Cloning and sequencing of a species-specific nucleotide fragment of *Borrelia burgdorferi* sensu stricto, which is repeated in several plasmids of the species

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**Abstract**

Among the etiological agents of Lyme disease, *Borrelia burgdorferi* sensu stricto strains carry a 16 kb plasmid, which did not hybridize to plasmids of *B. garinii* and *B. afzelii* strains. A 1271 bp DNA fragment of the 16 kb plasmid was cloned. It hybridized to several plasmids of this species (16, 27 and 55 kb). Sequencing of the cloned insert revealed a 327 bp ORF coding for a 14 kDa protein of unknown function, which could be expressed in *E. coli*. This ORF, conserved among *B. burgdorferi* sensu stricto strains, was carried by the same three plasmids.

**Keywords:** Species-specific nucleotide sequence; Repeated plasmid sequence; *Borrelia burgdorferi* sensu lato

**1. Introduction**

Lyme borreliosis is a multisystem disorder of humans and other mammals which involves the skin, musculoskeletal, cardiac and neurological systems. This disease has been identified throughout the world and is caused by spirochetes of the group *Borrelia burgdorferi* sensu lato (s.l.). Isolates have been divided into three species: *B. burgdorferi* sensu stricto (s.s.), *B. garinii* and *B. afzelii* [1]. Recently, a further phylogenetic delineation has been proposed, introducing *B. japonica* [2] and *B. andersonii* [3]. To date, however, no clinical isolates belonging to these two latter species have been found. The *B. burgdorferi* s.l. genome structure consists of an approximately 950 kb linear chromosome and of several circular and linear plasmids [4,5]. The size and number of plasmids vary among strains of *B. burgdorferi* s.l. [4,6]. Several genes, some of them encoding metabolic functions [7], have been mapped to plasmids. But little is known of the contribution of plasmids to the genetic specificity of *Borrelia* strains.

Several studies showed that *B. burgdorferi* s.s. seems preferentially involved in arthritic manifestations, *B. garinii* in neurologic manifestations and *B. afzelii* in late dermatologic complications [8-10]. No determinants of this species-related tropism have yet been identified. Our aim is to isolate and characterize species-specific sequences, in order to ultimately clar-
ify the tropism for different host tissues of *B. burgdorferi* s.l. strains, entailing their characteristic pathological manifestations in Lyme disease. Here, we report on a plasmid of *B. burgdorferi* s.s., which hybridized only to plasmids of the same species. A cloned plasmid sequence was found in several plasmids and contained an ORF for a 14 kDa protein.

2. Materials and methods

2.1. Bacterial strains

* Borrelia strains used in this study are listed in Fig. 1. They were grown in BSK medium at 37°C [11]. The growth was checked every 2 days by dark-field microscopy. *Escherichia coli* strains used as cloning host were DH5αF’ (F’, φ80dacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17(rK−,mK+), supE44, relA1, deoR, Δ(laczYA-argF)U169) (Promega) and LK111(pGP1-2).

2.2. DNA extraction and analysis

DNA was extracted following the plasmid enrichment method described by Barbour [4]. For pulsed field gel electrophoresis (PFGE), the conditions of the pulse time were as follows: 0.3 s, 45 min; 0.5 s, 1 h 45 min; 0.7 s, 1 h 45 min; 0.9 s, 45 min; and 5 s, 30 min. After PFGE, the 16 kb plasmid of the *B. burgdorferi* s.s. strain IP1 was cut out of the 1.2% agarose gel, eluted out of the agarose block in a dialysis bag under an electric field (160 V, 15 min). After phenol-chloroform extraction and isopropanol
Fig. 2. Specificity of recombinant plasmids pMC52 and pMC95 towards different *B. burgdorferi* sensu lato species involved in Lyme disease. DNA (1.5 μg) from strains B31 (lanes 1), IRS (lanes 2), IP1 (lanes 3), IP2 (lanes 4), IP3 (lanes 5) of *B. burgdorferi* sensu stricto species; from strains 20047 (lanes 6), N34 (lanes 7), G25 (lanes 8), P/Bi (lanes 9) of *B. garinii* species and from strains VS461 (lanes 10), Iper3 (lanes 11), UO1 (lanes 12) and UMO1 (lanes 13) of *B. afzelii* species were separated by PFGE in a 1.2% agarose gel. They were either stained with ethidium bromide and photographed under UV (A) or transferred on a nylon membrane and hybridized with radiolabeled pMC52 (B) or with radiolabeled pMC95 (C). DNA molecular weight markers (λ+λ cleaved by HindIII), given in base pairs (bp) are shown on the left.

