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Molecular Characterization of *Borrelia burgdorferi* Linear Plasmids by DNA Hybridization, PCR, Two-Dimensional Gel Electrophoresis, and Electron Microscopy

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Abstract: *Borrelia burgdorferi* sensu stricto is the predominant cause of Lyme disease. *B. burgdorferi* strains carry several linear and circular plasmids and this may be also used as a distinguishing property of the bacteria. The presence of a mixed population of linear and circular plasmids makes separation and characterization of each kind very difficult. In this study, 7 clinical strains of skin and CSF isolates were determined by clonal selection and plasmid profile of *B. burgdorferi* by electron microscopy and 2-dimensional agarose gel electrophoresis. The results demonstrate that the skin isolate (DK1) has 2 different phenotypes A and B, but the CSF isolate (DK6) has only 1 phenotype. Plasmid profiles from different strains of *B. burgdorferi* showed extreme complexity analyzed by conventional agarose electrophoresis. In conclusion, the DK1 strain has 10 plasmids in comparison to 6 plasmids in the DK6 strain. This could be due to a mixture of 2 phenotypes in the DK1 strain. Most of the plasmids in both strains were linear. This conclusion is supported by DNA hybridization, electron microscopy of plasmids, and amplification experiments with OspA-B specific primers.

Key Words: *Borrelia burgdorferi*, linear plasmid, plasmid profile, 2-dimensional gel electrophoresis, electron microscopy

Introduction

Borrelia burgdorferi is the spirochetal agent that causes Lyme borreliosis. Numerous approaches have been used to demonstrate the genetic and phenotypic diversity among *Borrelia* strains (1). It has been shown that the North American strain *Borrelia* B31 and the 2 Swedish strains G25 and G74 isolated from ticks have 2 morphologically distinct forms (2). *Borrelia burgdorferi* strains carry several linear and circular plasmids and this may be also used as a distinguishing property of the bacteria (3-9). Some strains of *B. burgdorferi* contain more than 10 plasmids, mostly of a linear structure with closed hairpin ends (3,10). *Borrelia* is unique among prokaryotes in having a small linear chromosome of 1 megabase pairs (11). The presence of linear plasmids has been reported in several other bacterial species such as *Yersinia pestis* (12), *Streptomyces* (13), *Nocardia opaca* (14), *Thiobacillus versutus* (15), and *Rhodococcus fascians* (16). Linear plasmids in prokaryotes have been shown to possess important functions (11,17). In *B. hermsii*, the agent of relapsing fever, several linear plasmids contain genes for antigenic variation (18). The genes for the outer surface proteins OspA and OspB in *B. burgdorferi* are carried by a linear plasmid of 49 kb size (19).

The presence of a mixed population of linear and circular plasmids makes separation and characterization of each kind very difficult. Different techniques have been used for the separation of linear and circular plasmids, like ultracentrifugation, gel electrophoresis, and electron microscopy. The electrophoretic mobility of plasmids in the gel is influenced not only by the molecular weight of the plasmid but also by their conformation. One example of this phenomenon is the fast mobility of the supercoiled form relative to the nicked relaxed open circular form of plasmids (20). We therefore decided to study the phenotype of skin and CSF isolates, clonal selection, and plasmid profile of *B. burgdorferi* by electron microscopy and 2-dimensional agarose gel electrophoresis and chose a 25 kb linear plasmid for further investigation.

Materials and Methods

Strains

Seven clinical strains of *B. burgdorferi* were obtained from Statens Seruminstitut, Copenhagen, Denmark (21). Genospecies was determined as described by Marconi et al. (22), and according to the OspA serotype classification determined by Wilske et al. (23) (Table 1). The bacteria were cultured in Barbour Stoenner-Kelly (BSK-II) medium (4) for 3 weeks at 33 °C.

Plasmid extraction

Plasmid DNA extraction was carried out by alkaline lysis as previously described (4,10). DNA concentration was determined spectrophotometrically by measuring the A_{260} (24).

