The characterization of telomeres in the linear plasmids of the bacterium *Borrelia burgdorferi*

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Summary

The replication of the telomeres of *Borrelia burgdorferi* 's 16 kb and 49 kb linear plasmids was investigated. Two dimensional agarose gel electrophoresis was used to analyze the replication intermediates of *B. burgdorferi* in an attempt to determine the mechanism of telomere replication. *Oxytricha* telomerase assays were used to investigate the possible existence of a telomerase-like enzyme in *B. burgdorferi* . The two dimensional agarose gels failed to identify specific replication intermediates; the telomerase assay either worked weakly or failed to work. This suggests that i) *B. burgdorferi* does not depend on telomerase to maintain its telomeres or ii) if telomerase exists, its substrate specificity differs significantly from that of *Oxytricha* telomerase.

Introduction

The genus *Borrelia*, which belongs to the spirochaete division of eubacteria, is peculiar because it consists of prokaryotic organisms with predominantly linear genomes (Barbour et al., 1990). *Borrelia burgdorferi*, the bacterial agent responsible for Lyme disease, was discovered to have double-stranded linear plasmids with covalently closed ends (Barbour and Garon, 1987). Strain B31 of *B. burgdorferi* is known to have three linear plasmids of sizes 49, 29 and 16 kb and two circular plasmids between 27 and 30 kb. Moreover, it is suspected that this organism has a linear chromosome of about 950 kb. It is known that the left ends of the 16 kb and 49 kb plasmids are A-T rich and identical in sequence except for a 30 bp insertion in the 49 kb plasmid. It is also known that 18 bases on the immediate left and right ends of the 16 kb plasmid are rich in A-T bases and identical in sequence except for at 3 positions (Barbour et al., 1990). Since this bacterial species has linear plasmids with the ability to self-replicate, it is likely that the ends of the DNA molecules are protected by telomeres. Barbour et al. (1990) have presented convincing evidence that conventional telomere structure is present at the chromosome termini of *B. burgdorferi* .

A central problem in DNA replication is the inability of DNA polymerase to replicate the ends of duplex DNA. This occurs because the enzyme requires an RNA primer which is later removed leaving a gap at the 5'end of the newly synthesized strand. Successive rounds of replication in linear DNA would lead to progressive shortening of the duplex if the problem were not overcome. All

bacteria other than spirochaetes have overcome this problem with a circular genome. Linear plasmids and chromosomes have also been able to overcome this dilemma with the evolution of telomeres.

Telomeres are required for chromosome stability and allow for the complete replication of the chromosomal DNA. The telomeres provide stability in two ways. They prevent the degradation of essential genetic information by serving as a protective cap against nuclease activity, and they prevent the fusion of broken chromosomal ends. Telomeres are also essential for the complete replication of the ends of a linear piece of DNA. Since DNA polymerase can only add bases onto a free 3' OH, RNA primers are required. When the primers are removed and replaced by DNA, the 5' end of the daughter strand resulting from lagging strand synthesis is unable to be replaced by DNA because there is no free 3' OH (Figure 1). Successive rounds of replication would lead to chromosome shortening and eventually senescence and cell death (Blackburn, 1991). If the replication of telomeres could be characterized in *B. burgdorferi*, it could be used as a possible target for chemotherapy.

We hypothesize that there are two possible models that *B. burgdorferi* might utilize to replicate its telomeres. The telomerase model, which has been characterized in the ciliates *Euplotes*, *Tetrahymena* and *Oxytricha*, involves the use of a ribonucleoprotein enzyme called telomerase (Shippen-Lentz and Blackburn, 1989; Blackburn, 1991). Telomerase has an internal RNA molecule which it uses as a template to direct DNA telomere synthesis (Shippen-Lentz and Blackburn, 1990). Telomerase binds to the 3' end of DNA and adds on its specific telomeric repeat by using an internal RNA molecule as a template (Figure 2). For example, in *Tetrahymena* the telomeric repeat added is GGGGTT and in *Euplotes* and *Oxytricha* the repeat added is GGGGTTTT (Blackburn, 1990). The telomeric repeats typically extend a few hundred base pairs beyond the end of the chromosome. In most of the organisms studied, the 3' ends of the chromosomes contain a G-rich strand that extends 12-16 nucleotides beyond the 3'-5' complementary strand (Blackburn, 1991).

The other model that could be responsible for the replication of the ends of the linear plasmids of *B. burgdorferi* is the mechanism used by poxviruses. Poxviruses are eukaryotic viruses that replicate in the cytoplasm of host cells. Their genome consists of a large double stranded linear piece of DNA. The vaccinia genome (185 kb) contains hairpin ends flanked by several short A-T rich tandem repeats. It is presumed that before viral replication can begin, a nick is

