Variation in the size of the *ospA*-containing linear plasmid, but not the linear chromosome, among the three *Borrelia* species associated with Lyme disease

D. SCOTT SAMUELS,* RICHARD T. MARCONI and CLAUDE F. GARON

Laboratory of Vectors and Pathogens, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840, USA

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The aetiological agents of Lyme disease form a phylogenetically heterogeneous group, composed of three species, *Borrelia burgdorferi*, *Borrelia garinii*, and group VS461. We have compared the sizes of the linear plasmid that carries the genes encoding the major outer-surface proteins OspA and OspB as well as the size and structure of the chromosome among the Lyme disease spirochaetes. We have found differences in the sizes of the *ospA*-containing plasmids, but not the linear chromosomes among the three species. The *ospA*-containing plasmid size of 50 kb in *B. burgdorferi* isolates is significantly smaller than the size of 55 kb in *B. garinii* isolates and 56 kb in group VS461 isolates. The chromosome was found to be linear in all three *Borrelia* species, but not significantly different in size.

Introduction

Lyme disease was first described as an outbreak of arthritis in Old Lyme, Connecticut, and nearby communities (Steere *et al.*, 1977). The infection presents a variety of clinical manifestations and is also found in Europe, Asia and Australia (Steere, 1989). The causative agent of Lyme disease was first isolated from its tick vector (Burgdorfer *et al.*, 1982) and then from human patients (Steere *et al.*, 1983; Benach *et al.*, 1983). It is a eubacterium, *Borrelia burgdorferi* (Johnson *et al.*, 1984), in the spirochaete phylum (Woese, 1987; Paster *et al.*, 1991). Isolates of *B. burgdorferi* were found to represent more than a single species by several criteria, including 16S RNA sequence (Adam *et al.*, 1991; Marconi & Garon, 1992*a, b*), DNA–DNA hybridization (Postic *et al.*, 1990; Baranton *et al.*, 1992), multilocus enzyme electrophoresis (Boerlin *et al.*, 1992), restriction fragment length polymorphisms (Postic *et al.*, 1990; Ståhlhammar-Carlemalm *et al.*, 1990; Adam *et al.*, 1991; Marconi & Garon, 1992*a, c*; Baranton *et al.*, 1992) and arbitrarily primed polymerase chain reaction (Welsh *et al.*, 1992). The species designation *B. burgdorferi* has been amended to delineate a group of North American and European isolates previously referred to as *B. burgdorferi sensu*

stricto (Baranton *et al.*, 1992; List No. 43, 1992), whilst a new species designation, *B. garinii*, delineates a group of European and Asian isolates previously referred to as group 20047 (Baranton *et al.*, 1992; List No. 43, 1992). A third species (Marconi & Garon, 1992*b*), composed of European and Asian isolates, is currently referred to as group VS461 (Baranton *et al.*, 1992). These three species represent three of the four genomic groups based on restriction fragment length polymorphisms of the *fla*, HSP60 and HSP70 genes (Wallich *et al.*, 1992). The three species can be differentiated by reactivity in Western blots with monoclonal antibodies (Jonsson *et al.*, 1992; Baranton *et al.*, 1992), in slot blots with 16S rRNA-directed probes (Marconi *et al.*, 1992) and in the polymerase chain reaction with 16S rDNA-directed primer sets (Marconi & Garon, 1992*c*). Another set of polymerase chain reaction primers can differentiate *B. burgdorferi* from the other two species (Rosa *et al.*, 1991).

B. burgdorferi has an unusual genome composed of a linear chromosome of about 950 kb (Ferdows & Barbour, 1989; Baril *et al.*, 1989; Davidson *et al.*, 1992; Casjens & Huang, 1993) and variable numbers of linear and circular plasmids that range in size from about 5 to 60 kb (Hyde & Johnson, 1984; Barbour & Garon, 1987; Barbour, 1988; Simpson *et al.*, 1990; Samuels & Garon, 1993). The linear and circular plasmids have copy numbers of about one per chromosome (Hinnebusch & Barbour, 1992; Casjens & Huang, 1993). Some of these plasmids are lost during *in vitro* cultivation (Schwan *et*

* Author for correspondence. Tel. +1 406 363 9224; fax +1 406 363 9204; e-mail scott@rml.niaid.nih.gov.

