ELSEVIER

FEMS Microbiology Letters 191 (2000) 199–203

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

A mutagenic PCR identifies isolates of *Borrelia garinii* responsible for Lyme borreliosis

Célia Mueller [a], Pierre Hutter [b], Roland Sahli [c], Anne-Gabrielle Bretz [a], Lina N. Toutoungi [a], Olivier Péter [a,*]

[a] *Infectious Disease and Immunology, Institut Central des Hôpitaux Valaisans, CH-1950 Sion, Switzerland*
[b] *Laboratory of DNA, Pathology, Institut Central des Hôpitaux Valaisans, CH-1950 Sion, Switzerland*
[c] *Centre Hospitalier Universitaire Vaudois, Institute of Microbiology, CH-1011 Lausanne, Switzerland*

Received 29 May 2000; received in revised form 17 August 2000; accepted 22 August 2000

Abstract

Borrelia garinii is one of the three major *Borreliae* responsible for Lyme borreliosis in Europe. We have characterized a protein of *B. garinii* (VS102) and a genomic fragment from the gene encoding this protein was cloned. The DNA sequence of the fragment showed high homology with a known gene of *B. burgdorferi* sensu stricto. The protein encoded by this gene in *B. burgdorferi* sensu stricto is a phosphocarrier protein (histidine-containing protein). A mutation T to G polymorphism at codon 57 was found to be specific to *B. garinii*. A PCR-based approach that allows the rapid detection of this mutation made it possible to specifically discriminate *B. garinii* from other *B. burgdorferi* genospecies with high sensitivity and specificity.

Keywords: *Borrelia garinii*; Lyme borreliosis; Mutagenic polymerase chain reaction; Phosphocarrier protein HPR

1. Introduction

Borrelia garinii, along with *B. burgdorferi* sensu stricto and *B. afzelii*, is one of the three major *Borreliae* responsible for Lyme borreliosis [1]. It is associated with the majority of neuroborreliosis cases in Europe [2–4]. *B. garinii* appears to be a very heterogeneous group which includes several serogroups [5] exhibiting divergences at the genetic level [1,6]. In Europe, *B. garinii* is the most common Borrelia species [7,8]. Its geographical distribution appears to be restricted to Europe and Asia, although seabirds, as a potential reservoir, may spread *B. garinii* between both hemispheres via a specific vector, *Ixodes uriae* [9]. It has been demonstrated that birds of the genus *Turdus* are reservoirs for *B. garinii* and *B. valaisiana*, based on direct isolation of these *Borrelia* species and xenodiagnostics [10].

After two-dimensional gel electrophoresis, followed by Western blot, two co-migrating proteins of about 11 kDa were identified on Immobilon membrane stained with amido black. One of these proteins showed a species-specific polymorphism, which we characterized at the DNA level. Our data suggest that this single nucleotide variation can be used as a molecular tool for the identification of *B. garinii* isolates.

2. Materials and methods

2.1. Borrelia isolates and other unrelated bacteria

All *Borrelia* isolates were grown in BSK II medium [11] at 34°C and were harvested during exponential-phase growth, washed twice in phosphate-buffered saline (pH 7.4) ([12], appendix B12) containing 5 mM $MgCl_2$. Pellets were resuspended in distilled water and frozen at −80°C until use. The 100 Eurasian and four American *Borrelia* isolates are listed in Table 1. They belong to six genospecies and originated from ticks ($n = 69$), rodents ($n = 8$), humans ($n = 25$) and birds ($n = 2$). All the isolates used were typed by restriction fragment length polymorphism (RFLP) of *rrf* (5S)–*rrl* (23S) intergenic spacer amplicons [6].

