Med Microbial Immunol (1995) 184:73–80

ORIGINAL INVESTIGATION

G. Will · S. Jauris-Heipke · E. Schwab · U. Busch
D. Rößler · E. Soutschek · B. Wilske · V. Preac-Mursic

Sequence analysis of *ospA* genes shows homogeneity within *Borrelia burgdorferi* sensu stricto and *Borrelia afzelii* strains but reveals major subgroups within the *Borrelia garinii* species

Received: 19 April 1995

Abstract The genes coding for the outer surface protein A (OspA) of 19 different *Borrelia burgdorferi* strains belonging to the seven OspA-serotypes 1–7, previously described [Wilske et al. (1993) J Clin Microbiol, 31: 340–350], have been investigated. *B. burgdorferi* sensu lato strains were chosen from various biological sources (ticks, human skin and cerebrospinal fluid) as well as different geographical origins (Germany, Slovenia, Austria, United States). The open reading frames of all *ospA* genes consist of 819–825 nucleotides corresponding to proteins of approximately 30 kDa. The *ospA* sequences obtained in this study and previous published studies were compared with the results from OspA serotyping with monoclonal antibodies. The classification into the seven OspA serotypes could be confirmed on a genetic basis (*ospA* genotypes 1–7) for all strains analyzed so far (n=29). In addition, one strain without OspA expression could be assigned to *ospA* genotype 2. Genetic stability could be proven for the *ospA* gene of *B. burgdorferi* strain PWudI after inoculation and reisolation from a gerbil. However, we found evidence for intragenic recombination by cluster analysis of *ospA* sequence data. Accordance of *ospA* genotype 1 strains with *B. burgdorferi* sensu stricto and *ospA* genotype 2 strains with *B. afzelii*, as well as the *ospA* genotype strains 3–7 with *B. garinii* was confirmed by pulsed-field gel electrophoresis of *Mlu*I-digested genomic DNA. *B. garinii* is not only more heterogenous in respect to the OspA-encoding genes, but shows moreover major subgroups formed by genotypes 4, 5 and 6 and genotypes 3 and 7, respectively. The latter group has not been described previously and is specifically recognized by an OspA-specific monoclonal antibody L32 1F7.

Key words *Borrelia burgdorferi, afzelii, garinii* · Outer surface protein A · *ospA* genes · *ospA* sequence analysis · Outer surface protein A serotypes

G. Will · S. Jauris-Heipke · E. Schwab · U. Busch
D. Rößler · E. Soutschek[1] · B. Wilske (✉) · V. Preac-Mursic
Abteilung Serologie, Max-von-Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie der Ludwig-Maximilians-Universität München, Pettenkoferstrasse 9a, D-80336 München, Germany Tel.: 49-89-5160 5231; Fax: 49-89-5160 4757

Present address:
[1] Mikrogen GmbH,
Westenstrasse 125/G, D-80339 München,
Germany

Introduction

Lyme borreliosis is a global tick borne disease caused by the spirochete *Borrelia burgdorferi* sensu lato [5, 11, 34]. The human disease is a multisystem disorder involving predominantly the skin, the nervous system and the joints [2, 34]. Lyme disease spirochetes have been reclassified into three different species [3, 13]. *B. burgdorferi* sensu stricto *B. afzelii* and *B. garinii*. In Asia *B. japonica* sp. nov. has been described [43].

The majority of Lyme disease spirochetes express a major plasmid-encoded outer surface protein A (OspA) [4, 6, 10] which varies considerably in phenotype and sequence [1, 7, 17, 33, 37, 39, 41, 45]. This protein is also a promising candidate for a vaccine [20, 32]. In a previous study we found a clear correlation between the classification into the different species and serotyping with monoclonal antibodies against OspA. OspA serotype 1 and 2 corresponded to two different species (*B. burgdorferi* sensu stricto and *B. afzelii*, respectively). OspA serotypes 3–7 belonged to a single species (*B. garinii*). In a limited number of strains, partial *ospA* sequence analysis confirmed the OspA serotypes [41].

OspA serotype 2 (*B. afzelii*) was primarily isolated from European patients with skin manifestations [35, 39, 41]. This was confirmed by the preferential reactivity of this type of strain with sera from patients with acrodermatitits chronica atrophicans [38]. Cerebrospinal fluid CSF and tick isolates are rather heterogeneous: *B. garinii* strains are more often isolated than *B. burgdorferi* sensu stricto or

B. afzelii [41]. In addition, skin isolates from patients with signs of dissemination were primarily classified as *B. burgdorferi* sensu stricto or *B. garinii* [35]. It is still controversially discussed whether strains causing neuroborreliosis differ from tick strains in subtypes of *B. garinii* [18, 41]. Therefore, it appears important to find reliable methods for subtyping strains within the species. The aim of this study was to analyze a broad panel of isolates for the genetic diversity of OspA as a tool for classification, and to prove whether there is an agreement of the *ospA* genotype with the previously described OspA serotype [41].

Here, we report the results of *ospA* sequence analysis of 19 strains including two representatives of all seven OspA serotypes previously described. Cluster analysis of the *ospA* genes revealed three main clusters corresponding to the three species. The considerable diversity of the *ospA* gene among *B. garinii* strains presented here, shows major subgroups which are in accordance to OspA serotype 4, 5, and 6, respectively, and a subgroup comprising of OspA serotype 3 and 7.