Abbreviation: TAFE, transverse alternating-field electrophoresis.

al., 1988; Barbour, 1988; Norris *et al.*, 1992). The linear plasmids have covalently closed hairpin ends (Barbour & Garon, 1987) with structures and sequences similar to those of African swine fever virus telomeres (Hinnebusch & Barbour, 1991). Only four genes have been mapped currently to plasmids; the remainder are on the chromosome (Old *et al.*, 1992; Casjens & Huang, 1993). The genes for the major outer-surface proteins OspA and OspB are on a 49 kb linear plasmid in B31 (Barbour & Garon, 1987) and a slightly larger plasmid in ACAI and R-IP90 (Jonsson *et al.*, 1992). The gene for OspD is on a 38 kb linear plasmid in B31 that is often lost upon *in vitro* cultivation (Norris *et al.*, 1992). The gene for OspC, a major immunodominant protein, is on a 26 kb circular plasmid in all three Lyme disease species (Marconi *et al.*, 1993a) and therefore represents the first *Borrelia* gene mapped to a circular DNA molecule.

Previously, heterogeneity in the size of the *ospA*-containing plasmid has been demonstrated (Barbour, 1988; Stålhammar-Carlemalm *et al.*, 1990; Jonsson *et al.*, 1992; Rosa *et al.*, 1992). This linear plasmid is found in virtually all Lyme disease spirochaete isolates. In the current study, we systematically addressed whether these differences in plasmid size correlated with species. In addition, we examined whether there were differences in the size and structure of the chromosome. Our results indicate that the chromosomes are linear and similar in size, but that the *ospA*-containing plasmids differ in size among the three *Borrelia* species associated with Lyme disease.

Methods

Bacterial strains and media. A total of 29 isolates of Lyme disease spirochaetes (12 *B. burgdorferi*, 9 *B. garinii* and 8 group VS461) were used in this study, 25 in the analysis of the *ospA*-containing plasmid and 17 in the analysis of the chromosome. These isolates have been identified to the species level and described previously (Marconi & Garon, 1992c; Baranton *et al.*, 1992). Bacteria were grown at 34 °C in BSK II medium (Barbour, 1984) with 0.56% (w/v) gelatin.

Preparation of DNA and agarose gel electrophoresis. For conventional gel electrophoresis, DNA was isolated as described by Barbour *et al.* (1985), fractionated on 0.2% agarose gels (SeaKem GTG, FMC Bioproducts) in recirculated 1 × TAE (40 mM-Tris, 20 mM-acetate, 1 mM-EDTA) at 12 V (0.4 V cm⁻¹) for 88 h, and stained with ethidium bromide as described previously (Samuels & Garon, 1993). For pulsed-field gel electrophoresis, DNA was prepared in agarose plugs essentially as described by Ferdows & Barbour (1989) except that the final concentration of agarose (Incert, FMC) was 1%. Plugs were stored in 0.5 M-EDTA (pH 8.0) at 4 °C and equilibrated before use in TE* (10 mM-Tris/HCl, pH 8.0, 10 mM-EDTA). DNA was fractionated on 0.8% agarose gels by transverse alternating-field electrophoresis (TAFE) in 0.25 × TBE (22 mM-Tris, 22 mM-borate, 0.5 mM-EDTA) using a GeneLine II system (Beckman) set at 13 °C. Electrophoresis was at 350 mA with pulse times of 60, 75, 90, 105 and 120 s for 12 h each (60 h total). Molecular mass standards were either λ DNA (incubated at 4 °C to form dimers) and λ DNA-Mono Cut Mix (New England Biolabs) or *Saccharomyces cerevisiae* strain YNN 295

chromosomal DNA (Beckman). Plasmid and chromosome sizes in kb were determined using an NA2 nucleic acid analyser (BRL) and were the means of three independent electrophoretic assays for each isolate. Molecular size difference between species were calculated by averaging the mean values for individual isolates of a particular species and performing independent *t*-tests, with significance judged at the 0.05 level. Molecular sizes are presented as the mean ($n = 5-9$ isolates of each species) $\pm$ SD.