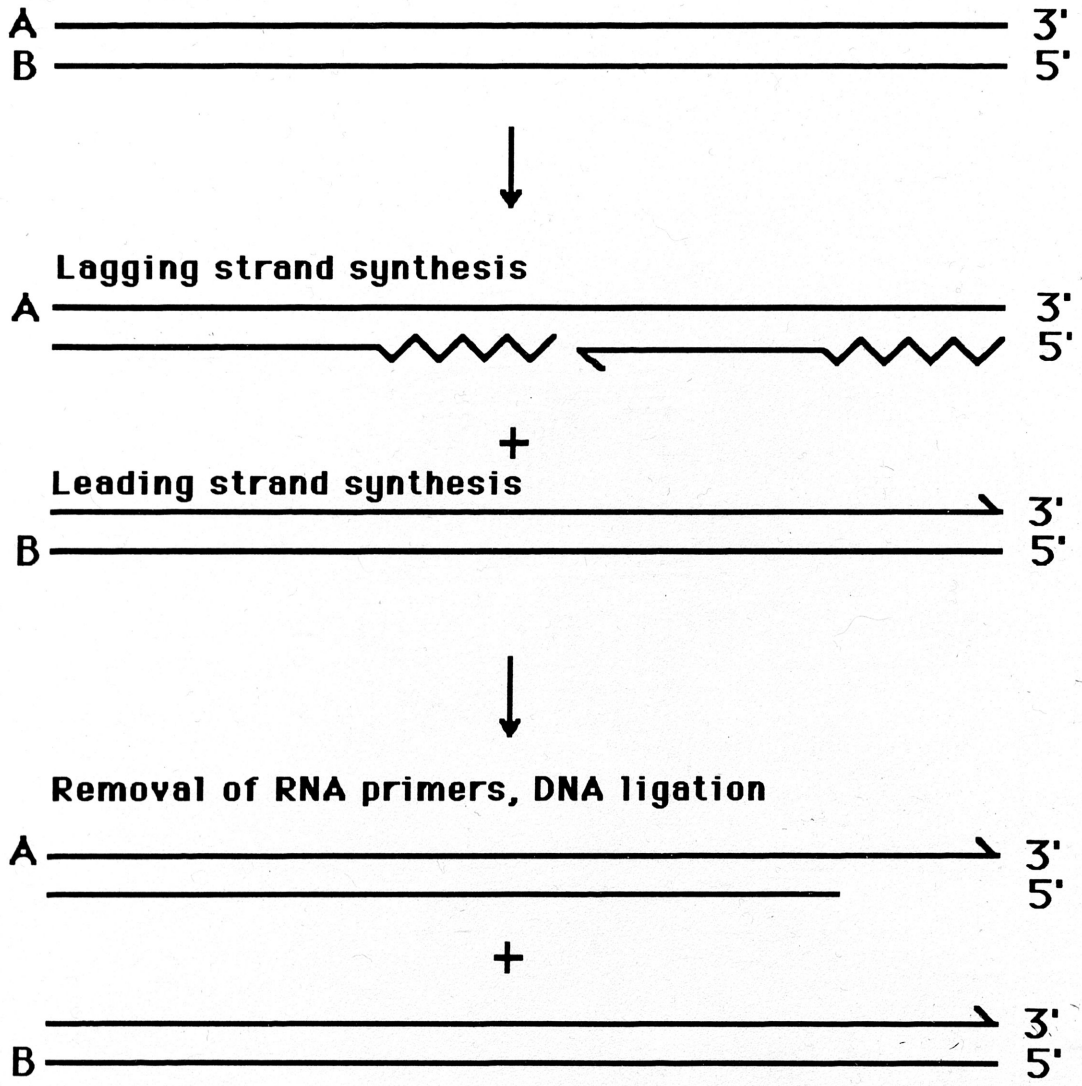

Figure 1. The problem with the complete replication of a linear piece of DNA with conventional methods of replication. Once complementary strand of strand A for DNA polymerase to use to fill in the gap. The chromosome will be shortened with every successive round of replication (adapted from Blackburn, 1991).