Table 1 *Borrelia burgdorferi* sensu lato strains analyzed for *ospA* genotype and comparison with OspA serotype. PWudI/6 was reisolated from gerbil; species was determinated by pulsed-field gel electrophoresis; OspA serotypes were determined as described [41]

Strain	Biological source	Geographic source	Species	*ospA*-genotype	OspA-serotype
PKa	CSF	Germany	*B. burgdorferi* s.s.	1	1
PBre	Skin	Germany	*B. burgdorferi* s.s.	1	1
T255	Tick	Germany	*B. burgdorferi* s.s.	1	1
297	CSF	USA	*B. burgdorferi* s.s.	1	1
PWudI	Skin	Germany	*B. afzelii*	2	2
PWudI/6	Gerbil	Germany	*B. afzelii*	2	2
PLe	Skin	Germany	*B. afzelii*	2	2
PLud	Skin	Germany	*B. afzelii*	2	2
PHo	CSF	Germany	*B. afzelii*	2	2
PLj7	Skin	Slovenia	*B. afzelii*	2	2
PBo	CSF	Germany	*B. afzelii*	2	0
PBr	CSF	Germany	*B. garinii*	3	3
PTrob	Skin	Solvenia	*B. garinii*	4	4
PHei	CSF	Germany	*B. garinii*	5	5
WABSou	Skin	Austria	not done	5	5
PWUdII	Skin	Germany	*B. garinii*	6	6
TN	Tick	Germany	*B. garinii*	6	6
TIs1	Tick	Germany	*B. garinii*	6	6
T25	Tick	Germany	*B. garinii*	7	7

Material and methods

Borrelia spp. strains and cultivation

All *B. burgdorferi* strains were cultivated in MKP medium [27] at 33°C. Designation and origin of *B. burgdorferi* sensu lato strains used in this study are given in Table 1.

Nucleic acid techniques

B. burgdorferi sensu lato strains were grown to a cell density of 10^9/ml. Cultures were harvested by centrifugation. Genomic DNA was isolated without fractionating plasmid and chromosomal DNA using the method of Langenberg et al. [23]. Total DNA of *Burgdorferi* sensu lato strains was analyzed by pulsed-field gel electrophoresis (PFGE) as described by Busch et al. [12]. For cloning and expression procedures *E. coli* strain JM109 [44] and plasmid pUC18 [36] were used. Cells were grown in L-broth at 37°C, plasmid containing strains were grown in media supplemented with ampicillin (50 mg/ml). Restriction enzyme digest, DNA ligation, transformation of competent cells, plasmid isolation, agarose gel electrophoresis and Southern blotting onto nitrocellulose membrane (Schleicher & Schuell) were performed by conventional techniques as described [31].

Oligonucleotides were synthesized on a "gene-assembler plus" (Pharmacia, Freiburg) and were purified on NAP10 columns (Pharmacia) as suggested by the manufacturer. PCR amplifications (50 µl volume) were carried out as described by the manufacturer (Cetus/Perkin-Elmer, Langen) using two different 5′ primers in combination with one 3′ primer [45] to obtain the coding sequences for the genes with and without leader sequence; 25 cycles (DNA denaturation at 94°C for 2 min, primer annealing at 45°C for 2 min and primer extension at 73°C for 4 min) were performed. Analysis of the PCR products was performed by gel electrophoresis (1.5% agarose gel containing 0.5 µg/ml ethidium bromide), *ospA*-positive PCR products were digested with appropriate restriction enzymes and ligated into pUC18 vector after extraction with chloroform and precipitation with ethanol. Recombinant plasmids were sequenced by the didesoxy chain-termination method using the dye Terminator-Taq cycle sequencing kit according to the instructions of the manufacturer (ABI 373 DNA-sequencer, Applied Biosystems GmbH, Weiterstadt). The cloning and sequencing of *ospA* genes of strains PKo and PBi has been described previously [45], their *ospA*-coding regions were PCR amplified and sequenced for internal control. For DNA sequencing, *ospA*-specific primers were used derived from sequences previously obtained.

Sequence analysis and nucleotide sequence accession numbers of *ospA*

For comparison of sequences the GCG (Vers. 7.0) software [16] was used. The amplified *ospA* nucleotide sequences of the *B. burgdorferi* sensu lato strains have been assigned the following EMBL/GenBank accession numbers: PKa1 (X80182), PBo (X80183), PWudI (X80184), PWudI/6 (X80185), PTrob (X80186), PHei (X80251), TN (X80252), PWudII (X80253), T25 (X80254), PBr (X80256), PLud (X85439), PHo (X85437), PBre (X85739), T255 (X855443), 297 (X85442), PLj7 (X85438), WABSou (X85441), TIs1 (X85440), and PLe (X85982).

Results

Determination of the species by PFGE

PFGE after *Mlu*I digestion allowed the classification of genomic DNA of all investigated *B. burgdorferi* sensu lato strains into the different *B. burgdorferi* sensu lato species (*B. burgdorferi* sensu stricto *B. afzelii* and *B. garinii*) according to their characteristic large restriction fragment pattern (LRFP) (see Table 1) as described by Belfaiza et al. [9]. *Mlu*I-digested genomic DNA of *B. burgdorferi* sensu stricto isolates PKa1 and PBre showed a characteristic DNA band with size of 135 kb, whereas *B. afzelii* iso-