* Corresponding author. Tel.: +41 (27) 603 47 90; Fax: +41 (27) 603 48 93; E-mail: olivier.peter@ichv.vsnet.ch

0378-1097/00/$20.00
PII: S0378-1097(00)00390-6

Table 1
Origins of *B. burgdorferi* sensu lato and of other bacterial strains used in this study

Isolate	Biological origin	Geographical origin	Isolate	Biological origin	Geographical origin
B. afzelii			FAR02	*I. uriae*	Denmark
934U	*Apodemus agrarius*	Korea	FIS01	*I. uriae*	Iceland
A100S	Human (skin)	The Netherlands	G25	*I. ricinus*	Sweden
A26S	Human (skin)	The Netherlands	HP3	*I. persulcatus*	Japan
A39S	Human (skin)	The Netherlands	Ip89[a]	*I. persulcatus*	CIS
A42S	Human (skin)	The Netherlands	Ip90	*I. persulcatus*	CIS
A45aS	Human (skin)	The Netherlands	M50	*I. ricinus*	The Netherlands
A51T	*Ixodes ricinus*	The Netherlands	M63[a]	*I. ricinus*	The Netherlands
A58T	*I. ricinus*	The Netherlands	N34	*I. ricinus*	Germany
A76S	Human (skin)	The Netherlands	NBS16[a]	*I. ricinus*	Sweden
ACA1	Human (skin)	Sweden	NE2	*I. ricinus*	Switzerland
DK3	Human (skin)	Denmark	NE83	*I. ricinus*	Switzerland
DK8	Human (skin)	Denmark	NT29[a]	*I. persulcatus*	Japan
F1	*I. ricinus*	Sweden	P/Br	Human (CSF)	Germany
IP3 (Iper3)	*Ixodes persulcatus*	CIS	PD89	Human (blood)	China
Iper	*I. persulcatus*	Japan	T25	*I. ricinus*	Germany
M55	*I. ricinus*	The Netherlands	TN	*I. ricinus*	Germany
M7	*I. persulcatus*	China	VS3[a]	*I. ricinus*	Switzerland
NE36	*Clethrion glareolus*	Switzerland	VS102[a]	*I. ricinus*	Switzerland
NE39	*C. glareolus*	Switzerland	VS156	*I. ricinus*	Switzerland
Pwud I	Human (skin)	Germany	VS244	*I. ricinus*	Switzerland
SMS1	*Apodemus flavicollis*	Sweden	VSBM	Human (CSF)	Switzerland
UM01	Human (skin)	Sweden	VSBP	Human (CSF)	Switzerland
VS25R-Or	*A. flavicollis*	Switzerland	VSDA	Human (CSF)	Switzerland
VS42R-R	*Apodemus sylvaticus*	Switzerland	*B. japonica*		
VS461[a]	*I. ricinus*	Switzerland	COW611c	*Ixodes ovatus*	Japan
B. burgdorferi sensu stricto			Fi340	*I. ovatus*	Japan
A44S	Human (skin)	The Netherlands	FiAE2	*A. speciosus*	Japan
B31[a]	*Ixodes dammini*	USA	FiEE2	*Eothenomys smithi*	Japan
CA-5	*Ixodes pacificus*	USA	HO14	*I. ovatus*	Japan
Charlie Tick	*I. dammini*	USA	IKA2	*I. ovatus*	Japan
Geho	Human (skin)	Germany	*B. lusitaniae*		
IP1	Human (CSF)	France	BR41	*I. ricinus*	Czech Republic
IP2	Human (CSF)	France	IR345	*I. ricinus*	Byelorussia
IP3	Human (CSF)	France	POTIB1	*I. ricinus*	Portugal
IRS	*I. ricinus*	Switzerland	POTIB2	*I. ricinus*	Portugal
M14	*I. ricinus*	The Netherlands	POTIB3	*I. ricinus*	Portugal
NE48	*I. ricinus*	Switzerland	*B. valaisiana*		
NE50	*I. ricinus*	Switzerland	AG1	*I. ricinus*	Switzerland
NE56	*I. ricinus*	Switzerland	AR-2	*I. ricinus*	The Netherlands
BE1 (P1G)	Human (synovial fluid)	Switzerland	F10.8.94	*I. ricinus*	Germany
VS2	*I. dammini*	USA	Frank	*I. ricinus*	Germany
VS44	*I. ricinus*	Switzerland	M19	*I. ricinus*	The Netherlands
VS73	*I. ricinus*	Switzerland	M52	*I. ricinus*	The Netherlands
VS82	*I. ricinus*	Switzerland	M53	*I. ricinus*	The Netherlands
VS106	*I. ricinus*	Switzerland	M57	*I. ricinus*	The Netherlands
VS108	*I. ricinus*	Switzerland	NE168	*I. ricinus*	Switzerland
VS115	*I. ricinus*	Switzerland	NE218	*Turdus merula*	Switzerland
VS134	*I. ricinus*	Switzerland	NE223	*T. merula*	Switzerland
VS146	*I. ricinus*	Switzerland	VS116	*I. ricinus*	Switzerland
VS161	*I. ricinus*	Switzerland	Other bacterial strains tested		
VS219	*I. ricinus*	Switzerland	*Staphylococcus aureus*		
B. garinii			*Staphylococcus epidermidis*		
387	Human (CSF)	Germany			
935T[a]	*I. persulcatus*	Korea	*Escherichia coli*		
A19S[a]	Human (skin)	The Netherlands	*Salmonella* sp.		
A77C	Human (CSF)	The Netherlands	*Shigella sonnei*		
AR-1	*I. ricinus*	The Netherlands	*Klebsiella pneumoniae*		
BITS	*I. ricinus*	Italy	*Pseudomonas aeruginosa*		
FAR01	*Ixodes uriae*	Denmark			