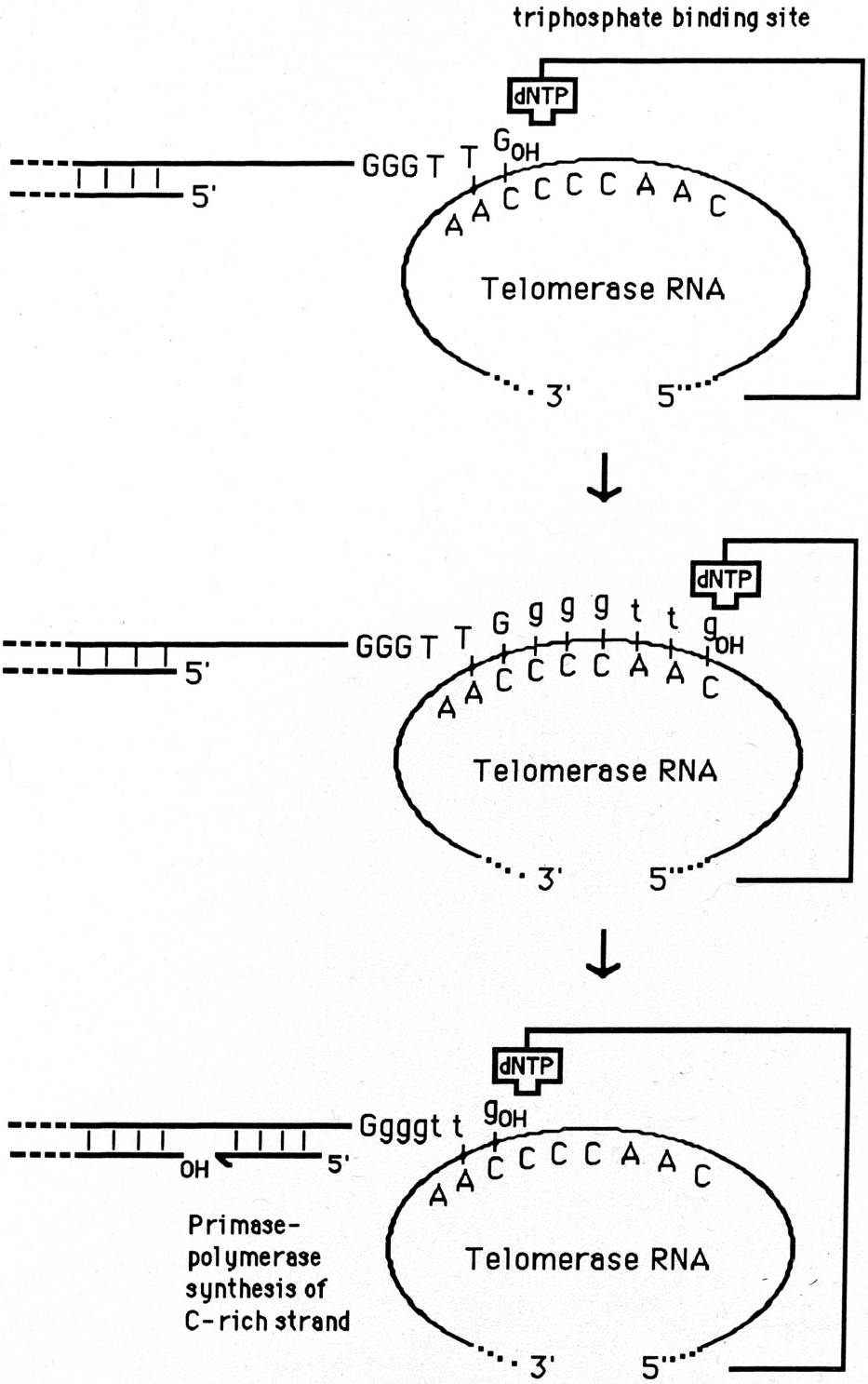

Figure 2. The internal RNA template of telomerase base pairs with the protruding 3' end of a linear piece of DNA. Complementary bases are then added onto the 3' end of the substrate DNA. This process is repeated several times generating a long stretch of telomeric repeats (adapted from Blackburn, 1991).

introduced into one of the DNA strands near the terminal hairpin (see Figure 3). The hairpin opens up creating a 3' OH for DNA polymerase to use as a primer. The bottom complementary strand is then synthesized using the top, opened up strand as a template. Both strands now contain inverted repeats that can fold back on themselves to form hairpins. The double stranded DNA at one end of the chromosome is separated into single stranded DNA so that the inverted repeats can base pair with themselves to form hairpins. This creates a 3'OH on the bottom strand which can be used as a primer for DNA polymerase. There are two possible mechanisms that DNA polymerase can use to synthesize the rest of the DNA. The DNA polymerase could continue using leading strand synthesis and synthesize the DNA around the hairpin loop at the right end of the DNA until it encounters the top terminal hairpin. Or, the DNA polymerase could use standard leading and lagging strand synthesis to copy the original DNA. Once DNA replication is complete, a cruciform structure forms within the concatamer and nicks are introduced to resolve the concatamer into monomers (Traktman, 1990).

In order to distinguish between these two models of telomere replication, we decided to use two dimensional agarose gel electrophoresis to analyze replication intermediates, and a telomerase assay to determine the possible existence of a telomerase enzyme. The exact mechanism of replication would be dependent on the types of replication intermediates detected. If the telomerase assay worked well on *B. burgdorferi* DNA substrate, it would suggest the possible presence of a telomerase-like enzyme in *B. burgdorferi*.

Results

Two Dimensional Agarose Gel Electrophoresis

Two dimensional agarose gel electrophoresis is a technique that is capable of distinguishing between different types of replication intermediates. The first dimension is run at a low voltage (1V/cm), in a low percentage agarose gel (0.4 %) and in the absence of ethidium bromide. This separates the DNA according to size. The second dimension is run at a high voltage (5V/cm), in a high percentage agarose gel (1%) and in the presence of ethidium bromide (0.5 ug/mL). The ethidium bromide intercalates between the DNA bases and stiffens the DNA. The second dimension separates the DNA according to shape (Brewer and Fangman, 1987; Bell and Byers, 1983; Huberman, unpublished material).

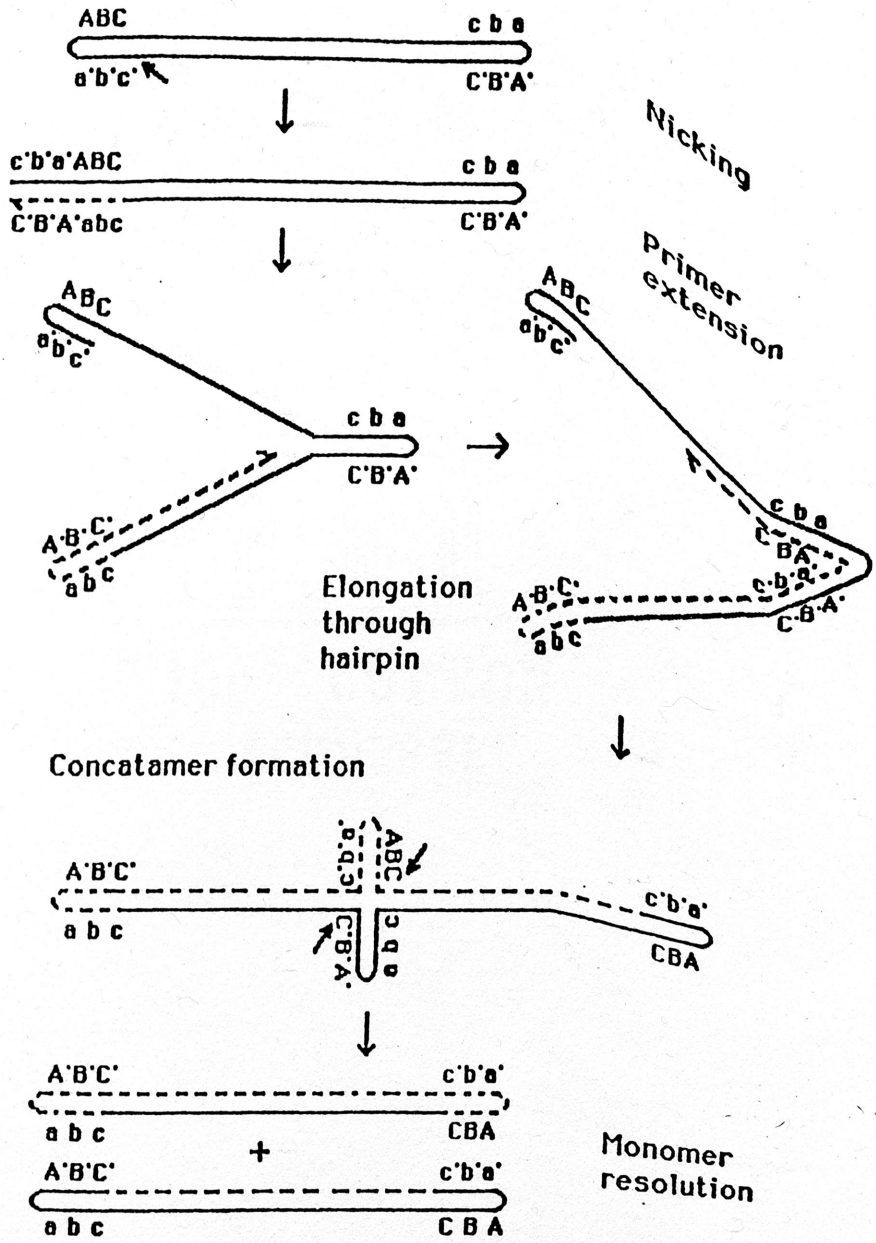

Figure 3. Mechanism of poxvirus replication. The small arrows indicate the sites of nicking (adapted from Traktman, 1990).