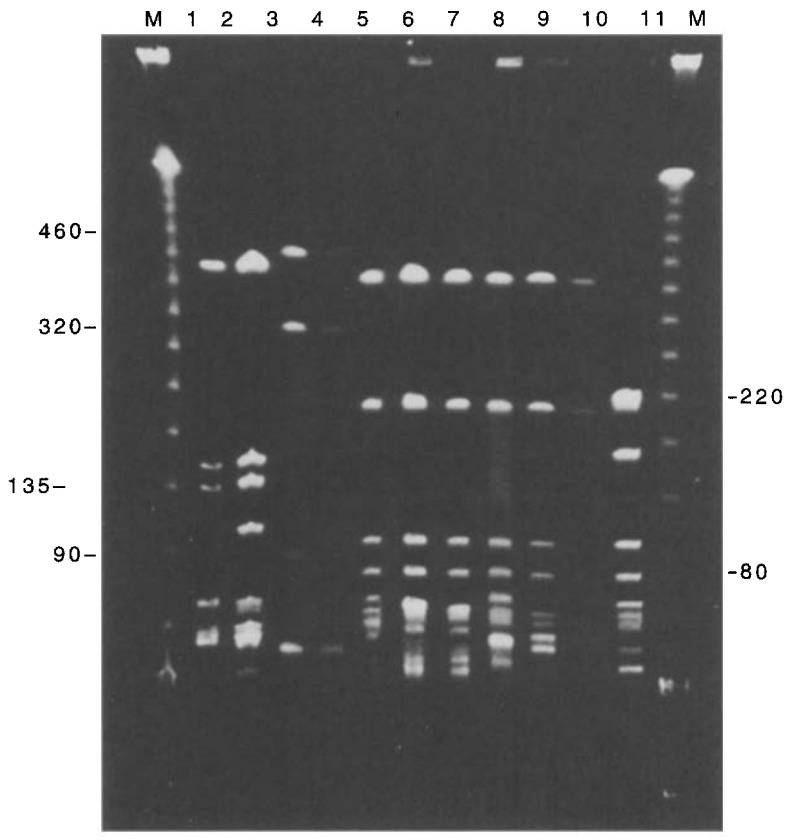

Fig. 1 Pulsed-field gel electrophoresis of *Mlu*I restriction digest of whole *Borrelia* DNA. *Lane M* Lambda concatemer, 48.5 kb; *B. burgdorferi* sensu stricto strains PKa1 and PBre (*lanes 1* and *2*); *B. afzelii* strains PWudI and PLud (*lanes 3* and *4*); *B. garinii* strains PBr, PBi, PTrob, PHei, PWudII, TIs1 and T25 (*lanes 5–11*, respectively). Characteristics bands for *B. garinii* (80, 220 kb) are indicated on the *right*. On the *left side*, characteristic bands for *B. afzelii* (90, 320, 460 kb) and for *B. burgdorferi* sensu stricto (135 kb) are marked

lates PWudI and PLud are indicated by 90-, 320- and 460-kb bands. In contrast, *B. garinii* isolates PBr (OspA serotype 3), PBi (serotype 4), PTrob (serotype 4), PHei (serotype 5), PWudII (serotype 6), TIs1 (serotype 6), and T25 (serotype 7), were characterized by DNA bands of 80 and 220 kb (Fig. 1).

Cloning and sequencing of the *ospA* genes

The *ospA* genes of 19 different *B. burgdorferi* sensu lato strains (listed in Table 1) have been PCR amplified, cloned and sequenced. Their open reading frames consist of 822–825 nucleotides, which corresponds to proteins of 272–273 amino acids (aa), respectively (Fig. 2).

The *ospA* sequences of all investigated *B. burgdorferi* sensu stricto (OspA serotype 1) and *B. afzelii* (serotype 2) type strains showed a gap of 3 nucleotides at position 521–523 (PKo numbering) if compared to *B. garinii* strains. A similar 3-bp deletion was detectable in the *ospA* of *B. garinii* OspA serotype 4 strains (PBi and PTrob) at position 518–520 (PKo numbering) and *B. garinii* OspA serotype 5 strains (PHei and WABSou) at position 622–624. In contrast, *B. garinii* serotype 3 (PBr), serotype 6 (PWudII, TN, TIs1) and serotype 7 (T25) strains did not show any characteristic deletion within the overall sequence of *ospA*.