[a]Sequenced isolates.

Seven other unrelated bacterial strains, shown in Table 1, were tested and used as a control of PCR specificity.

2.2. Protein separation

Proteins of the isolate VS102 (*B. garinii*) were separated by two-dimensional gel electrophoresis. In the first dimension immobilized pH gradient strips (Pharmacia-LKB, Uppsala, Sweden), with a gradient from pH 3 to 10, were used. The second dimension was performed on polyacrylamide gel (gradient from 9 to 16%). After transfer to Immobilon P (polyvinylidene difluoride (PVDF) membrane, Millipore, Bedford, MA, USA) the membrane was stained with amido black. The protein was sequenced with an automated protein microsequencer from the PVDF membrane (SWISS-2DSERVICE, sequencing service, Geneva, Switzerland). Molecular techniques were carried out (see Section 3) as described in Maniatis et al. [12].

2.3. Mutagenic PCR

A bacterial suspension (20 or 50 μl, protein concentration 1 mg ml^{-1}) was heated for 5 min at 95°C for lysis. The PCR reaction was carried out in a 50-μl reaction mixture containing 2 μl of bacterial lysate (Taq: 2.5 U, nucleotides 200 μM). The upstream primer was 5′-ATGGTAAAAAAAGAAGC-3′ and the downstream primer was 5′-CCTCACCCTCAGCACATATCAAAAGCTTTTTACCAG-3′. A Biometra T-Gradient (Biometra, Göttingen, Germany) was used and cycling conditions were as follows: 95°C for 15 min followed by 29 cycles (94°C for 1 min, 50°C for 1 min, 72°C for 1 min) and a final elongation at 72°C for 5 min. The PCR product (10 μl) was electrophoresed through a 3% agarose gel (Agarose STG, Eurobio, Les Ulis, France) in 1×TBE ([12], appendix B23), for 20 min at 100 V.

2.4. Digestion with PvuII

A total of 40 μl PCR products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany). 15 μl of purified PCR products were incubated with 2 U of *Pvu*II (Gibco BRL, Life Technologies, Basel, Switzerland) for 1 h at 37°C, with an additional 2 U of enzyme for another 2 h of incubation. Products of digestion (8 μl) were analyzed through a 3% agarose gel (Agarose STG Eurobio) in 1×TBE for 30 min at 90 V.

2.5. Sequencing PCR

Sequencing of the mutagenic PCR fragment was performed with upstream primer 5′-TGTAAAACGACGGCCAGTATGGTAAAAAAAGAAGC-3′ and downstream primer 5′-AAATCCTTCTAAAATTCA-3′ used to amplify DNA. The amplification reactions were optimized and carried out in Biometra T-Gradient. The cycling conditions were: 95°C for 15 min followed by 5 cycles of (95°C for 1 min, 50°C for 1 min, 72°C for 1 min) and 30 cycles of (95°C for 1 min, 43°C for 1 min, 72°C for 1 min, 72°C for 1 min).