Two-dimensional gel electrophoresis

DNA samples were examined by electrophoresis in 0.3% agarose (Sea Kem, FMC Bio Products, Rochland, ME, USA) gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Gels were run at 50 V for 5 min and then at 14 V for 18-24 h, followed by staining with 0.5-1 g/ml ethidium bromide. After visualization of plasmid bands with a UV transilluminator (TM-20 UV, Inc. Sangarbier, CA, USA) the gel was exposed to the same UV light for 5 or 15 min. The lanes of plasmids were cut from the gel and placed in 90° position from the first dimension in a new gel holder and the second dimension gel (0.3%) was poured. Ethidium bromide to a final concentration of 0.5-1 mg/ml was added to the TAE buffer and the gel was run at 14 V for 18 h. As a characterized control of circular and linear plasmids, supercoiled pUC18 and phage lambda DNA digested with HindIII, respectively, were applied.

Preparation of DNA probe and hybridization

A 32 mer oligo from the published sequence of the OspA gene (GenBank accession number X14407 (25) was designed from base number 139-170

5´-GGAGAATATATTATGAAAAAATATTTATTGGG- 3´.
DNA labeling with DIG and hybridization was carried out according to the manufacturer's instructions (Boehringer Mannheim, Germany).

PCR analysis

In order to obtain PCR primers specific for OspA and OspB, primers from the conserved region of OspA and OspB sequence were designed (25). PR1 5´-GGAGAATATATTATGAAAAAATATTTATTGGA-3´, PR2, 5´-CGTTTTTAATTTCATCAAGT-3´, PR3 5´-GGGGGTTTACTTATATATTATTTTAAAGC-3´. Primers 1 and 2 amplify the OspA gene and primers 1 and 3 amplify the total sequence of OspA-B operon. PCR was performed as previously described (21).

Clonal selection of *B. burgdorferi* in solid medium

Solid medium for growth of borrelia was prepared by adding special agar-Nobel (Difco Laboratories, MI, USA) to 100 ml of BSKII broth medium to a final concentration of 1%. Different dilutions of low passage *B. burgdorferi* DK1 were cultured in BSKII-Agar in 70% N_2 - 20% H_2 - 10% CO_2 and incubated at 33 ºC for 3 weeks. Plates were examined for the presence of colonies by dissecting microscope with transmitted light. After growth of spirochetes on solid medium several well-isolated colonies

Table 1. *B. burgdorferi* strains, origin, and passage number.

Strain	Passage number	Source[a]	OspA serotype[b]	Genotype[b]
DK1	32	Skin, (EM)	1	*B. afzelii*
DK2	11	Skin, (ACA)	2	*B. afzelii*
DK3	11	Skin, (ACA)	2	*B. afzelii*
DK4	10	Skin, (EM)	2	*B. afzelii*
DK5	12	Skin, (ACA)	2	*B. afzelii*
DK6	12	CSF, (LMR)	4	*B. garinii*
DK7	30	Skin, (ACA)	1	*B. burgdorferi* senso stricto

[a]EM, erythema migrans; ACA, acrodermatitis chronica atrophicans; CSF, cerebrospinal fluid; LMR, lymphocytic meningoradiculitis.[b] Determined by comparison with OspA typing system of Wilske et al. (28).

were selected and subcultured into liquid BSKII medium, incubated in 33 ºC for 3 weeks. Cultures were examined by dark field microscopy and cells were collected and DNA was extracted for PCR analysis as mentioned above.

Electron microscopy of Borrelia

Borrelia strains DK1 and DK6 grown in BSKII medium at 33 ºC were examined by electron microscopy. Three milliliters of the medium was centrifuged at 5000 × g for 15 min and 200 l of the harvested cells was either fixed for 30 min in 200 l of 3% glutaraldehyde buffered in 0.1 M cacodylate with 4.5% glucose or suspended in 200 l of PBS. After centrifugation for 10 min at 5000 × g the pellet was suspended in 200 l of PBS. A Formvar-carbon-coated copper grid was placed on top of a drop containing the organisms for 2 min, subsequently dried and negative stained with 3% phosphotungstic acid (pH 6.8) for 1 min, air dried and photographed in a Philips 201 transmission electron microscope. The *Borrelia* was characterized according to the number of flagella and the cell diameter.

Electron microscopy of plasmids DNA from the DK1 strain

Linear plasmid samples were prepared for electron microscopy as previously described (3,26).