Analysis of the deduced aa sequences

The deduced aa sequences (overall sequence) of the various *ospA* genes investigated in this study share identities between 74.7–100% (Table 2). Within the *B. burgdorferi* sensu stricto and *B. afzelii* group OspA nearly reaches homogeneity (98.5–100%), whereas among strains of the *B. garinii* group (OspA serotypes 4–7) OspA appeared to be heterogenous (79.6–100%). In detail, *B. garinii*-type strains of the same OspA serotype share a striking identity on the molecular level of the OspA sequences presented here (99.3% within OspA serotype 4, 99.3% within OspA serotype 5 and 96.7–100% within OspA serotype 6 strains). Highest diversity (78.4–82.1%) was observed with OspA of *B. garinii* strains PBr and T25 (OspA serotype 3 and 7, respectively); they differ clearly if compared to the other *B. garinii* stains. These results show agreement between *ospA* genotypes (presented here in this study) and their respective phenotypes (OspA serotypes), as previously determined with monoclonal antibodies [41]. For ease of presentation and due to identities observed within the OspA aa sequences, the alignment to create Fig. 2 was carried out only with a set of 12 of the 19 investigated strains covering typical representatives of all three species and all seven OspA sero/genotypes (Fig. 2, OspA serotype in parentheses).

```
          1                                                  50                                                 100
PBr     (3) ----------.---------- ---------- ------g--- ---------- ----m----- ---------s ---------- a--------- sq--nq----
T25     (7) ---------- ---------- ---------- ---------- ---------- ----e----- ---------- --------v- aa-------- -----q-k--
297     (1) ---------- ---------- ---------- ---------- --------n- --d-i----- ---------- --------v- a----v---- ----gq----
PBre    (1) ---------- ---------- ---------- --------n- --------n- --d-i----- ---------- --------v- a----v---- s---gq----
PWudI   (2) ---------- ---------- ---------a ---------- ---------- ---------- i--------d --------t- ---------- ----------
PLj7    (2) ---------- ---------- ---------a ---------- ---------- ---------- i--------d --------t- ---------- ----------
PBi     (4) ---------- ---------- ---------- ---------- ---------- ----m----- ---------s ----t----- s--------- ----------
PTrob   (4) ---------- ---------- ---------- ---------- ---------- ----m----- ---------t ----t----- s--------- s---------
PHei    (5) ---------- ---------- ---------- ------g--- ---------- ----m----- ---------- ----t----- t----v---- ----------
WABSou  (5) ---------- ---------- ---------- ------g--- ---------- ----m----- ---------- ----t----- t----v---- ----------
PWudII  (6) ---------- ---------- ---------- ------g-t- ---------- ----e----------------- ----t----- t----v---- -----q-k--
TIs1    (6) ---------- ---------- ---------- ------g--- ---------- ----m----- ---------- ----t----- t----v---- ----------
Cons.       MKKYLLGIGL ILALIACKQN VSSLDEKNSV SVDLPGEMKV LVSKEKDKDG KYSLKATVDK LELKGTSDKN NGSGVLEGEK DDKSKAKLTI ADDLSKTTFE

          101                                                150                                                200
PBr     (3) ---------- -r--n----- ---------- k----v---- ---------- -n--s----- ---g-a---- -tdg-e-k-t -t-------- ----------
T25     (7) ---------- -----l---- ---------- k----v---- ---------- qn--s----- ---s------ -t---e-k-t -ea------- ---e------
297     (1) ---------- ---------- ---------- -v---i---- d--------- ----s----- ---g-v---- -t--.----v ---------- --------s-
PBre    (1) ---------- ---a------ ---------- -v---i---- d--------- ----s----- ---s-v---- -t--.----v ---------- --------s-
PWudI   (2) l--------- -r--s----t ----m----- ---a--m--e ---k-----m ---------- ---n-----k v-n-.-v--e ---------- e-a-------
PLj7    (2) l--------- -r--s----t ----m----- ---a--m--e ---k-----m ---------- ---n-----k v-n-.-v--e ---------- e-a-------
PBi     (4) ---------- ----n----- -i-----a-- -------l-- ---------- ---------- ---d-a---- ----.----k -t----v--- h-pn------
PTrob   (4) ---------- ----n----- -i-----a-- -------l-- ---------- ---------- a--d-a---- ----.----k -t----v--- h-pn------
PHei    (5) ---------- -----l---- ---------- -i-----v-- ---------- ---k------ ---d------ ---------k -t-------- ----------
WABSou  (5) ---------- -----l---- ---------- -i-----v-- ---------- ---k------ ---d------ ---------k -t-------- ----------
PWudII  (6) ---------- -----l---- ---------- -t-----v-- ---------- ----s----- ---d------ ---------k -t----v--- --l-------
TIs1    (6) ---------- -----l---- ---------- -i-----v-- ---------- ----s----- ---d------ ---------k -t----v--- --l-------
Cons.       IFKEDGKTLV SKKVTSKDKS STEEKFNEKG ELSEKTITRA NGTRLEYTEI KSDGTGKAKE VLK-FTLEGT LAADGKTTL- VKEGTVTLSK NISKSGEVTV

          201                                                250                  274
PBr     (3) --------p- d----e-k-d -------k-- q-p------- -n-----n-n r--na---sp a---d-a---a---
T25     (7) e-k-----p- d--s-t---- -------k-- q--------- -n-------n t---k---sp a---d--a--a---
297     (1) e-----ssa- ----a--n-g ------t--- ----d----- -n-----q-- -n--k---s- ---tk---i-----
PBre    (1) e-----ssa- ----a--n-g ------t--- ----d----- -n-----q-- -n--k---s- ---tk---i-----
PWudI   (2) -----n---- ---------- ---------- ---t------ q--------- ---------- ----------
PLj7    (2) -----n---- ---------- ---------- ---t------ q--------- ---------- ----------
PBi     (4) e---sns--- -----k---n ---------- ----ni---- ---------- --------n- ----------
PTrob   (4) e---sns--- -----k---n ---------- ----ni---- ---------- --------n- ----------
PHei    (5) --d---ss.g n--s-t---g -------k-r t--------- -------n-- --------k- ---t--k-------
WABSou  (5) --d---ss.g n--s-t---g -------k-r q--------- -------n-- --------k- ---t--k---d---
PWudII  (6) --d-s----- -----k---- ---------- q---n----- ---------- --------k- ---t--k---d---
TIs1    (6) --d-s----- -----k---- ---------- q---n----- ---------- --------k- ---t---k--d---
Cons.       ALNDTDTTQA TKKTGAWDSK TSTLTISVNS KKTKQLVFTK EDTITVQKYD SAGTNLEGTA VEIKTLDELKNALK
```

Fig. 2 Alignment of the deduced OspA amino acid sequence of 12 strains. OspA sero/genotype is indicated in parentheses, missing amino acid positions are represented as points, identical aa as dashes