Southern blot analysis. Fractionated DNA was vacuum-blotted onto GeneScreen membranes (DuPont NEN) using a VacuGene system (Pharmacia LKB) and UV-crosslinked using a Stratalinker (Stratagene) according to the manufacturers' instructions. The membrane was probed with a 5' end-labelled oligonucleotide (5' dGGCTGCTAACA-TTTTGCTTACATGC 3') which represents nucleotides 46-70 of the coding region of *ospA* from *B. burgdorferi* isolate B31 (Bergström *et al.*, 1989). This oligonucleotide probe specifically recognizes the *ospA* gene from all isolates from each of the three Lyme disease-associated species tested (Marconi *et al.*, 1993b; R. T. Marconi, D. S. Samuels, T. G. Schwan, M. E. Konkel & C. F. Garon, unpublished data). Hybridization was at 37 °C as described previously (Marconi *et al.*, 1992). The membranes were washed twice in 2 × SSC/0.1% SDS at 37 °C and once in 0.1 × SSC/0.1% SDS at 37 °C, and exposed to XAR film (Kodak) with intensifying screens at -70 °C.

Results

Species variation in the size of the ospA-containing plasmid

The size of the *ospA*-containing linear DNA molecule, the largest of the plasmids in most isolates, was determined by constant-field electrophoresis in 0.2% agarose gels (a representative gel is shown in Fig. 1a), which have a linear range of separation from about 10 kb to over 60 kb (Sambrook *et al.*, 1989). The *ospA* gene was identified by Southern blotting with an oligonucleotide probe to a conserved region (a representative blot is shown in Fig. 1b). The size was determined by regression from the size of intact and restricted λ DNA markers on at least two electrophoretic assays. We found that the *ospA*-containing plasmid from *B. burgdorferi* isolates (1352, 20004, Illinois 1, 3028, IP2A, 25015, B31, CA3 and Sh-2-82) had a mean size of 50.2 ± 0.8 kb. The *ospA*-containing plasmid from *B. garinii* isolates (G1, G25, VS102, R-IP90, FRG, 20047, 153, G2 and UO1) was larger with a greater variance (mean size of 55.5 ± 1.8 kb). The *ospA*-containing plasmid from group VS461 isolates (R-IP3, R-IP21, VS461, PGau, J1, UMO1 and ECM1) had the largest mean size and variance (56.4 ± 2.3 kb) of the Lyme disease spirochaetes. The size of the plasmid from *B. burgdorferi* isolates was significantly smaller than that from *B. garinii* isolates ($P = 2 \times 10^{-8}$) and group VS461 isolates ($P = 1 \times 10^{-8}$). However, the sizes of the plasmids from the latter two species were statistically indistinguishable ($P = 0.4$). The mean sizes determined for each species are consistent with those previously reported for individual isolates. Direct contour length measurements by electron microscopy have

Fig. 1. Size of the *ospA*-containing plasmid among the *Borrelia* species associated with Lyme disease. (a) Ethidium bromide-stained 0.2% agarose gel of DNA from representative isolates of *B. burgdorferi* (1352, 20004, Illinois 1, 3028, IP2A and 25015), *B. garinii* (G1, G25, VS102 and R-IP90), and group VS461 (R-IP3, R-IP21 and VS461). Electrophoresis conditions were designed to maximize separation in the size range of the *ospA*-containing plasmid and therefore some smaller plasmids were not retained on the gel. (b) Southern blot of the gel shown in (a) probed with an oligonucleotide from the *ospA* gene.

revealed the size of the largest linear plasmid of *B. burgdorferi* isolate B31 to be 49 kb (Barbour & Garon, 1987) or 53 kb (Stålhammar-Carlemalm *et al.*, 1990), and those of the *B. garinii* isolates NE83 and NE2 to be 58 and 57 kb, respectively (Stålhammar-Carlemalm *et al.*, 1990). Pulsed-field gel electrophoresis has indicated that the *osp* operon-containing plasmid of *B. burgdorferi* isolate B31 is smaller than those of the *B. garinii* isolate R-IP90 and the group VS461 isolate ACAI (Jonsson *et al.*, 1992), and that the *osp* operon-containing plasmid of the *B. garinii* isolate G2 is larger than those of the *B.*

Fig. 2. Size and structure of the chromosome among the *Borrelia* species associated with Lyme disease. Ethidium bromide-stained TAFE gel (0.8% agarose) of DNA from representative isolates of *B. burgdorferi* (Sh-2-82, 297, 20004, 27985, 3028, Veery and Illinois 1), *B. garinii* (VS102, R-IP90, G1, G25 and FRG), and group VS461 (R-IP21, VS461, R-IP3, J1 and B023). TAFE conditions were designed to maximize separation in the size range of the chromosome and therefore linear plasmids were not retained on the gel. S.c. indicates linear yeast chromosome molecular mass markers.