Different patterns on the gel thus indicate the type of replication intermediates present (see Figure 4). On the second dimension gel, linear pieces of DNA will run as a diagonal whereas replication intermediates will be retarded more and hence, will run above the diagonal. The darkest spot at the 1n position will correspond to linear DNA since it is present in a much greater abundance. The replication intermediates will form an arc or line originating at the 1n linear DNA and ending at a position indicative of size 2n. For instance, in panel A of Figure 4, the origin of replication starts outside the restriction fragment (1kb) that was probed for. Hence a Y shaped structure is created. As the replication fork just begins to move through the restriction fragment, which is still largely linear duplex DNA, it migrates through the gel to the position just above the 1 kb mark. As replication continues, the Y gets larger. The shape that will be retarded the most in the gel is the structure with three equal length arms or when the Y has moved half way through the restriction fragment. Since this replication intermediate is retarded the most, it will appear at the top of the arc at a position midway between 1 kb and 2 kb. As replication continues, the replication intermediate assumes a more linear shape and hence travels faster through the gel. When replication is almost complete (bottom left corner of panel A), the size of the molecule is almost 2 kb. When replication is complete, two 1 kb fragments will be produced which will appear at the 1 kb spot. Hence an arc shaped pattern like this indicates a Y -shaped intermediate. If the origin of replication originates inside the restriction fragment, a bubble shaped intermediate will be generated. In panel B (Figure 4), the intermediate that is retarded the most in the gel is the shape with the largest bubble. This is the fragment where replication is almost complete and hence has a size of 2 kb. The other two panels in Figure 4 show other possible replication intermediates and their corresponding two dimensional patterns. Two Y shaped structures could meet within the restriction fragment (Panel C) or there could be an origin of replication at an asymmetrical point within the restriction fragment (Panel D) (Brewer and Fangman, 1987). Hence, by using two dimensional agarose gel electrophoresis, we should be able to determine the type of replication intermediates present in replicating *B. burgdorferi* . If we probed for the telomeres, we would be able to determine the mechanism of telomere replication.

Borrelia burgdorferi (strain 532), which has a doubling time of 12 hours, was grown for varying periods of time ranging from 1.9 to 4.9 days. DNA was

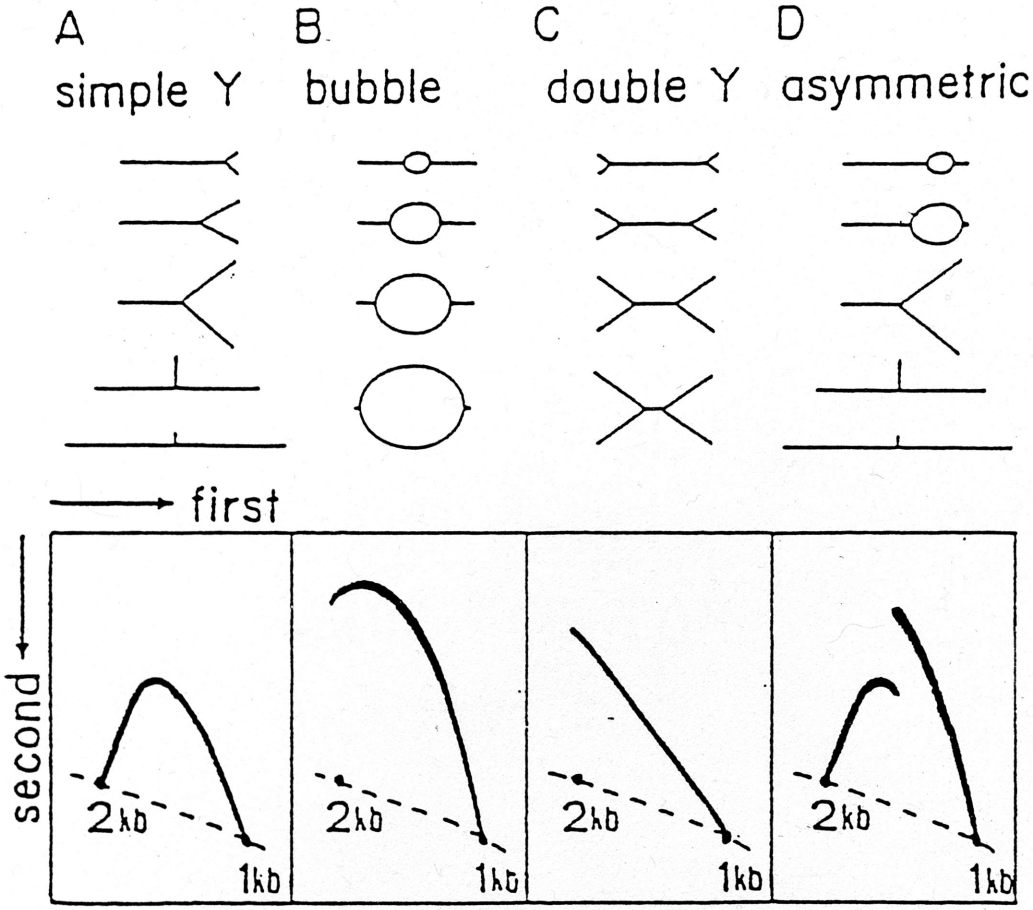

Figure 4. Replication intermediates generated from a 1 kb restriction fragment with the origin of replication at different locations. The panels show the corresponding migration of the replication intermediates on a two dimensional agarose gel. The dashed line indicates the location of linear molecules (adapted from Brewer and Fangman, 1987).

