1983; Bedinger *et al.*, 1989).

As shown in Fig. 3, we suggest that the first step is the rare extrusion of the inverted sequences to form an unstable cruciform by intrastrand base pairing, which causes the DNA polymerase to pause. Many studies of



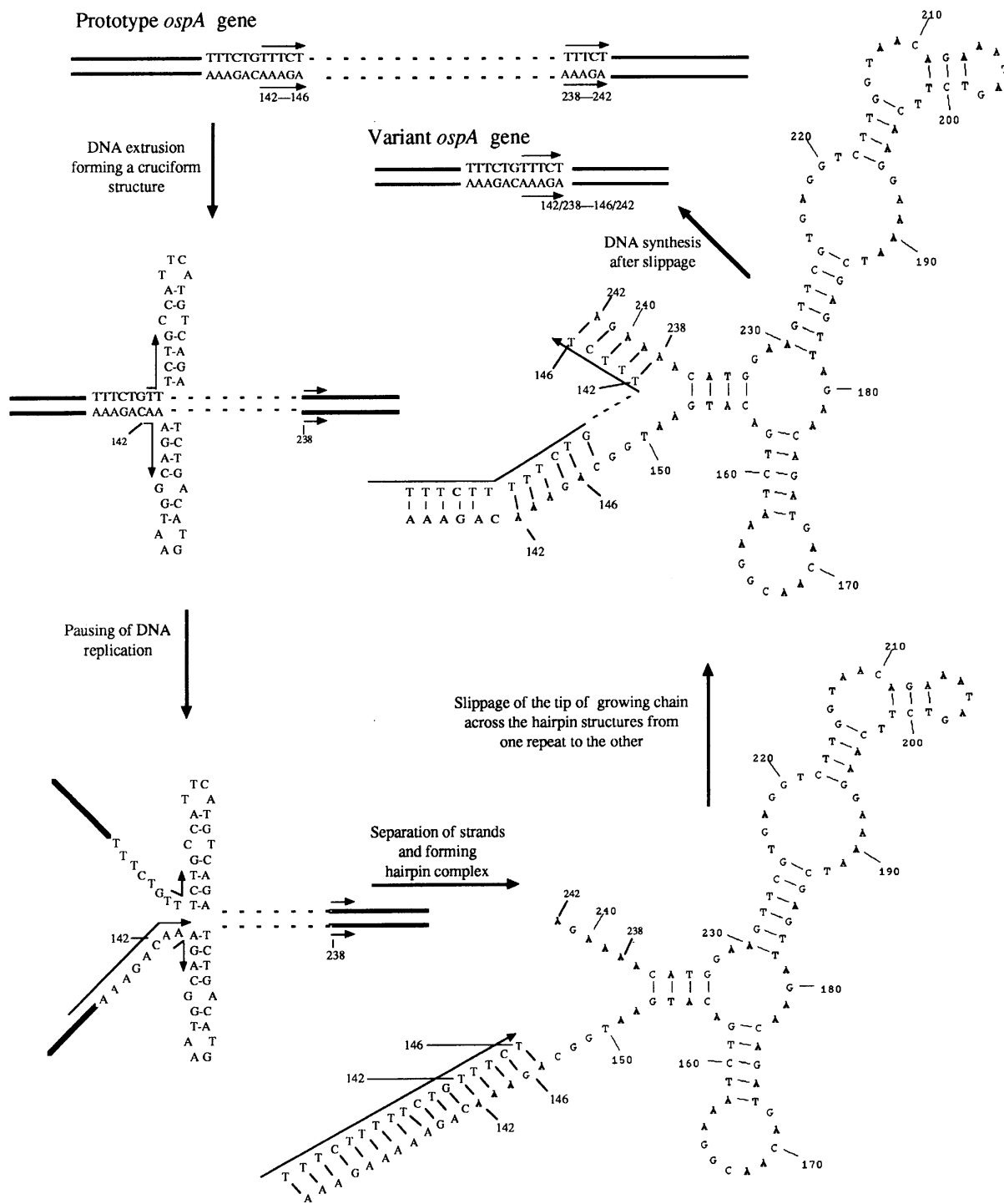
**Fig. 2.** Comparison of the *ospA* genes of *B. afzelii* strains R9 and XJ23. A 96 bp-long DNA deletion from the R9 *ospA* gene was found at nt 142–237; the upstream and downstream sequences flanking the deleted DNA were almost identical to that of XJ23 except four bases changed from T to C at nt 441, A to G at nt 543, G to C at nt 574, and A to G at nt 691. The pair of 6-mer direct repeats (AAAGAC) in tandem at nt 136–147 is underlined, and overlapping it by four bases an 18-mer inverted and complementary repeat is indicated by broken arrows at nt 144–161. In addition, a 5-mer aaaga indicated by small characters was found at the beginning of the deleted DNA (nt 142–146) and just following the deleted DNA (nt 238–242).