Precipitation, DNA was resuspended in TE (10 mM Tris pH 8; 1 mM EDTA). Purity of the plasmid was ascertained by agarose gel electrophoresis, before being radiolabeled by random priming and used for hybridization. Colony blotting, Southern blotting and probe hybridizations were performed according to standard procedures [12]. DNA was amplified with DNA polymerase of *Thermus brockianus* (Dynazyme, Techgen) for 35 cycles under the following conditions: 1 min at 96°C; 1 min at 54°C; 1 min at 72°C. The last cycle was terminated by an elongation time of 10 min at 72°C. Both DNA strands were completely sequenced following the instructions of the supplier of DNA sequencing kit (USB, Sequenase Version 2.0).

2.3. Expression of the ORF of the borrelial insert cloned in pMC52

Specific labeling of the ORF product was achieved
in E. coli using the T7 expression system [13]. The
borrelial DNA insert was first isolated from the
pBluescript SK+ vector by XbaI and PstI hydrolysis
and subcloned in the same sites of the pT5-7 vector
just downstream of the bacteriophage T7 gene φ10
promoter. The recombinant pT7-5 was introduced

1 TACCAACATGAATAATATCTGATATATTTAGAAAAACAAAAAACCATAGTTAATTTTTAAAAAC
2 73 CAGTCCAAATATATGTTTTTATATAAAATAATTCCTCCTTAAATGTTTTCGTTTCAACAATAAGACTATC
3 143 AAACAAATGCAGAAAACTAAACACTTCTACTCTACTATTAAATCGTTAAAAATA
4 214 TTTCTTTGCAATTAAGCAATTTAGAATATATATATATAGAACATATTATTATTTGTTAATAATTTAAAA
5 285 ATTACTGGGCGCATTATTGGAAAAATTATTAATATATATATAGAACATATTATTATTTGTTAATAATTTAAAA
6 349 CTA TCT TCA CAC TTA ATA ATT CTG ATT TAC ACA CTA AAC AAC ATT GAC CTA AAT
7 7 Leu Ser Ser His Leu Ile Ile Leu Ile Tyr Thr Leu Asn Asn Ile Asp Leu Asn
8 403 TCA AAA AAT ATG GGA TAC TAT AGT AGG GGC TTT ATA CGC GTT GGC TTT ACT TTT
9 25 Ser Lys Asn Ile Gly Tyr Tyr Ser Arg Gly Phe Ile Arg Arg Ala Phe Thr Phe
10 457 AAC ATA GAT AGA TAT GGC TAT ACT AGT AAA GAT ATT GAA ATA GAC ATA GAC TTA
11 43 Asn Ile Tyr Asp Tyr Gln Cys Thr Ser Lys Asn Ile Glu Ile Asp Ile Asp Leu
12 511 TTA ATA AAG TAT CTC GAT TTT TTA TAA AAC AAT CTA AAA ATT ATA ACT AAT AAA
13 61 Leu Ile Lys Tyr Leu Asp Phe Leu Glu Asn Asn Leu Ile Thr Asn Lys
14 565 TAT AAA GTA AAA AAA AAT ATA TTC AAA CTT TAC ATA ATC AAT TAT CCT TTA
15 79 Tyr Lys Val Glu Lys Asn Ile Phe Lys Leu Tyr Tyr Ile Ile Asn Tyr Pro Leu
16 619 AAA ATA TGT TAC ACA AAA ATT ATG AAC TAC TAT AAA TAG ACTATAATAGATATTTAAA
17 97 Lys Ile Cys Thr Lys Thr Ile Met Asn Tyr Tyr Lys
18 677 AAGAGAAACATTTAGATATATTACAAAGGTGTGTTTTCCTCCCTTAATCAGTTTAAAAGGTGTAT
19 3
20 748 TGGGCGTATACCATATATATATTATTATATCCCCATATTAAATTTTTTTATCAGGTTGCAAATGTTTAT
21 819 AGTGTCATTGCCCTAAATGAAAGAACATATGAAAAATATATATATATATGCAAGAATTAAAAAG
22 890 ATACAGCGTCATAGCCTAAATATGAAAGAACATATGAAAAATATATATATATGCAAGAATTAAAAAG
23 961 TCCCTAGAAAGAGTTTTATATATAATATAAAAAGACTACTACGTATTTATATATGAGAATATTAAAAAAATTTT
24 1032 ACAAAACCATCATTACTACTAATATTATATAACATCATTATATTATAAATAGCTTTATGCAAAATTTAGAAATATT
25 1103 GTTTTACGTTATAAAAATATATCAGCTATATTTATATATATATATATATATATATATATATATATATATATATATATATAT
26 1174 AATTCAATTGGATGGGAATATACCTCATGTCATTACGCCTTTACACTACGTATTTGTTAATAATATATATATATATATATATATAT
27 1245 AAGTTAACATTCTTGTCAAAAAACTA