Table 2 OspA amino acid sequence identities (in %) of the 19 *Borrelia burgdorferi* sensu lato strains investigated in this study. Strains of the same species are boxed

Genotype	1	1	1	1	2	2	2	2	2	2	2	3	4	4	5	5	6	6	6	7
Strain	PKa1	PBre	T255	297	PKo	PWudI	PLe	PLud	PHo	PLj7	PBo	WudI/6	PBr	PBi	PTrob	PHei	WAB-Sou	PWudII	TN	Tis1	T25
PKa1	–	98.5	99.6	100	77.3	77.3	76.9	76.9	75.8	77.3	77.3	77.3	78.0	80.2	79.9	80.9	80.5	81.0	81.0	80.2	78.3
PBre		–	98.2	98.9	76.9	76.9	76.6	76.6	75.5	76.9	76.9	76.9	77.3	79.9	79.5	80.2	79.8	80.6	80.6	79.5	78.3
T255			–	99.2	76.9	76.9	76.6	76.6	75.5	76.9	76.9	76.9	77.7	79.9	79.5	80.5	80.2	80.6	80.6	79.9	78.0
297				–	77.7	77.7	77.3	77.2	76.2	77.7	77.7	77.7	78.3	80.6	80.2	80.9	80.5	81.0	81.0	80.2	78.8
PKo					–	100	99.6	99.6	98.5	100	100	100	76.6	83.2	82.8	79.8	79.4	81.3	81.3	81.3	76.2
PWudI						–	99.6	99.6	98.5	100	100	100	76.6	83.2	82.8	79.8	79.4	81.3	81.3	81.3	76.2
PLe							–	99.2	98.2	99.6	99.6	99.6	76.2	82.8	82.4	79.4	79.0	81.0	81.0	81.0	75.8
PLud								–	98.1	99.6	99.6	99.6	76.2	82.8	82.4	79.4	79.0	81.0	81.0	81.0	75.8
PHo									–	98.5	98.5	98.5	75.1	81.7	81.3	78.3	77.9	79.9	79.9	79.9	74.7
PLj7										–	100	100	76.6	83.2	82.9	79.8	79.4	81.3	81.3	81.3	76.2
PBo											–	100	76.6	83.2	82.8	79.8	79.4	81.3	81.3	81.3	76.2
WudI/6												–	76.6	83.2	82.8	79.8	79.4	81.3	81.3	81.3	76.2
PBr													–	80.6	79.9	80.2	80.6	79.6	79.6	80.3	87.2
PBi														–	99.2	86.0	85.7	87.9	87.9	89.0	78.8
PTrob															–	85.7	85.3	87.6	87.6	88.7	78.3
PHei																–	99.2	90.8	90.8	92.7	80.2
WABSou																	–	91.6	91.6	93.4	80.6
PWudII																		–	100	96.7	82.1
TN																			–	96.7	82.1
Tis1																				–	81.0
T25																					–

All OspA protein sequences are almost identical within the N terminus (aa 1–36), except the *B. afzelii* strains (genotype 2) which show a change from valine to asparagine at position 30 (see Fig. 2). OspA sero/genotype 5 strains (PHei and WABSou) are characterized by a deletion of an aa at position 209, OspA serotype 1, 2 and 4 strains by a deletion at aa 175 (Fig. 2). Comparison of the aa sequences of *B. garinii* OspA sero/genotypes if compared with OspA of the other strains. In the region between aa 130–274, sequence motif characteristics are clearly visible corresponding to each of the seven sero/genotypes investigated. By this method, the *ospA* genotype of *B. afzelii* strain PBo, which does not express OspA, could be determined. It corresponds to *ospA* genotype 2. This was reconfirmed as OspA pheno/serotype 2 after analysis of recombinantly expressed PBo-OspA with monoclonal antibodies (data not shown).

The genetic stability of the *ospA* gene could be demonstrated for the *B. afzelii* strain PWudI. After infection of gerbil with this strain and reisolation of the *Borrelia* organism after 1 week, the *ospA* gene was isolated, cloned and sequenced. The DNA sequence of the reisolate (designated as WudI/6) showed 100% identity to the *ospA* gene of the original infecting strain PWudI. This finding confirms earlier described investigations which were made in immunocompetent mice [8, 26]. Sequence analysis of the *ospA* genes of *B. afzelii* strain PKo and *B. garinii* strain PBi from early (PKo: 5; PBi: 7) and late passages (PKo: 302; PBi: 298) confirmed this stability also in the in vitro system (data not shown).

Cluster analysis of the *ospA* gene of all 19 strains investigated in this study and *ospA* sequences described earlier [10, 14, 17, 20, 22, 37, 45] shows the strong homogeneity of *ospA* within the *B. burgdorferi* sensu stricto group (sero/genotype 1) and the *B. afzelii* group (sero/genotype 2) (Fig. 3). The heterogeneity within the *ospA* of *B. garinii* type strains is clearly visible. Separation of strains PBr and T25 (sero/genotype 3 and 7, respectively), PBi and PTrob (sero/genotype 4) and PHei and WABSou (both sero/genotype 5) demonstrates the striking heterogeneity within this group. The high diversity of *ospA* of strains PBr and T25 delimits these from all other *B. garinii*-type strains. Following these results, the *B. garinii* group delineates into major subgroups (Fig. 3) covering the OspA sero/genotype 3, 7 pair and OspA sero/genotype 4, 5, and the remainder 6-type strains.