2.6. Nucleotide sequence accession numbers

The full-length sequence of the gene encoding the phosphocarrier protein (histidine-containing protein) of *B. garinii* (VS102 isolate) has been assigned the GenBank accession number AF291154 and partial sequences of the same gene in *B. garinii* isolates: VS3 (AF291150), 935T (AF291151), NT29 (AF291152), NBS16 (AF291153), IP89 (AF293454), A19S (AF293455), M63 (AF293456) and in *B. afzelii* VS461 reference strain (AF291155).

3. Results and discussion

Following two-dimensional gel electrophoresis and transfer onto Immobilon membrane, the membranes were stained with amido black. A cluster of two spots was observed at 11 kDa. The larger spot with an estimated p*I* of 5.7 was sequenced. The N-terminal sequence of the first 14 amino acids was determined and subsequently confirmed from a second preparation. From this sequence we designed a degenerate oligonucleotide probe which was used on Southern blots of several restriction digests (*Eco*RI, *Eco*RV, *Bgl*II, *Pst*I and *Hin*dIII) of genomic DNA from *B. garinii*. An *Eco*RI and a *Hin*dIII fragment each showed strong homology to the probe. Both fragments were cloned in plasmids and sequenced.

Results from DNA sequencing indicated a very high homology with a known sequence of *B. burgdorferi* sensu stricto (TIGR database) [13] at positions 569 283–569 544, referred to as BB557 HPR (histidine-containing protein). This protein is a phosphocarrier protein (phosphoenolpyruvate:phosphotransferase system) well conserved in bacteria (e.g. *E. coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae*), and involved in fructose uptake. Using primers tailed with the M13–21 sequence, we amplified the relevant gene segment from several *Borrelia* isolates. This segment's sequence was then determined in five *B. garinii*, one *B. burgdorferi* sensu stricto and one *B. afzelii* isolates. Comparative analysis of all seven sequences showed 95% identity between these *Borrelia* isolates (Fig. 1). All five *B. garinii* isolates had an identical substitution at nucleotide 169, where a G was found instead of a T as in the other two species. This substitution leads to an amino acid change at position 57, where in *B. garinii* an alanine replaces the serine residue, typically found in the other isolates. Because of the lack of a restriction site at positions 167–170, we designed a mutagenic PCR system as reported in Fig. 1, in order to screen a larger number of *Borrelia* isolates. The downstream primer contained a base substitution creating a restriction site recognized by