Fig. 3. Nucleotide sequence of a species-specific 1271 bp fragment from the 16 kb plasmid of B. burgdorferi sensu stricto strain IP1. The translation initiation codon ATG is indicated by an arrow, and the stop codon by dots. Putative -10 promoter sequence is boxed. Sequence location for PCR primers 1, reverse 2 (R2), 3, and reverse 4 (R4) are underlined. The sequence of the borrelial DNA insert of pMC52 has been submitted to the GenBank Nucleotide Sequence Data Library under accession number U12332. A request for accession number is under way for the nucleotide sequence of the insert of pMC95 (Fig. 1C) and of another recombinant of the 16 kb plasmid, hybridizing exclusively to the 16 kb plasmid of B. burgdorferi s.s. strains.
at 37°C, centrifuged, and resuspended samples were fractionated by SDS-14% polyacrylamide gel electrophoresis (PAGE). Proteins were either stained with Coomassie brilliant blue or revealed by autoradiography.

3. Results and discussion

3.1. Species specificity of a 16 kb plasmid of B. burgdorferi sensu stricto

The plasmid profiles of B. burgdorferi s.l. strains and related spirochetes (listed in the legend to Fig. 1) were determined by PFGE. Interestingly, the five analyzed strains of B. burgdorferi s.s. contained a 16 kb plasmid which had no counterpart of equivalent size in strains of the other species, suggesting that this plasmid might be specific to B. burgdorferi s.s. (Fig. 1A, lanes 1–3).

This possibility was tested by hybridizing the 16 kb plasmid of B. burgdorferi s.s. strain IP1, excised from an agarose gel, and labeled by random priming, with DNA of spirochetes transferred by Southern blotting. As shown by autoradiography in Fig. 1B, the 16 kb plasmid hybridized to several plasmids of strains belonging to the B. burgdorferi s.s. species: the 16, 27, 32 and 55 kb plasmids of strain B31; the 16, 27, 29, 32, and 55 kb plasmids of strain IP1; and the 16, and 55 kb plasmids of strain IRS. No hybridization occurred to any plasmids of the B. garinii, B. afzelii, B. japonica, B. hermsii, B. parkeri, and B. turicatae strains.

Results presented here (Fig. 1B) and by Hinnebusch and Barbour [14] suggest the whole 16 kb plasmid to be specific of the B. burgdorferi s.s. species. Xu and Johnson [6], studying a large number of strains, also reported the presence of a 18 kb plasmid in B. burgdorferi s.s. strains (supposedly corresponding to the 16 kb plasmid in the present work), while no plasmid of equivalent size was found in B. garinii and B. afzelii strains. Another species-specific plasmid has been described in a newly delineated Borrelia species [20].

Hinnebusch and Barbour [14] reported hybridization of the left telomere of a 16 kb plasmid of B. burgdorferi s.s. strain B31 to several plasmids of strains belonging to the same species. By using pri-
mers corresponding to the 16 kb plasmid telomere of strain B31 (5'-C TTGAAATACTAAACTT-3'; 5'-TGATAATCAATAAACCTG-3'; [15]), we could amplify by PCR a DNA fragment of the expected size (124 bp) from a preparation of the 16 kb plasmid of strain PI1 (result not shown). This suggests the 16 kb plasmid of strain PI1 to be linear and homologous to the one described in strain B31.

3.2. Cloning of species-specific DNA sequences from the 16 kb plasmid

For the isolation of specific sequences, the purified 16 kb plasmid was restricted with S au3A, DNA fragments were ligated to BamHI linearized pBluescript II SK+, and introduced by transformation into E. coli DH5αF'. Recombinants were transferred to a nylon membrane and screened for a positive colony hybridization signal with randomly radiolabeled EcoRI cleaved total DNA of B. burgdorferi s.s. strain PI1, in the absence of hybridization with DNA of B. garinii (strain N34) and B. afzelii (strain UO1) [12].