then prepared from each batch of cells and subjected to Hind III restriction enzyme digestion. The digests were run on a 1D agarose gel (0.4 %) for about 18 hours and then transferred to nitrocellulose membrane by standard Southern transfer. The blot was then probed with an Osp B probe, a PCR generated gene fragment located at an internal site on the 49 kb plasmid. The resulting autoradiograph showed that the probe hybridized to a 1.2 kb restriction fragment (Figure 5). This is consistent with the 49 kb Hind III restriction map because Osp B is located on a 1.2 kb restriction fragment. This procedure was performed to prove that the DNA isolated belonged to *Borrelia burgdorferi* since only *B. burgdorferi* contains the Osp B gene. The procedure also showed that the Osp B internal probe was specific for the 1.2 kb fragment and that it did not hybridize to several fragments. This is important for the second dimension of the gel because we only want a signal produced from one restriction fragment.

Knowing that a 10% innoculum of *B. burgdorferi* reaches stationary phase at 96 hours (Rawlings, personnal communication, 1992), we analyzed DNA from the log phase of growth (1.9, 2.3, 3.0 and 3.8 days) for replicating DNA. The DNA was digested with Hind III at 37° C for 1 hour and then run on a 1D gel for approximately 18 hours. The band was then cut out and rotated 90° to form the 'well' of the second dimension. The second dimension agarose gel (1%) was then poured around the band. The second dimension was run for about 4-5 hours at 4° C in the presence of ethidium bromide (0.5 ug/mL). The gel was then transferred by standard Southern transfer to nitrocellulose membrane and then hybridized with the Osp B internal probe. We anticipated that the internal probe would allow us to generate some arc patterns which would provide a positive control and proof of replicating DNA. We then hoped to strip the nitrocellulose membrane and probe it with a telomere probe. The telomere probe is an 18 base end-labelled synthetic oligonucleotide specific for the left end of the 16 kb and 49 kb linear plasmids. Unfortunately, none of the Osp B probed second dimension gels generated arcs. Even after 3 weeks of exposure, the second dimension gels resulted in a single spot corresponding to the 1.2 kb fragment (Figure 6 and Figure 7). Telomere probed blots likewise failed to detect replication arcs.

Telomerase Assay

To determine whether *Borrelia burgdorferi* uses a telomerase enzyme to replicate and elongate its telomeres, we synthesized an oligonucleotide from the 3'

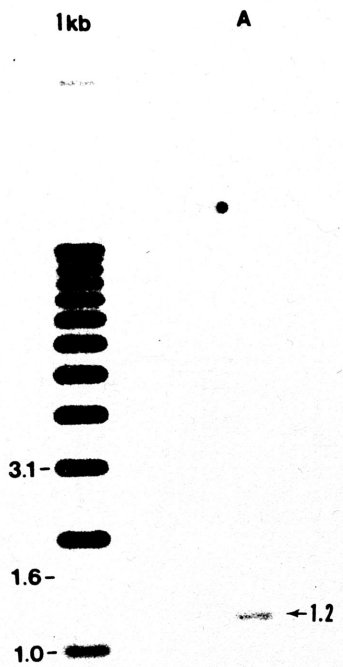

Figure 5. Autoradiograph showing a 1kb ladder and the hybridization of the Osp B internal probe to a 1.2 kb restriction fragment (24 hour exposure, 1 intensifying screen, -70°C). Lane A contains Hind III digested genomic *B. burgdorferi* DNA (92 hour culture) run on a 0.4% agarose gel at 1V/cm for 18 hours. This confirms that the DNA was isolated from *B. burgdorferi* and indicates that the probe specifically hybridizes to a 1.2 kb fragment.

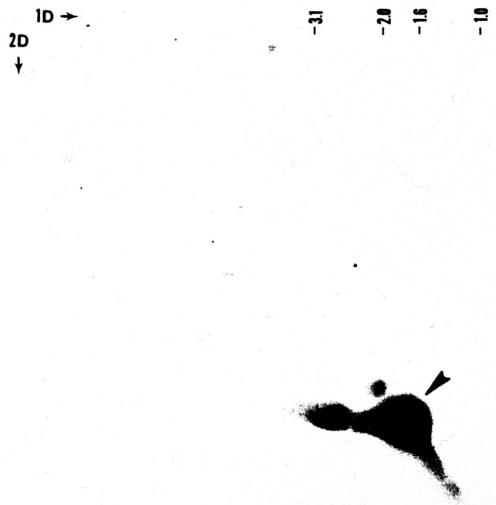

Figure 6. Autoradiograph (3 week exposure, 1 intensifying screen, -70°C) showing a two dimensional gel of Hind III digested genomic *B. burgdorferi* DNA (92 hour culture). The Osp B internal probe was used. The large spot indicated by the arrow represents linear DNA (1.2kb). The tail pointing to the bottom left of the picture represents sheared 1.2 kb fragments. The tail contains restriction fragments from 1.2 kb to the lowest detectable size. The second large spot is probably due to a partial digest. It is not clear what the other signals represent. If replication intermediates were detected, they would appear as an arc originating at the 1.2 kb sized spot and terminating around a size of 2.4 kb (1D gel: 0.4% agarose run at 1V/cm for 18 hours; 2D gel: 1% agarose, 0.5 ug/mL ethidium bromide run at 5V/cm for 4-5 hours).