Results and Discussion

We took advantage of the special properties of linear plasmids to analyze and characterize the complex mixture of linear and circular plasmids found in isolates of *B. burgdorferi*. To study the phenomenon of UV-EtBr induced nicking of DNA and to demonstrate the feasibility of the 2-dimensional system we first performed a model experiment with standard supercoiled circular plasmids (pUC18), and Hind III fragments of lambda DNA (Figure 1) results showed the feasibility of the technique (details in the figure legend). Figure 2 illustrates the extreme complexity of plasmids extracted from several different strains of *B. burgdorferi* analyzed by conventional agarose electrophoresis. Evidently this technique is not

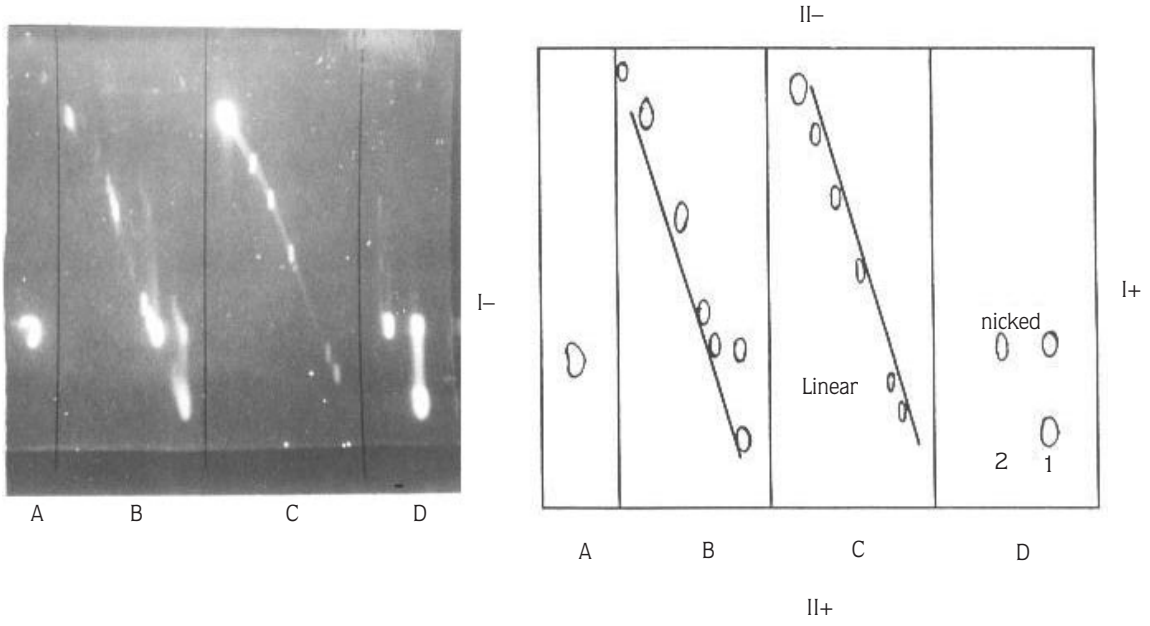

Figure 1. The effect of UV-EtBr on supercoiled circular plasmid (pUC18) in 5 and 15 min irradiation to UV respectively shows in lane 1 of sample D 2 differently migrating forms of the plasmid, after 5 min exposure to UV 1 slow migrating corresponding to non-nicked plasmid and 1 nicked form of plasmid (with arrow). After 15 min exposure to UV light only the completely converted nicked form of plasmid is formed (sample D lane 2). Lambda DNA digested with HindIII restriction enzyme separated by 2-dimensional technique is shown in sample C. All fragments move in a straight line without any upper band. Sample B is a mixture of samples D and C separated by the 2-dimensional technique; note the ability of 2-dimensional gel electrophoresis to separate the nicked and intact and circular DNA. The arrow indicates the nicked form of supercoiled circular plasmid. Sample A, EcoRI linearized pUC18, which has the same mobility of linear DNA, illustrating that nicked and EcoRI digested pUC18 attain the same mobility.