Two such recombinant colonies were further studied. The first recombinant plasmid, called pMC52, was extracted, purified, radiolabeled by random priming and hybridized on a Southern blot of PFGE separated DNA from strains of the three species of B. burgdorferi s.l. pMC52 hybridized to the 16 kb plasmid, and to one or two additional plasmids of B. burgdorferi s.s. strains: the 27 kb plasmid of strain IRS, or the 27 and 55 kb plasmids of the B31, IP1, IP2 and IP3 strains, and did not hybridize to DNA of B. garinii and B. afzelii strains (Fig. 2B). A second recombinant plasmid, called pMC95, was species-specific but hybridized only to the 16 kb plasmid (Fig. 2C).

Plasmid-associated repeated sequences have been found in several B. burgdorferi s.l. species. They involve gene families identified on circular plasmids [7,16–19], on linear plasmids [20] or on both types of plasmids [21]. Yet, these sequences are not species-specific (they are found in different B. burgdorferi s.l. species) and do not concern the 16 kb plasmid of B. burgdorferi s.s.

3.3. Characterization of the borrelial insert of pMC52

Being repeated on several plasmids, the borrelial insert of pMC52 was further studied. The nucleotide sequence and deduced amino acid sequence of this 1271 bp insert are shown in Fig. 3. It has a 22.3% G+C content. No homology could be found with any nucleotide or amino acid sequence deposited in sequence databases (release October 1996). No consensus or motif sequences were found (Prosite, GCG). Since no particular structures (inverted repeats, hairpins, etc.) could be found in the cloned sequence, its presence on different plasmids seems not to be due to transposons or other movable genetic elements.

An open reading frame of 327 nucleotides (coding for a polypeptide of 108 amino acids with a calculated molecular mass of 13 116 Da) could be found. The analysis of the sequence upstream of the ORF

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Table 1

Hybridization of different parts of the borrelial insert of pMC52 with plasmids of B. burgdorferi sensu stricto strains

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Probe of borrelial DNA*</th>
<th>ORF (nt 328–654)</th>
<th>3' non-coding end (nt 699–1092)</th>
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<tbody>
<tr>
<td>Strain</td>
<td>Size (kb)</td>
<td>Whole insert (nt 1–1271)</td>
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<td>B31</td>
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<td>16</td>
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*Borrelial sequences of recombinant plasmid pMC52 (numbered in Fig. 3) were radiolabeled by random priming. Total pMC52 DNA or PCR products spanning the indicated nucleotides were used. Probes were hybridized with DNA of B. burgdorferi s.s. strains previously separated by PFGE and transferred to nylon membrane. Presence or absence of hybridization is indicated by + or −, respectively.
did not reveal any obvious sequences compatible with Borrelia [22,23] or E. coli promoters, except for a −10 consensus sequence (nt 258–263, boxed in Fig. 3). The presence on the insert of an ORF was confirmed by its expression in E. coli. For this purpose, specific labeling of the ORF product was achieved using the T7 expression system [13]. After induction, a protein of 14 kDa was expressed (Fig. 4, lane A), whereas no protein was detected in the control strain carrying pT7-5 (Fig. 4, lane B).

This ORF is conserved among B. burgdorferi s.s. strains as indicated by the fact that the 327 bp PCR amplified ORF sequence (using primers 1 and R2, Fig. 3), hybridized (as the whole insert) to 16, 27 and 55 kb plasmids of B. burgdorferi strains B31 and IP1, as well as to the 16 and 27 kb plasmids of strain IRS (Table 1). Two PCR primers (3 and R4, Fig. 3) were then chosen outside the ORF to test if the 3′ non-coding end of the insert is also repeated on several plasmids. PCR using primers 3 and R4 amplified a 395 bp DNA fragment, which after labeling hybridized to plasmids of 16 kb and 55 kb of strains B31 and IP1, and to the 16 kb plasmid of strain IRS. No hybridization was observed with the 27 kb plasmid of any of these strains (Table 1). The extent of sequences repeated in different plasmids seems thus to vary. Isolation of the repeated sequences in the different plasmids and comparison of their sequences will reveal the extent of homology and possible mechanism of occurrence.

In conclusion, as a first step towards an understanding of the preferential pathological manifestations caused by each species involved in Lyme disease, we showed the 16 kb plasmid of a B. burgdorferi s.s. strain to be specific to this species. We have identified a DNA fragment which is repeated in several plasmids of this species and which encoded a 14 kDa protein. The development of additional species-specific diagnostic tools, based on these sequences, will also be useful in the study of the geographical distribution and etiological role of this species in different manifestations of the disease.

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