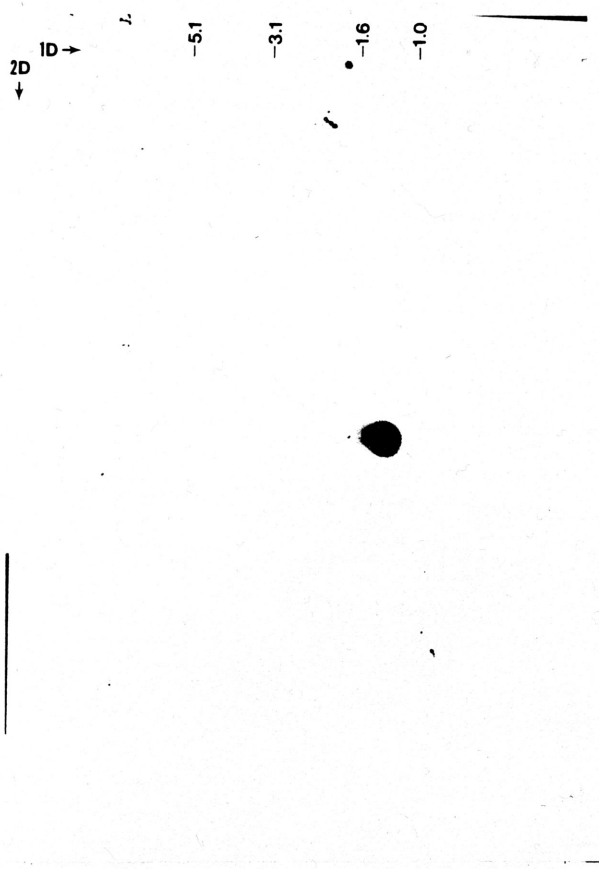

Figure 7. Autoradiograph (2 week exposure, 1 intensifying screen, -70°C) showing a two dimensional gel of Hind III digested genomic *B. burgdorferi* DNA (73 hour culture). The Osp B internal probe was used. The single spot represents linear DNA of the 1.2 kb restriction fragment (1D gel: 0.4% agarose run at 1V/cm for 18 hours; 2D gel: 1% agarose, 0.5 ug/mL ethidium bromide run at 5V/cm for 4-5 hours).

left end of the 16 kb and 49 kb linear plasmids and tested to see if *Oxytricha* telomerase would use the *B. burgdorferi* 3'end as a substrate for telomere elongation. Although it is known that the natural substrate of *Oxytricha* telomerase is G-C rich, this telomerase has been known to use a variety of telomere-specific substrates, including A-T rich sequences (Shippen-Lentz, personnal communication, 1993).

The synthetic oligonucleotide was gel purified to remove shorter oligonucleotide impurities and then used in the telomerase assay. A telomerase cocktail, *Oxytricha* cell extract and 100 ng of the *B. burgdorferi* oligonucleotide were incubated at 30° C for 1 hour. The reaction was then stopped with telomerase stop reaction, extracted with phenol/chloroform and precipitated with ammonium acetate and ethanol. The DNA was then resuspended in xylene cyanol dye and loaded onto a 10% polyacrylamide gel. As controls, *Oxytricha* oligonucleotide, human oligonucleotide and no oligonucleotide were also used in the telomerase assay and loaded onto the gel. The *Oxytricha* oligonucleotide would serve as a positive control, the human oligonucleotide would serve as a weak positive control and the no oligonucleotide would serve as a negative control. The resulting autoradiograph (Figure 8) showed that the *Oxytricha* telomerase was either not working very efficiently or not working at all on the *Borrelia burgdorferi* oligonucleotide. There are some faint bands in the *B. burgdorferi* lane but these faint bands are also present in the no oligonucleotide lane and can be attributed to DNA polymerase activity (Shippen-Lentz, personnal communication,1993).

Experimental Procedures

Growth of *Borrelia burgdorferi*

Cultures of *Borrelia burgdorferi* (Strain 531) and growth media were obtained from Julie Rawlings (Texas Department of Health, Austin). *Borrelia burgdorferi* (10% innoculum) was incubated at 31°C in 6% rabbit serum and 9 mL of BSK-H (Barber-Stoner-Kelley media; Sigma) media for varying amounts of time between 1.9-4.9 days. At specific times, the bacteria were spun down at 5K using a clinical centrifuge and washed twice with 1X PBS [0.057 M Na_2HPO_4, 0.018 M KH_2PO_4, 0.072 M NaCl, pH 7.2). The bacteria were then stored at -70°C until further use. Bacteria were grown in a tissue culture incubator and handled in a tissue culture hood.

Figure 8. Autoradiograph (22 hour exposure, 1 intensifying screen, -80°C) showing the products of the *Oxytricha* telomerase assay run on a 10% polyacrylamide gel at 500V for 4 hours. Lane A: no oligonucleotide (negative control), Lane B: *B. burgdorferi* 16 kb plasmid 3'end, Lane C: Human oligonucleotide $((T_2A_6)_3)$, Lane D: *Oxytricha* oligonucleotide $((T_4G_4)_3$ - positive control), Lane E: kinased *Oxytricha* oligonucleotide (indicates location of input oligonucleotide). Lanes leaked and narrowed down due to salt. *Oxytricha* telomerase adds on a repeat of GGGGTTTT. This is evident in Lane D. The dark bands correspond to successive additions of the repeat GGGGTTTT and the light bands correspond to DNA lengths inbetween the 8 base repeats. For example, telomerse could add one G and then stop. Since the human oligonucleotide is a weak substrate, there are weak bands present in Lane C. There are no strong bands in Lane B so telomerase is either unable to use the *B. burgdorferi* oligonucleotide as a substrate or was able to use it weakly. Lanes A and B have faint bands which are probably due to DNA polymerase activity (Shippen-Lentz, personnal communication, 1993).

DNA Preparation

Pelleted bacterial cells were washed in 1.0 mL TNE [10 mM Tris-HCl, pH 8.0, 10 mM EDTA] and repelleted. The cells were resuspended in 135 µl of TNE and then 135 µl of TNE containing 2% Triton X-100 and 30 µl of freshly prepared lysozyme (5 mg/mL) were added. The reaction was incubated at 37°C for 30 minutes. Proteinase K (20 mg/mL) was added to the mixture and then incubated at 65°C overnight. The DNA preparation was stored at -20°C until used (Ficht, personnal communication, 1992).