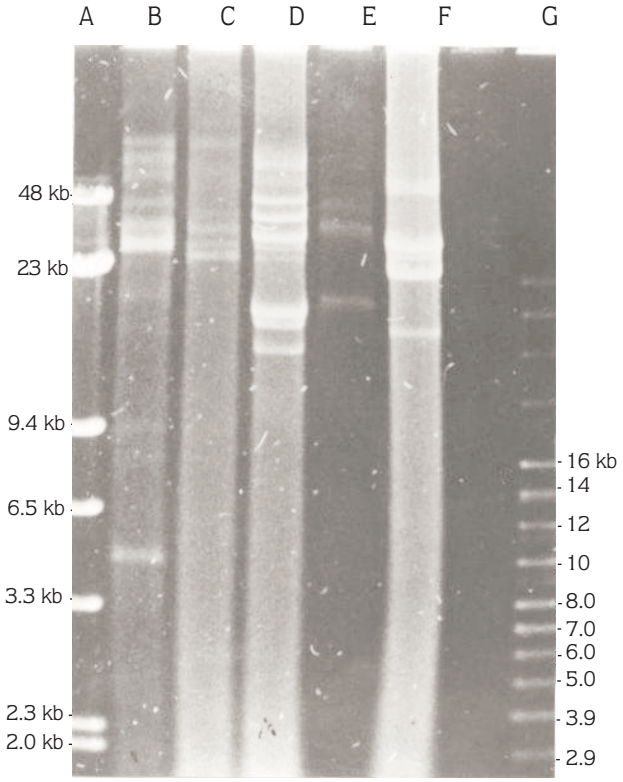

Figure 2. Plasmids profiles from different strains of *Borrelia burgdorferi.* The DK1 strain (B), DK5 strain (C), DK6 strain (D), DK 2 strain (E), DK7 strain (F), and a supercoiled circular molecular weight marker (G). Linear molecular markers (A) (HindIII fragments of Lambda DNA). Samples were separated in 0.3% gel at 14 ºC for 20 h and then stained with ethidium bromide.

able to discriminate between linear and circular plasmids. The 2-dimensional technique was used to analyze plasmids extracted from 3 different *B. burgdorferi* isolates: DK1, DK6, and DK7. Figure 3 shows the 3 selected *B. burgdorferi* strains analyzed by the 2-dimensional system. A clear segregation of plasmids with different properties is observed. Plasmids that move along the diagonal line (unaffected by the UV-EtBr nicking) are prevalent in all 3 isolates. The DK7 strain has 4 plasmids dislocated from the diagonal line probably representing 4 circular plasmids. DK6 has no circular plasmid and in DK1 only 1 circular plasmid is affected by UV-EtBr nicking.

We assume that plasmids located along the diagonal line represent special linear plasmids. It is clear that by adding more plasmid DNA to the gel we are able to visualize more plasmid. DK1 has more than 10 plasmids in more concentrated plasmid DNA analysis. It is possible that this is the result of extraction of total plasmid from 1 culture containing 2 morphologically distinct *borrelia*. DK6 has only 5 linear plasmids.

The OspA gene is known to be located on a linear plasmid of 49 kb (25). To demonstrate the identity of one linear plasmid moving along the diagonal line we performed a hybridization analysis with a DNA probe from an OspA gene and it showed that the probe hybridized to the 49 kb plasmids on the diagonal line illustrated in Figure 4. PCR analysis of the 25 kb linear plasmid extracted from the diagonal line with the OspA specific probe produces a fragment of expected size corresponding to the primer span in the original OspA sequence and shows sequence variation between OspA of the DK1 and DK6 strains. It has been shown previously that OspA-B genes are located in linear plasmids of different sizes (27).

Examination of the DK1 strain with transmission electron microscopy showed 2 morphologically different cell types: A) a smaller blunt-ended type with a width of 0.2-0.3 m and 7-8 flagella at each end; and B) a larger type with a tapered end and with width of 0.3-0.4 m and 12-14 flagella at each end. The DK6 strain showed only 1 phenotype with 7-8 flagella at each end (Figure 5). Previous studies have shown that ticks are able to transmit 2 different phenotypes of *B. burgdorferi.* Cells of strain B31 isolated in the USA from *Ixodes dammini* and strain G25 isolated in Sweden from *Ixodes ricinus* have similar but not identical ultrastructures. One tick isolate was found to consist of 2 morphologically different spirochetes, for which cell size and shape as well as number of flagella varied (2,28). *I. dammini*, with its broad host range, has the potential to acquire multiple microorganisms (29).

Recently it has been demonstrated, by using a polymerase chain reaction, that DNA from more than 1 of the 3 Borrelia species associated with Lyme disease in Europe was present in the biological fluids of Lyme disease patients (30). Simultaneous infections with 2 different borrelia could have different explanations; the tick was infected with more than one species because of the infection of the host with different borrelia. A second possibility is successive infectious interrupted larval meals. A third possibility is an infectious larval meal by a previously transovarially infected larva. The fourth possibility is a mixed infection acquired transovarially.