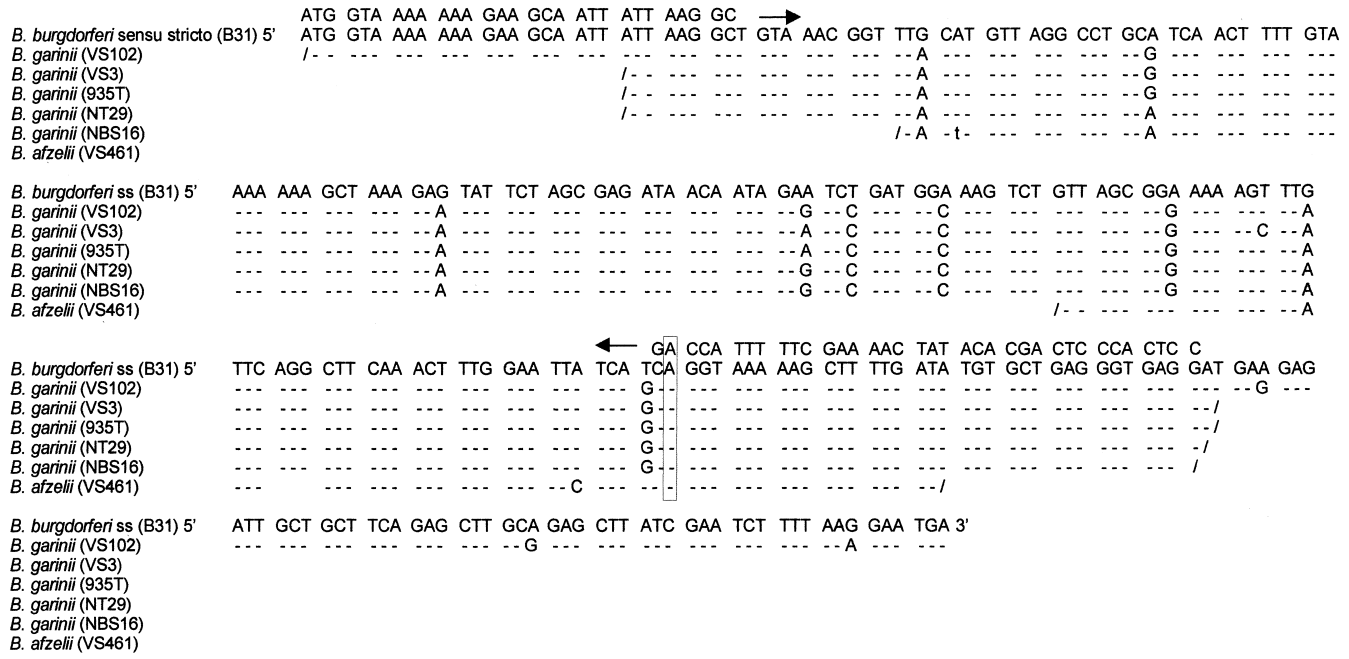

Fig. 1. Complete sequence of the phosphocarrier HPR gene of *B. burgdorferi* sensu stricto (B31). Polymorphisms observed in *B. garinii* isolates (VS102, VS3, 935T, NT29, NBS16) and *B. afzelii* (ACA1) are shown. Sequences followed by arrows correspond to the primers designed for mutagenic PCR. The box represents the critical nucleotide used for mutagenic PCR.

*Pvu*II (3′-GTC/GAC-5′). Thus amplification of the sequence from *Borrelia* isolates carrying a G at position 169, subsequently digested by *Pvu*II, generates two fragments of 169 bp and 36 bp, respectively. The isolates with undigested amplicon showed a single fragment of 205 bp. We analyzed a total of 104 isolates by PCR belonging to six species of *B. burgdorferi* sensu lato (Table 1). Using this system we were able to confirm the presence of the G polymorphism (Fig. 2) in all but two (Ip89, A19S) of the *B. garinii* isolates ($n = 31$). These two isolates were specifically chosen with respect to their particular RFLP of *rrf–rrl* intergenic spacer amplicons [6]. The other isolates were identical to *B. burgdorferi* sensu stricto (25 *B. burgdorferi* sensu stricto, 25 *B. afzelii*, 12 *B. valaisiana*, six *B. japonica* and five *B. lusitaniae*). Three additional *B. garinii* isolates were sequenced including Ip89 and A19S, in order to confirm the unusual polymorphism of these two isolates. The specificity of the PCR was confirmed using strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Salmonella* sp., *Shigella sonnei*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, showing no amplification.

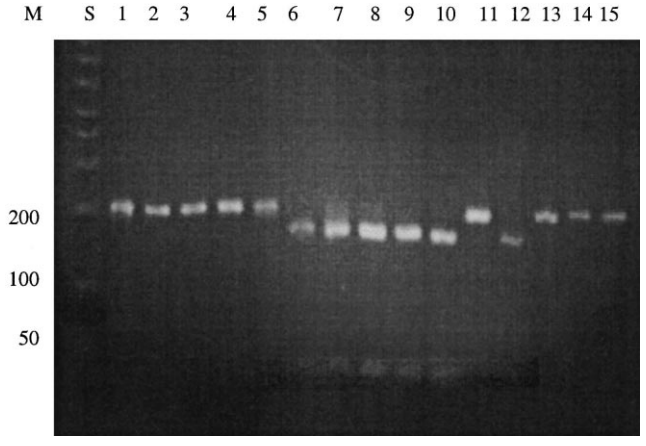

Fig. 2. Amplicons digested with *Pvu*II and electrophoresed in 3% agarose gel. Lane M: molecular size (in bp). Lane S: DNA ladder 50–2000 bp. Lanes 1–5: *B. burgdorferi* sensu stricto (VS82, VS106, VS108, VS115, VS146). Lanes 6–10: *B. garinii* (387, A77, M63, FAR01, FAR02). Lane 11: *B. afzelii* (A26S). Lane 12: *B. garinii* (M50). Lanes 13 and 14: *B. afzelii* (AS8T, F1). Lane 15: *B. valaisiana* (NE168).