Restriction enzyme digest

8.4 µl of *B. burgdorferi* DNA preparation was digested with 24U/µl of Hind III and Buffer B (Boehringer Mannheim) at 37°C for 1 hour. The reaction was stopped with DNA dye [50% glycerol, 20 mM EDTA, pH 7.4, 0.1% xylene cyanol, 0.1% bromophenol blue, 0.1 % SDS].

Two dimensional agarose gel electrophoresis

First dimension: 0.4 % Agarose gel (SeaKem ME) in 0.5X TBE [0.045 M Tris borate, 0.001 M EDTA pH 8.0, pH 8.0] run in 0.5X TBE at 1V/cm for about 18 hours. The 1kb marker lane was cut out and stained with 0.5 µg/mL ethidium bromide and visualized under UV light. The DNA digest band of interest was cut out and trimmed to fit the second dimension gel. The second dimension gel was poured around the 1D band (adapted from Brewer and Fangman, 1987; Bell and Byers, 1983; Huberman, unpublished material).

Second dimension: 1% Agarose gel containing ethidium bromide (0.5 µg/mL) in 1X TBE (4°C) run in 1X TBE containing ethidium bromide (0.5 µg/mL) at 5V/cm for 4-5 hours at 4°C (adapted from Brewer and Fangman, 1987; Bell and Byers, 1983; Huberman, unpublished material).

Southern Transfer

The gel was soaked in 1.5 M NaCl, 0.5 N NaOH for 45 minutes, rinsed in water, soaked in 1M Tris, 1.5 M NaCl for 30 minutes and then for another 15 minutes. A nitrocellulose filter (Schleicher and Schuell Pure Nitrocellulose) cut to the size of the gel was wetted in ddH_2O and soaked in 10X SSC [1.5 M NaCl, 0.15 M Na citrate, pH 7.0] for 5 minutes. The gel was placed upside down on a wick and the nitrocellulose membrane was placed on top of the gel. The transfer was carried out overnight with 10X SSC. The membrane was soaked in 6X SSC, dried

and UV irradiated to crosslink the DNA to the nitrocellulose membrane (Maniatis et al., 1982).

Polymerase Chain Reaction

A Perkin Elmer Cetus DNA Thermal Cycler was used for PCR. Synthetic primers were ordered for the *B. burgdorferi* Osp B gene (Advanced DNA Technologies Laboratory- BSBE, Texas A&M). *Taq* polymerase buffer (1X), $MgCl_2$ (25mM), 4dNTPs, 10μM of each primer (Osp B 571(+) and Osp B 1292(-)), Genomic *B. burgdorferi* DNA (1ng - strain 532) and *Taq* polymerase (2.5 U; Perkin Elmer Cetus) were incubated for 30 cycles. An agarose gel (0.8%) of the PCR products resulted in a single band of about 746 bp consistent with the Osp B gene fragment product desired.

Probe preparation

Random Primed DNA labeling kit (Boehringer Mannheim) was used to label the PCR generated Osp B internal probe. 50 ng of PCR product (denatured at 100°C for 10 minutes), 50 μCi[α^{32}P]dATP (Amersham), 1:1:1 mixture of dGTP, dTTP and dCTP, and Klenow enzyme were incubated at 37°C for 30 minutes. The reaction was stopped with oligo-labeling stop mix [20 mM NaCl, 20 mM Tris pH 7.5, 2mM EDTA and 0.25% SDS]. Unincorporated nucleotides were removed by G-25 sephadex spin column purification and the probe was counted using a scintillation counter.

The telomere probe was obtained by end-labeling (Boehringer Mannheim-DNA Tailing Kit) a synthetic oligonucleotide (pTL16 - sequence: 5' GTA TAG AGT ATT TTG ACT CAA AAC TTT ACC C 3') ordered from the Advanced DNA Technologies Laboratory. 200 μCi[α^{32}P]dATP (Amersham) was dried down and resuspended in 4.5μl of H_2O and then mixed with Tailing buffer 1, $CoCl_2$, 50 ng of pTL16 oligo and Terminal Transferase and incubated at 37°C for 20 minutes. The reaction was stopped with oligo labeling stop mix and unincorporated dNTPs were removed with a G-25 sephadex spin column. The probe was counted using a scintillation counter.

Prehybridization/Hybridization

Nitrocellulose filters (Schleicher and Schuell Pure Nitrocellulose) were prehybridized at 65°C for 1 hour in 20 mL of a prehybridization/hybridization mix

[6X SSC, 1% SDS, 0.1% $NaPP_i$, 100μg/mL Herring sperm carrier DNA and 1% Blotto (Carnation Non-Fat Milk)].

Labeled Osp B probe ($1x10^6$cpm/mL) was added to 10 mL Prehybridization/Hybridization mix and hybridized at 65°C overnight. Blots were washed with Wash I [4X SSC, 0.1% SDS, 0.1% $NaPP_i$] for 45 minutes and Wash II [1X SSC, 0.1% SDS, 0.1% $NaPP_i$] two times for 30 minutes.

End-labeled telomere probe (pTL16)($1x10^6$cpm/mL) was added to 10 mL Prehybridization/Hybridization mix and hybridized at 63°C for 3 hours. Blots were washed with Wash II [1X SSC, 0.1% SDS, 0.1% $NaPP_i$] for 15 minutes at room temperature and then twice at 63°C for 5 minutes each.

Blots were then exposed to autoradiographic film (XAR film- Kodak) with one intensifying screen (FisherBiotech) at -70°C overnight and then put back on film for varying lengths of time depending on the strength of the signal observed.