Discussion

The outer surface protein A OspA has been investigated in many aspects. It was the first outer surface protein described [6] and its immunological diversity has been intensively studied [5, 33, 40, 41]. There is a growing body of evidence that OspA is involved in the pathogenesis of Lyme borreliosis. OspA may play a role in invasion of extracellular matrices, adhesion processes and as a factor stimulating the production of cytokines [15, 21, 24]. In addition, variation of this protein may be involved in evasion

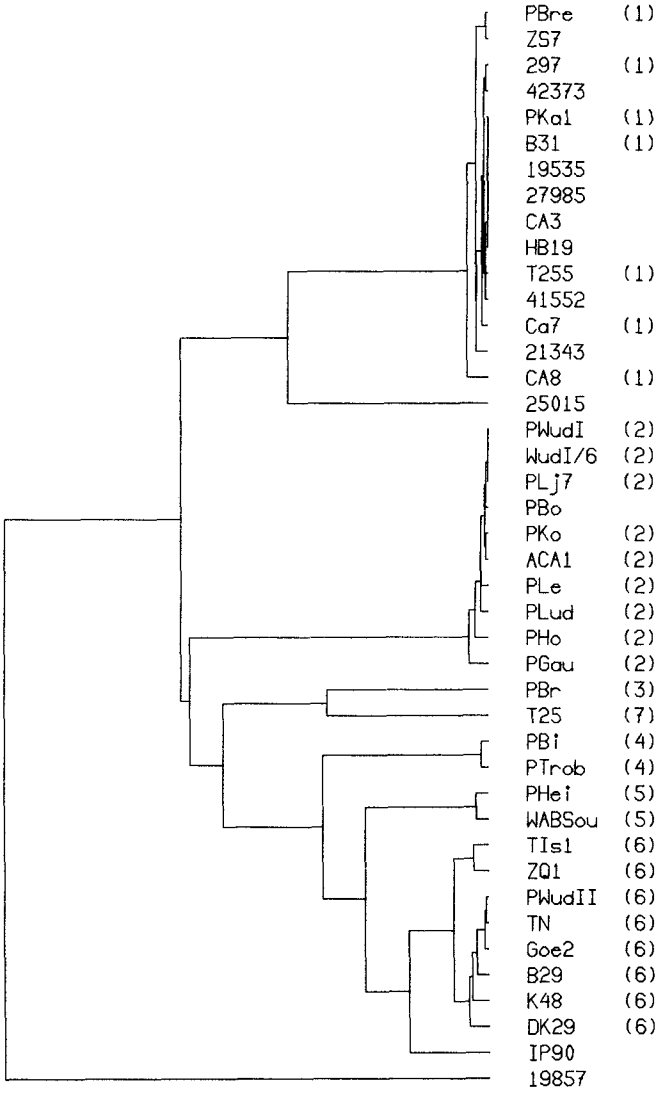

Fig. 3 Cluster analysis of *ospA* DNA sequences (from position 70-822/825 omitting the coding region for the signal sequence) from 19 strains analyzed in this study and 23 sequences already published [10, 14, 17, 20, 22, 37, 45]

of the borrelia from the host's immunoresponse [25, 28, 30]. Sequence analysis of *ospA* has been used as a tool for classification [14, 17, 22, 41, 45] or investigation of possible recombination events [17].

In the study presented here, the seven OspA serotypes previously described [41] could be confirmed on the molecular basis by sequence analysis of the complete *ospA* genes. The sequence data obtained in our laboratory added another 19 *ospA* sequences to the 21 sequences already published. This allowed cluster analysis of *ospA* with a significantly higher number of strains. Using this comprehensive analysis we found the following points.

The *ospA* tree forms three main clusters which are in agreement with grouping into the three species *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* as derived from PFGE pattern of *Mlu*I-digested DNA. This corroborates earlier studies with fewer strains performed by Wallich at al. [37] and Dykhuizen et al. [17], and also reconfirms our results previously obtained with strains analyzed for *ospA* partial sequences and 16SrRNA [41].

The *B. garinii* group turned out to be very heterogeneous in contrast to the homogeneity of *B. burgdorferi* sensu stricto and *B. afzelii* (Fig. 3). Two strains included in this study (PBr and T25) formed a new subgroup within the *B. garinii* cluster. Interestingly, OspA of these two strains is specifically recognized by monoclonal antibody L32 1F7. However, they differ considerably in their *ospA* genotype, which confirms the immunological classification into two different serotypes (3 and 7). Strains PBi and PTrob form another group more related to the other *B. garinii* strains than to PBr and T25. Strains PBi and PTrob, previously assigned to OspA serotype 4, are specifically recognized by monoclonal antibody L32 1G3 and they form a separate cluster within the *B. garinii* group. Of note was that this was also shown by sequence analysis of the p 83/100 gene [29].

OspA sero/genotype 5 strains PHei and WABSou form another special group. The *ospA* sequence analysis performed by Dykhuizen et al. [17] suggested that ancestors of PHei underwent intragenic recombination. We found another strain, WABSou, with nearly the same *ospA* sequence as PHei, thus showing the same evidence of a recent recombination event. These two strains have been both isolated from human tissue, but from different geographic regions (Göttingen, Germany; Vienna, Austria). The question arises as to whether this type of recombination occurred at different places, or prior to the geographic distribution of the strains. Our findings and those of Dykhuizen et al. confirm that recombination of *ospA* do not only take place under laboratory conditions, as previously shown by Rosa et al. [28]. Thus, recombination events may occur more often than expected, adding another problem for development of a OspA vaccine in addition to OspA heterogeneity.