burgdorferi isolates B31 and Sh-2-82 (Rosa *et al.*, 1992). Conventional gel electrophoresis has also shown that the approximately 50 kb (linear) plasmid of several *B. burgdorferi* isolates is smaller than those from *B. garinii* and group VS461 isolates (Barbour, 1988). Some isolates contain a linear plasmid larger than the *ospA*-containing plasmid (about 63 kb in the case of VS461) (Fig. 1 and data not shown).

The size of the linear chromosome is constant across species

The chromosome has been shown to be a linear molecule of about 950 kb in *B. burgdorferi* by its ability to enter pulsed-field electrophoresis gels (Ferdows & Barbour, 1989; Baril *et al.*, 1989) and its linear physical map (Davidson *et al.*, 1992; Casjens & Huang, 1993). By the criterion of entry into the gel, the chromosomes from all Lyme disease spirochaetes examined were linear (a representative gel is shown in Fig. 2), as were the chromosomes from the non-Lyme disease spirochaetes *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. coriaceae* and *B. anserina* (Marconi *et al.*, 1993c). The size of the

chromosome was interpolated by regression from the size of intact yeast chromosomes on three electrophoretic assays. The chromosome from *B. burgdorferi* isolates (Sh-2-82, 297, 20004, 27985, 3028, Veery and Illinois 1) had a mean size of 964 ± 11 kb. The chromosome from *B. garinii* isolates (R-IP90, G1, G25, FRG and VS102) had a smaller mean size and less variance (957 ± 4 kb). The chromosome from group VS461 isolates (R-IP21, VS461, R-IP3, J1 and BO23) was also smaller and had less variance than the *B. burgdorferi* chromosome, with a mean size of 958 ± 2 kb. Although the size of the chromosome from the *B. burgdorferi* isolates was slightly larger and more variable than the chromosomes from *B. garinii* and group VS461 isolates, the difference was statistically indistinguishable ($P = 0.2$). These values for chromosome size are similar to, but slightly larger than, those determined by clamped homogeneous electrode field (CHEF) pulsed-field gel electrophoresis and physical mapping, and they are consistent in that the *B. burgdorferi* chromosome was found to be slightly larger and more variable in size than those of the other two species (S. Casjens, personal communication).

Discussion

We have found that the three recently defined *Borrelia* species associated with Lyme disease have differences in the sizes of their *ospA*-containing plasmids but not their linear chromosomes. The *ospA*-containing plasmid from *B. burgdorferi* was statistically smaller than the corresponding DNA molecules in the other species. However, the size differences are not great enough to be of diagnostic value (with the possible exception of differentiating *B. burgdorferi* isolates from *B. garinii* and group VS461 isolates on the basis of *ospA*-containing plasmid size). Most isolates have unique plasmid profiles (Fig. 1a and data not shown), as demonstrated previously (Barbour, 1988; Schwan *et al.*, 1988; Ståhlhammar-Carlemalm *et al.*, 1990; Jonsson *et al.*, 1992; Norton Hughes *et al.*, 1992). Plasmid profiles were not, however, correlated with the phylogenetically established species (Fig. 1a and data not shown). The role of the 63 kb linear plasmid in VS461 and other isolates should be pursued, as this plasmid represents approximately 5% of the genome.

We initially attempted to size the *ospA*-containing plasmids by pulsed-field gel electrophoresis (TAFE) and found values larger than those obtained with constant-field gel electrophoresis (data not shown). This incongruity in molecular size was reported by Norton Hughes *et al.* (1992) and may also affect the chromosome size determination. We believe that conventional gel electrophoresis yields a more accurate size for plasmids because the values are consistent with those obtained by

direct electron microscopy contour length measurement (Barbour & Garon, 1987; Ståhlhammar-Carlemalm *et al.*, 1990). Perhaps the partial retardation of linear plasmid migration in pulsed-field gels is caused by the covalently closed ends (Barbour & Garon, 1987), in a similar fashion to the retardation of circular plasmid migration (Ferdows & Barbour, 1989; Marconi *et al.*, 1993c; W. O. Granath, L. Lubke & C. F. Garon, unpublished data).

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