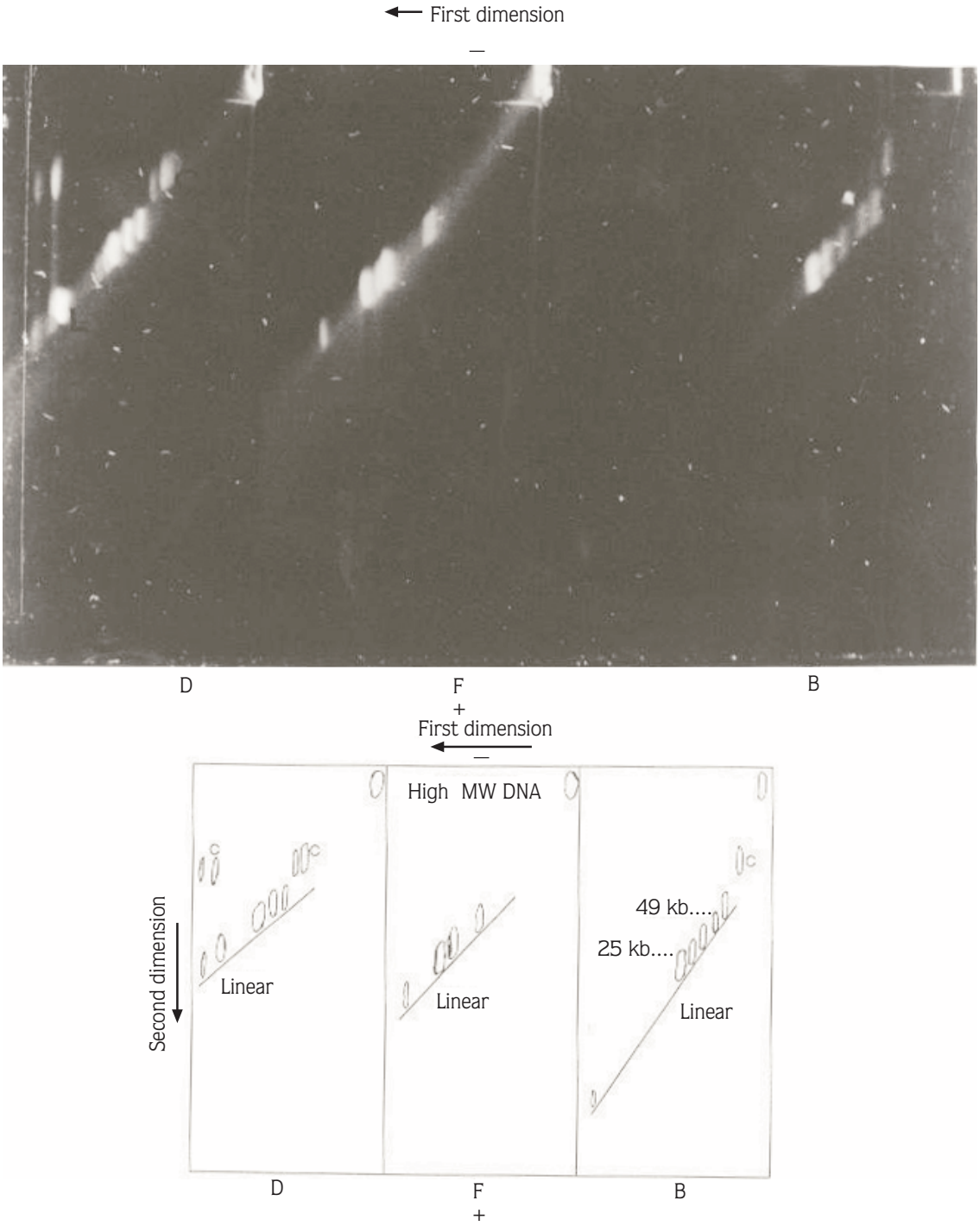

Figure 3. Two-dimensional gel electrophoresis of 3 strains of *B. burgdorferi*. (D) The DK7 strain plasmids in which UV-EtBr is nicked is indicated by arrows. These UV-EtBr nicked plasmid forms most likely represent circular plasmid indicated by the letter c, whereas the plasmid moves in a diagonal line are linear as a result in this strain; there are 4 plasmids with upper bands revealing the nicked form of circular plasmids and 4 bands without upper bands revealing linearity of the plasmids. Sample (F) The DK6 strain as all the plasmids moving in a diagonal line indicating that this strain contains only linear plasmids and there is no upper band in the plasmids and DK1 (sample B) all of the plasmids except one move in a diagonal line indicating the low content of circular plasmids, the largest plasmid in this strain is circular with an upper band but linear plasmid including a 49 kb plasmid (encoding the OspA-B genes) has no upper band.