deletions stimulated by palindromic sequences have shown that deletion end points tend to occur in short direct repeats, and that DNA rearrangement by strand slippage leads to deletion of one of the repeats and the palindromic sequences between the repeats (Ehrlich *et al.*, 1993; Pinder *et al.*, 1997). The recombination event presented did not seem to follow this rule. Although there were imperfect short direct repeats (AAAGC at nt 142–146, and AAAGG at nt 162–166) flanking the 18 bp palindromic structure, the recombination did not occur between them. Instead of that, it occurred with another direct repeat (AAAGC at nt 238–242) 76 bp downstream from the 18 bp palindrome. The unusual recombination could be explained by strand separation and formation of a hairpin complex. The low G + C content of *Borrelia* genomic DNA might help the formation of the unusual secondary structure. The hairpin loop was considered to form after cruciform formation because this might cause pausing of DNA replication. However, if the lagging-strand synthesis in RC replication is slow and/or initiated relatively infrequently along the newly synthesized rolling strand, the hairpin loop formation could occur without prior cruciform formation. The formation of the hairpin complex may have brought the short direct repeats closer together and facilitated replication across the fork from the leading to the lagging strand by base-pairing between the two short direct repeats. Three direct repeats (AAAGAA/AAAGAC/AAAGAC nt 130–146) in tandem at the upstream site of the deletion

appeared to play a key role in the nascent strand slippage across the replication fork. After the strand slippage, further DNA synthesis led to loss of the hairpin complex and one of the short direct repeats.

### OspA variation and pathogenicity

It is not clear how important this type of strand slippage event has been in genome evolution. Monoclonal or polyclonal antibodies to OspA or OspB have been shown to have roles in the selection of variant spirochaetes *in vitro* (Sadziene *et al.*, 1992) and *in vivo* (Fikrig *et al.*, 1995). Although without direct evidence, strain R9 had been immediately analysed by SDS-PAGE and found to express a variant OspA when it was isolated from the patient (Li *et al.*, 1994), and this kind of mutant was never isolated from the environment, whereas *Borrelia* strains with a complete *ospA* gene were generally isolated from various sources (Will *et al.*, 1995), suggesting that the OspA variation of strain R9 may have occurred during human infection and the mutant strain may have been selected by human immune pressure. All *ospA* genes of the three Lyme disease *Borrelia* species (*B. burgdorferi* s. s., *B. afzelii* and *B. garinii*) were shown to have sequence conservation within the N terminus (Will *et al.*, 1995). This implied that the slippage model could in theory be used by Lyme borreliac strains. However, the findings that strain FP1,



**Fig. 3.** Model of the DNA rearrangement by strand slippage mechanism. The deleted DNA is indicated by a broken line and the sequences flanking the deleted DNA are indicated by a thick line. The short direct repeats at both termini of the deletion are shown and indicated by short arrows, and the growing chain is represented by a long thin arrow. See text for further details.

which had similar features to strain XJ23 (Li *et al.*, 1994), and other strains (Will *et al.*, 1995) isolated from Lyme disease patients, had no *OspA* or *OspB* changes, and that *Borrelia* *OspA* was stable in the re-isolated

strains from chronically infected immunocompetent mice (Persing *et al.*, 1994), indicate that the rearrangement in the R9 *ospA* gene was not a universal avoidance mechanism.

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