Our results have determined that DNA sequencing of *B. garinii* phosphocarrier protein HPR (histidine-containing protein) showed 95% identity with the other species of *B. burgdorferi* sensu lato. Moreover, a single nucleotide substitution in the gene encoding this protein changed amino acid 57 from a serine in *B. burgdorferi* sensu stricto, *B. afzelii*, *B. valaisiana*, *B. lusitaniae* and *B. japonica* to an alanine in *B. garinii*. Our data indicate that this DNA sequence variation, occurring in a well conserved region, may be used as a reliable marker to identify most *B. garinii* isolates. Additional PCR systems for identification of *B. garinii*, similar to the one reported here, may prove useful to type some particular isolates or to confirm genotyping in this frequent and particularly heterogeneous species.

Acknowledgements

We thank all the colleagues who provided us with sev-

eral *Borrelia* isolates. We thank Alexia Couturier-Maret for excellent technical assistance and Jean-Claude Piffaretti for critical review of the manuscript and helpful suggestions. This work was supported by the Swiss National Foundation for Scientific Research (Grant 3200-052739) and the Foundation for research and development of the Institut Central des Hôpitaux Valaisans. The automated DNA sequencer was provided by the E. Boninchi Foundation to Pierre Hutter.

References

[1] Baranton, G., Postic, D., Saint Girons, I., Boerlin, P., Piffaretti, J.C., Assous, M. and Grimont, P.A.D. (1992) Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. 42, 378–383.

[2] Assous, M.V., Postic, D., Paul, G., Névot, P. and Baranton, G. (1993) Western blot analysis of sera from Lyme borreliosis patients according to the genomic species of the Borrelia strains used as antigens. Eur. J. Clin. Microbiol. Infect. Dis. 12, 261–268.

[3] Anthonissen, F.M., De Kesel, M., Hoet, P.P. and Bigaignon, G.H. (1994) Evidence for the involvement of different genospecies of Borrelia in the clinical outcome of Lyme disease in Belgium. Res. Microbiol. 145, 327–331.

[4] Péter, O., Bretz, A.G., Postic, D. and Dayer, E. (1997) Association of distinct species of *Borrelia burgdorferi* sensu lato with neuroborreliosis in Switzerland. Clin. Microbiol. Infect. 3, 423–431.

[5] Wilske, B., Jauris-Heipke, S. and Lobentanzer, R. et al. (1995) Phenotypic analysis of outer surface protein C (OspC) of *Borrelia burgdorferi* sensu lato by monoclonal antibodies: relationship to genospecies and OspA serotype. J. Clin. Microbiol. 33, 103–109.

[6] Postic, D., Assous, M.V., Grimont, P.A.D. and Baranton, G. (1994) Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of *rrf* (5S)-*rrl* (23S) intergenic spacer amplicons. Int. J. Syst. Bacteriol. 44, 743–752.

[7] Hubalek, Z. and Halouzka, J. (1997) Distribution of *Borrelia burgdorferi* sensu lato genomic groups in Europe, a review. Eur. J. Epidemiol. 13, 951–957.

[8] Saint-Girons, I., Gern, L. and Gray, J.S. et al. (1998) Identification of *Borrelia burgdorferi* sensu lato species in Europe. Zbl. Bakteriol. 287, 190–195.

[9] Olsen, B., Duffy, D.C., Jaenson, T.G.T., Gylfe, A., Bonnedahl, J. and Bergström, S. (1995) Transhemispheric exchange of Lyme disease spirochetes by seabirds. J. Clin. Microbiol. 33, 3270–3274.

[10] Humair, P.-F., Péter, O., Wallich, R. and Gern, L. (1995) Strain variation of Lyme disease spirochete isolated from *Ixodes ricinus* ticks and rodents collected in two endemic areas in Switzerland. J. Med. Entomol. 32, 433–438.

[11] Barbour, A.G. (1984) Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. 57, 521–525.

[12] Maniatis, T.E., Fritsch, E.F. and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[13] Fraser, C.M., Casjens, S. and Huang, W.M. et al. (1997) Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature 390, 580–586.