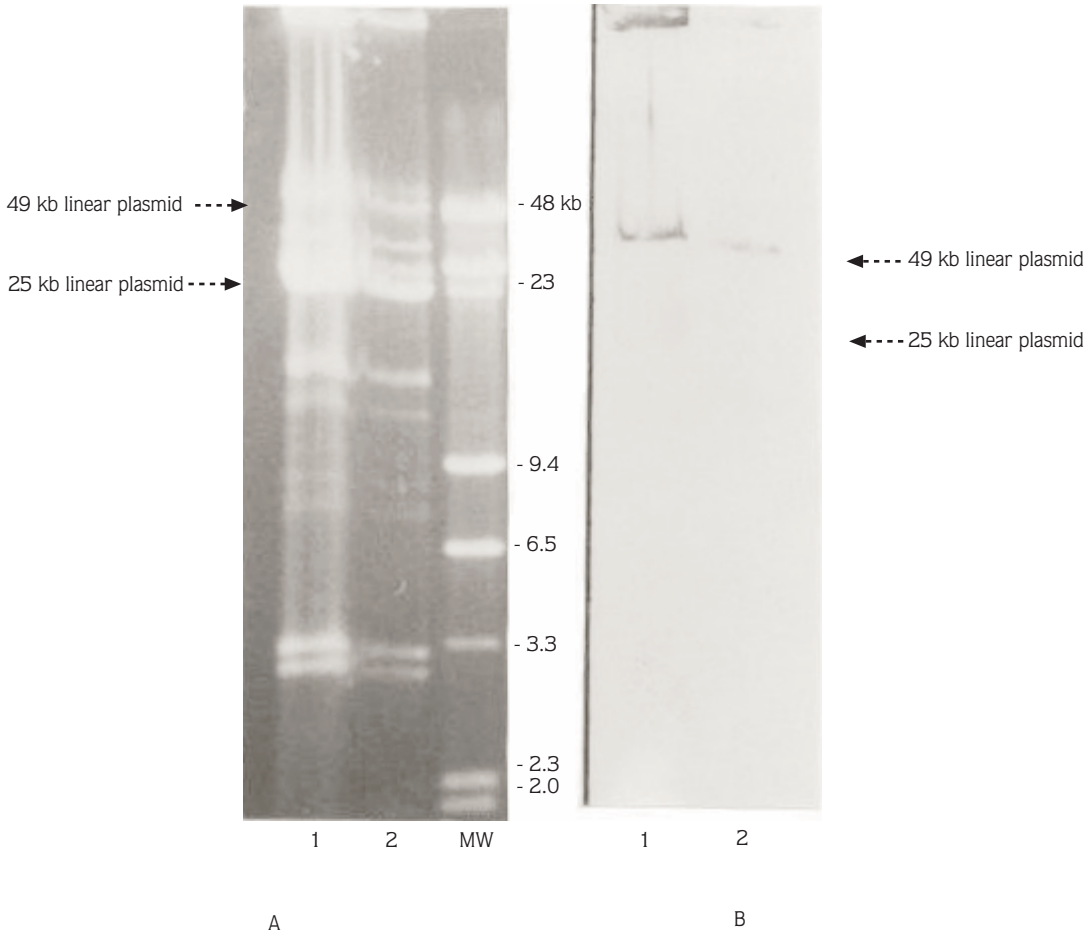

Figure 4. Hybridization of total plasmid extracted with the OspA specific probe.
A: Lanes 1 and 2 are total plasmid extracted from the DK1 strain, lane 1 contains more DNA material for detection of low copy number plasmids. Lane 2 is the same DNA with fewer amounts for clear separation of plasmid bands. 49 and 25 kb plasmids are shown with arrows. Picture b shows hybridization of OspA specific probe to 49 kb linear plasmid.

