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 multisystem 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 (≥70 mol %) A+T content in their DNA (*Barbour & Hayes, 1988*). A striking peculiarity of this genus is their genome structure, consisting of linear chromosomes 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...
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 (b). 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 borreliosis 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 SS–23S rRNA intergenic spacer amplicons (Postic et al., 1994) (unpublished data). The borrelial strains used were R9 and XJ23. 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 BamHI site of pUC118 with a DNA-ligating kit (Takara) and then transformed into competent *E. coli* DH5α cells using a cold CaCl₂ 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 18 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′-CAATTTTCTATTTGGTATTGTGTATTA-3′) designed based on the *ospA* sequence of R9) annealed to the 5′ end 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 dye-deoxy 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′-TGTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-CAGGAAACAGCTATGAC-3′) purchased from Takara, and the *ospA* specific primers AR1 (5′-AGCTTTTCCCGGTTATTAATC-3′), nt 486–467, AF1 (5′-GTAAAACGACGGCCAGT-3′), nt 486–467, and O31a (5′-TGTAAAACGACGGCCAGT-3′), nt 486–467.

strains were grown in media supplemented with ampicillin at a final concentration of 100 µg ml⁻¹.

**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 electrobias. The mAbs used were OspA-specific mAbs P31c and O31a (Masuzawa et al., 1991).

**DNA methodology.** Preparation of the plasmid enrichment DNA from borrelial 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 BamHI site of pUC118 with a DNA-ligating kit (Takara) and then transformed into competent *E. coli* DH5α cells using a cold CaCl₂ 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 18 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).

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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 product was directly sequenced with primers A8, AR1, AR1, 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 there 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 (AGACGGTAAGTACGTCT) 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 borrelial strains. However, the findings that strain FP1,
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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