Oligonucleotide Gel Purification

Synthetic oligonucleotides of the 3' left end of the 16 kb and 49 kb linear plasmids of *B. burgdorferi* (L3- sequence: 3' ATA TTA (A/T)AA A(A/T)(T/A) AAT CAT 5' (with the brackets signifying an equal probability of A or T) were ordered from the Advanced DNA Technologies Laboratory (BSBE, Texas A&M). 210 μg of L3 was mixed in a 1:1 ratio with 100% formamide and run on a 20% polyacrylamide gel [0.6X TBE, 20% Acrylamide, 1% bisacrylamide, 50% urea (8M)]. The gel was run at 600V until the BPB dye had run 20 cm from the well. DNA was visualized with a TLC fluorescent plate by back shadowing and the darkest band, corresponding to the pure 18 base oligonucleotide, was cut out. The gel was diced, mixed with TE [10 mM Tris, pH 7.5, 1 mM EDTA] and rocked overnight at room temperature. Half of the resin from a C18 sep-pak column was packed into a 1mL syringe, wetted with 100% methanol and rinsed with 1-2 column volumes of TE. The eluted DNA was loaded onto the column, rinsed with 3-5 volumes of water and eluted in a 50% methanol, 50% H_2O mixture. The DNA was then dried down in a speed vac and resuspended in H_2O (Shippen-Lentz, personnal communication, 1993).

Telomerase Assay

Telomerase assay cocktail [1X Telomerase buffer, 100μM dTTP, 1.25 μM [α^{32}P]dGTP, 33 mM potassium glutamate, 89mM EGTA,11mM $MgCl_2$], *Oxytricha* cell extract and 50-100 ng of input oligonucleotide substrate were

incubated at 30°C for 1 hour. Telomerase buffer (10X) contains 500mM Tris, pH 8.0, 19 mM Spermidine, 10 mM DTT and 1M NaOAc. The reaction was stopped with Telomerase Stop buffer [21 mM EDTA, 10 mM Tris pH 8.0]. The DNA was phenol:chloroform extracted and then precipitated with NH_4OAc, glycogen and 95% ethanol. After washing with 70% ethanol and drying with a speed vac, the DNA was resuspended in xylene cyanol dye, denatured at 100°C for 2 minutes, loaded onto a 10% polyacrylamide gel and run in 0.6X TBE at 500 V for approximately 4 hours. Control reactions consisted of *Oxytricha* oligonucloetide, human oligonucleotide, no oligonucleotide and kinased *Oxytricha* oligonucleotide (no telomerase reaction). The kinased oligonucleotide indicates the location of the input *Oxytricha* oligonucleotide (Shippen Lentz, personnal communication, 1993; Shippen-Lentz and Blackburn, 1989). The polyacrylamide gel (10%) contained 8M urea, 0.6X TBE and 10% acrylamide/bisacylamide (29:1).

Discussion & Conclusion

There are several possible explanations for the failure of the two dimensional agarose gel technique. Since the Osp B internal probe failed to detect any replication intermediates even after 3 weeks of exposure, it is possible that the concentration of replicating DNA is significantly lower than the concentration of linear DNA. Several more weeks of exposure might possibly be required for the detection of replication intermediates. In fact, after 3 weeks of exposure, other signals started appearing but these could not be satisfactorily discriminated against the increase in background. Another possible reason for the failure of the technique, is that for some unknown reason, the DNA that I isolated from the *B. burgdorferi* cultures might not have been replicating. I would suggest either enriching for replication intermediates or synchronizing the *B. burgdorferi* into mid-log phase. Another solution might be to use a more sensitive signal/signal detection system that does not require such a long period of time to produce a signal. I recently did an experiment to test the extent of Hind III digestion on the *B. burgdorferi* DNA for varying amounts of time. It is apparent that digestion is complete after 1 hour. Hence, it is possible that the restriction enzyme or contaminants of the restriction enzyme preparation degraded the replication intermediates. A solution to this would be to run a partial digest and then analyze the digest with two dimensional agarose gel electrophoresis. If the restriction enzyme is responsible for digesting the replication intermediates, the partial

digest will allow the detection of the replication intermediates. Finally, since I never managed to actually see replication intermediate arcs on the two dimensional gels that I ran, it is possible that I made an error in the technique. This possibility could be solved by obtaining a positive control from one of the authors of the 2D technique papers and attempting the experimental procedure again.

We cannot assume that because the two dimensional technique failed to detect replication intermediates that *B. burgdorferi* must use some other method to replicate its telomeres, because even the Osp B internal probe failed to detect replication intermediates that would be predicted for any replicating DNA.

The telomerase assay resulted in weak bands in the *B. burgdorferi* oligonucleotide lane. Since the weak bands are also present in the negative control (no oligonucleotide) lane, they are probably due to DNA polymerase activity (Shippen-Lentz, personnal communication, 1993). It could be argued that the reason the telomerase assay failed to work was because the *Oxytricha* telomerase cannot use the *B. burgdorferi* oligonucleotide as a substrate. This does not prove that there is no *B. burgdorferi* telomerase. But since it is known that the *Oxytricha* telomerase can use a wide range of sequences as substrates, so only if a strong reaction was detected could we speculate the possible existence of a *B. burgdorferi* telomerase. Since *Oxytricha* telomerase failed to add telomeric repeats onto the *B. burgdorferi* oligonucleotide, it implies that there probably is no telomerase in *B. burgdorferi* . If there is a telomerase-like enzyme present in *B. burgdorferi* , it must be very different from the *Oxytricha* telomerase in its substrate specificity.

In conclusion, I would like to say that despite the fact that my experiments did not succeed like I had anticipated, I feel I have learnt a variety of useful experimental techniques and benefitted from being exposed to a laboratory for a year. I suggest that the telomerase assay be repeated to obtain a prettier gel with the *Oxytricha* oligonucleotide positioned in a lane next to the *B. Burgdorferi* oligonucleotide to prove that the repeats added onto the *B. burgdorferi* are not 8 base repeats characteristic of *Oxytricha* telomerase. I also suggest that the two dimensional agarose gel technique be attempted on a positive control that is known to generate replication intermediate arc patterns. Once it is known that the technique is working, *B. burgdorferi* cells should be synchronized in mid-log phase, replication intermediates should be enriched for and partial restriction enzyme digests should be used for the two dimensional agarose gel analysis.

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