The possible association of certain OspA types with different disease manifestations is another important aspect. In Europe, strains isolated from skin belong primarily to the species *B. afzelii*, whereas strains isolated from CSF are very hetergeneous [13, 18, 35, 39, 41]. An open question is whether the heterogeneity of CSF isolates simply reflects the heterogeneity of tick strains [18] or whether strains with certain OspA types (i.e., type 4) are more prone to cause neuroborreliosis than others [41]. However, since there is a close relationship between the OspA- and Ospc type, the type of OspC may also play a role in pathogenesis of the disease [42]. The same may be true for Osp proteins. The finding of Fuchs et al. [21] that OspA is a plasminogen receptor indicates an important role of OspA for dissemination of the borrelia in the host. Thus, molecular differences of OspA may be associated with different potentials to disseminate, implicating the importance of molecular and immunological analysis of OspA for identification of factors involved in pathogenicity. In addition, our results have important implications for the development of diagnostic antigens concerning OspA on the one hand and for *ospA* as a target for PCR on the other.

Acknowledgements We would like to thank W. Burgdorfer for providing us with *B. burgdorferi* strain B31, Russell Johnson for sending us strain 297 and Elisabeth Aberer for sending us strain WAB-Sou. We also thank Cecilia Hizo-Teufel, Claudia Schweizer, Erika Honka and Jutta Teepe for excellent technical assistance, and Mrs. Bergerhausen for photographic help. This work was supported by grants from the Max von Pettenkofer Institute. We express our thanks to the head of the institute, Prof. Dr. G. Ruckdeschel, for the generous support.

References

1. Adam T, Gassmann GS, Rasiah C, Göbel UB (1991) Phenotypic and genotypic analysis of *Borrelia burgdorferi* isolates from various sources. Infect Immun 59:2579–2585
2. Åsbrink E, Hovmark A (1985) Successful cultivation of spirochetes from skin lesions of patients with erythema chronicum migrans Afzelius and acrodermatitis chronica atrophicans. Acta Pathol Microbiol Immunol Scand [B]93:161–163
3. Baranton G, Postic D, Saint Girons I, Boerlin P, Piffaretti J-C, Assous M, Grimont PAD (1982) Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int J Syst Bacteriol 42: 378–383
4. Barbour AG (1988) Antigenic variations of surface proteins of Borrelia species. Rev Infect Dis 10:399–402
5. Barbour AG, Burgdorfer W, Hayes SF, Peter O, Aeschlimann A (1983) Isolation of a cultivable spirochete from *Ixodes ricinus* ticks of Switzerland. Curr Microbiol 8:123–126
6. Barbour AG, Tessier SL, Todd WJ (1983) Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect Immun 41:795–804
7. Barbour AG, Heiland RA, Howe TR (1985) Heterogeneity of major proteins in Lyme disease Borreliae: a molecular analysis of North American and European isolates. J Infect Dis 152: 478–484
8. Barthold SW, De Souza WS, Janotka JL, Smith AL, Persing DH (1993) Chronic Lyme borreliosis in the laboratory mouse. Am J Pathol 143:959–971
9. Belfaiza J, Postic D, Bellenger E, Baranton G, Girons IS (1993) Genomic fingerprinting of *Borrelia burgdorferi* sensu lato by pulsed-field gel electrophoresis. J Clin Microbiol 31:2873–2877
10. Bergström S, Bundoc VG, Barbour AG (1989) Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochete *Borrelia burgdorferi*. Mol Microbiol 3:479–486
11. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwald E, Davis JP (1982) Lyme disease – a tick-borne spirochetosis? Science 216:1317–1319
12. Busch U, Teufel CH, Boehmer R, Wilske B, Preac-Mursic V (1995) Molecular characterization of *Borrelia burgdorferi* sensu lato strains by pulsed-field gel electrophoresis. Electrophoresis (in press)
13. Canica MM, Nato F, Du Merle L, Mazie JC, Baranton G, Postic D (1993) Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scan J Infect Dis 25:441–448
14. Caporale DA, Kocher TD (1994) Sequence variation in the outer-surface-protein genes of *Borrelia burgdorferi*. Mol Biol Evol 11:51–64
15. Comstock LE, Fikrig E, Shoberg RJ, Flavell RA, Thomas DD (1993) A monoclonal antibody to OspA inhibits assocation of *Borrelia burgdorferi* with human endothelial cells. Infect Immun 61:423–431
16. Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12:387–395
17. Dykhuizen DE, Polin DS, Dunn JJ, Wilske B, Preac-Mursic V, Dattwyler RJ, Luft BJ (1993) *Borrelia burgdorferi* is clonal: implications for taxonomy and vaccine development. Proc Natl Acad Sci USA 90:10163–10167
18. Eiffert H, Ohlenbusch A, Christen H-J, Thomssen R, Spielman A, Matuschka F-R (1995) Nondifferentiation between Lyme disease spirochetes from vector ticks and human cerebrospinal fluids. J Infect Dis 171:476–479
19. Fellinger W, Redl B, Stöffler G (1992) Sequence of the complete osp operon encoding two major outer membrane proteins of a European *Borrelia burgdorferi* isolate (B29). Gene 120:127–128
20. Fikrig E, Barthold SW, Persing DH, Sun X, Kantor FS, Flavell RA (1992) *Borrelia burgdorferi* strain 25015: characterization of outer surface protein A and vaccination against infection. J Immun 148:2256–2260
21. Fuchs H, Wallich R, Simon MM, Kramer MD (1994) The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. Proc Natl Acad Sci USA 91:12594–12598
22. Jonsson M, Noppa L, Barbour AG, Bergström S (1992) Heterogeneity of outer membrane proteins in *Borrelia burgdorferi*: comparison of osp operons of three isolates of different geographic origins. Infect Immun 60:1845–1853
23. Langenberg W, Rauws EAJ, Widjojokusomo A, Tytgat GNJ, Zanen HC (1986) Identification of *Campylobacter pyloridis* isolate by restriction endonuclease DNA analysis. J Clin Microbiol 24:414–417
24. Ma Y, Weis JJ (1993) *Borrelia burgdorferi* outer surface lipoproteins OspA and OspB possess B-cell mitogenic and cytokine-stimulatory properties. Infect Immun 61:3843–3853
25. Marconi RT, Konkel ME, Garon CF (1993) Variability of *osp* genes and gene products among species of Lyme disease spirochetes. Infect Immun 61:2611–2617
26. Persing DH, Mathiesen D, Podzorski D, Barthold SW (1994) Genetic stability of *Borrelia burgdorferi* recovered from chronically infected immunocompetent mice. Infect Immun 62:3521–3527
27. Preac-Mursic V, Wilske B, Schierz G (1986) European *Borrelia burgdorferi* isolated from humans and ticks: culture conditions and antibiotic susceptibility. Zentralbl Bakteriol Hyg A 263: 112–118
28. Rosa PA, Schwan T, Hogan D (1992) Recombination between genes encoding major outer surface proteins A and B of *Borrelia burgdorferi*. Mol Microbiol 6:3031–3040
29. Rössler D, Eiffert H, Jauris-Heipke S, Lehnert G, Preac-Mursic V, Schlott T, Soutschek E, Teepe J, Wilske B (1995) Molecular and immunological characterization of the p83/100 protein of various *Borrelia burgdorferi* sensu lato strains. Med Microbiol Immunol 184:23–32
30. Sadziene A, Rosa PA, Thompson PA, Hogan DM, Barbour AG (1992) Antibody-resistent mutants of *Borrelia burgdorferi*: in vitro selection and characterization. J Exp Med 176:799–809
31. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York
32. Simon MM, Schaible UE, Kramer MD, Eckerskorn C, Müller-Hermelink HK, Wallich R (1991) Recombinant outer surface protein A from *Borrelia burgdorferi* induces antibodies protective against spirochetal infection in mice. J Infect Dis 164:123–132
33. Stanek G, Jurkowitsch B, Köchl C, Burger I, Khanakha G (1990) Reactivity of European and American isolates of *Borrelia burgdorferi* with different monoclonal antibodies by means of a microimmunoblot technique. Zentralbl Bakteriol 272:426–436
34. Steere AC (1989) Medical progress-Lyme disease. N Engl J Med 321:586–596
35. Van Dam AP, Kuiper H, Vos K, Widjojokusumo A, De Jongh BM, Spanjaard L, Ramselaar ACP, Kramer MD, Dankert J (1993) Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. Clin Infect Dis 17:708–717
36. Viera J, Messing J (1982) The pUC plasmids, a M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268