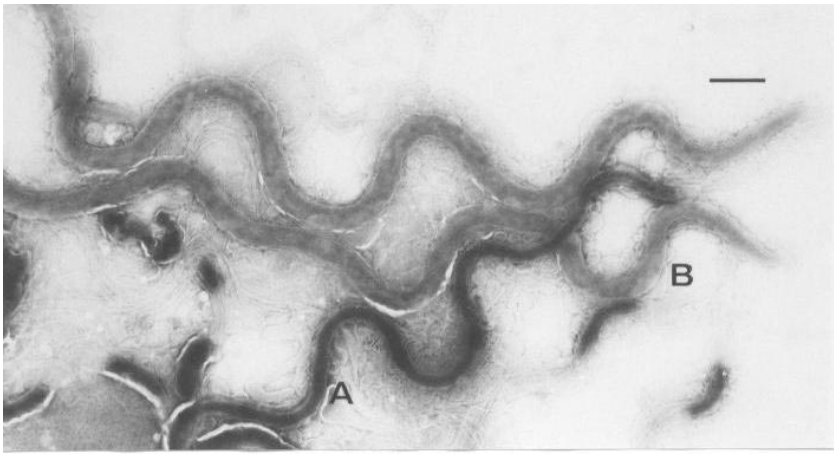

Figure 5. Electron microscopy of unfixed, negative stained DK1 strain (skin isolate). This strain consists of 2 morphologically distinct borrelia A. small and B. larger *borrelia*. Bar 1 m. Magnification 10,260 x.

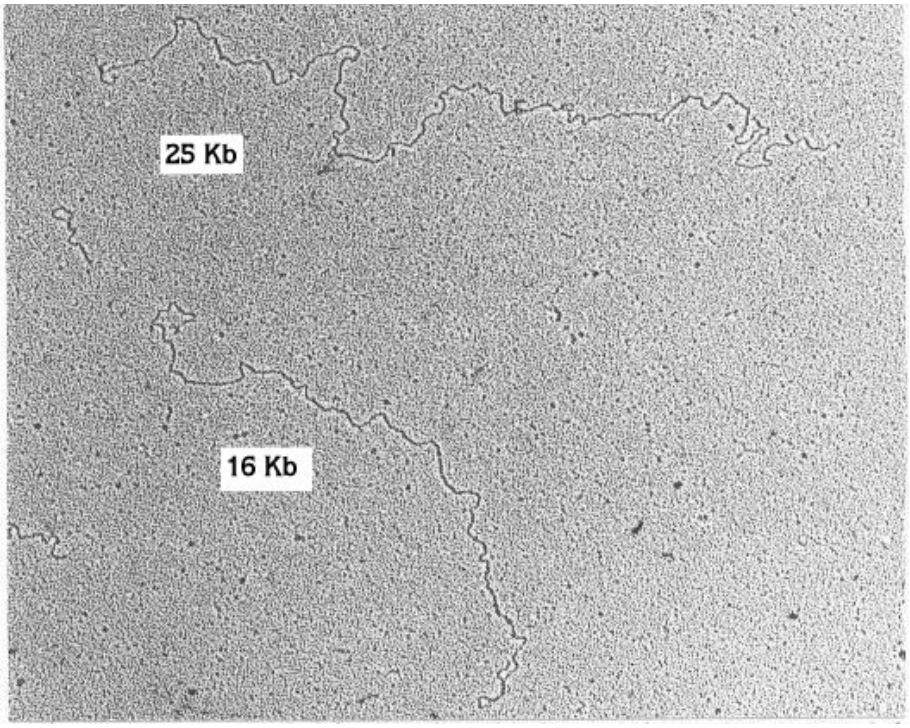

Figure 6. Electron micrograph of 25 kb plasmid extracted from DK1 strain, magnification 52,000x.

Clonal selection of the DK1 strain by cultivation on BSKII-agar produced 2 different colonies with average diameter of 0.5 to 2 mm, with diffused borders that penetrated into the agarose. Colonies were visible after 2 weeks of incubation but 3 weeks of incubation were needed to isolate colonies from plates. Subculture of several colonies, and examination of cells by electron microscopy and PCR amplification of 2 phenotypes with OspA and OspB specific primer showed that phenotype (A) did not contain the OspA and OspB genes. The dominant phenotype (B) encodes OspA and OspA-B genes.

Electron microscopy of total plasmid extracted from DK1 strain has shown that most of the plasmids in this strain are linear. Electron microscopy of the putative 25 kb plasmid characterized as linear plasmid in 2-dimensional gel electrophoresis revealed 25 kb linear duplex molecules (Figure 6).

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