37. Wallich R, Helmes C, Schaible UE, Lobet Y, Motor SE, Kramer MD, Simon MM (1992) Evaluation of genetic divergence among *Borrelia burgdorferi* isolates by use of OspA, fla, HSP60, and HSP70 gene probes. Infect Immun 60:4856–4866
38. Wilske B, Preac-Mursic V, Schierz G, Gueye W, Herzer P, Weber K (1988) Immunochemical analysis of the immune response in late manifestations of Lyme borreliosis. Zentralbl Bakteriol Mikrobiol Hyg [A]267:549–558
39. Wilske B, Preac-Mursic V, Schierz G, Kühbeck R, Barbour AG, Kramer M (1988) Antigenic variability of *Borrelia burgdorferi*. Ann NY Acad Sci 539:126–143
40. Wilske B, Preac-Mursic V, Fuchs R, Bruckbauer H, Hofmann A, Zumstein G, Jauris S, Soutschek E, Motz M (1990) Immunodominant proteins of *Borrelia burgdorferi*; implications for improving serodiagnosis of Lyme borreliosis. In: Neu HC (ed) New antibacterial strategies. Proceedings of an international Symposium. Brocket Hall, Herfordshire, 30 June–3 July 1990. Churchill Livingstone, Edinburgh London Melbourne, pp 47–63
41. Wilske B, Preac-Mursic V, Göbel UB, Graf B, Kauris S, Soutschek E, Schwab E, Zumstein G (1993) An OspA serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal antibodies and OspA sequence analysis. J Clin Microbiol 31:340–350
42. Wilske B, Jauris-Heipke S, Lobentanzer R, Pradel I, Preac-Mursic V, Roessler D, Soutschek E, Johnson RC (1995) Phenotypic analysis of the 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
43. Yanagihara Y, Kawabata H, Masuzawa T (1994) Characterization of *Borrelia japonica* isolated from *Ixodes ovatus* in Japan. In: Cevenini R, Sambri V, La Place M (eds) Advances in Lyme borreliosis research. Proceedings of the VI international conference on Lyme borreliosis, 19–22 June 1994. Societa Editrice Esculapio, Bologna, pp 27–30
44. Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC18 vectors. Gene 33:103–119
45. Zumstein G, Fuchs R, Hofmann A, Preac-Mursic V, Soutschek E, Wilske B (1992) Genetic polymorphism of the gene encoding the outer surface protein A (OspA) of *Borrelia burgdorferi*. Med Microbiol Immunol 181:57–70