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Molecular analysis of the channel-forming protein P13 and its paralogue family 48 from different Lyme disease *Borrelia* species

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The aetiological agent of Lyme disease, *Borrelia burgdorferi* cycles between its tick vector and mammalian hosts, implying that it can sense different environments and consequently change the expression of genes encoding several surface-associated proteins. The genome of the type strain *B. burgdorferi* B31 has revealed 175 different gene families. The *p13* gene, situated on the chromosome, encodes a channel-forming protein that belongs to the gene family 48 consisting of eight additional paralogous genes. The heterogeneity of the P13 protein from different Lyme disease *Borrelia* strains was investigated. The predicted surface-exposed domains are the most heterogeneous regions and contain probable epitopes of P13. The membrane-spanning architecture of P13 was determined and a model for the location of this protein in the outer membrane is presented. The transcription of the paralogues of gene family 48 during *in vitro* culturing and in a mouse infection model was also analysed. The *bba01* gene is the only *p13* paralogue present in all three Lyme-disease-causing genospecies; it is stable during cultivation *in vitro* and the BBA01 protein was expressed in all *Borrelia* strains investigated. Conversely, paralogues *bbi31*, *bbq06* and *bbh41* were only detected in *B. burgdorferi* and the corresponding plasmids harbouring *bbi31* and *bbh41* were lost during *in vitro* passage. Finally, *p13* and *bbi31* are the only members of gene family 48 that are transcribed in mice, suggesting their importance during mammalian infection.

INTRODUCTION

Borrelia burgdorferi is a tick-transmitted spirochaete that causes Lyme borreliosis, a disease characterized by dermatological, rheumatological, cardiac and neurological manifestations (Steere, 1989). There are three genospecies of *Borrelia* that can cause Lyme borreliosis in humans, *B. burgdorferi*, *B. afzelii* and *B. garinii*. *B. burgdorferi* has an unusual genome consisting of a linear chromosome of 910 kb and 21 plasmids (12 linear and 9 circular) giving an additional 611 kb (Casjens *et al.*, 2000; Fraser *et al.*, 1997). Previous studies have shown that several *B. burgdorferi* plasmids are important for infectivity (Hefty *et al.*, 2002; Purser *et al.*, 2003; Purser & Norris, 2000; Schwan *et al.*, 1988a; Thomas *et al.*, 2001). Culture passages of *B. burgdorferi* are associated with loss of plasmids (Barbour, 1988) and thereby loss of infectivity (Labandeira-Rey & Skare, 2001; Schwan *et al.*, 1988a; Xu *et al.*, 1996). The *B. burgdorferi* B31 genome contains 175 gene families. Most of the paralogous genes within these families are situated on plasmids. Those gene families that already have been investigated include *bdr* (Carlyon *et al.*, 2000; Zuckert *et al.*, 1999), *bmp* (Aron *et al.*, 1994; Gorbacheva *et al.*, 2000; Ramamoorthy *et al.*, 1996; Simpson *et al.*, 1994), *elp* (Akins *et al.*, 1999; Hefty *et al.*, 2001), *erp* (Stevenson *et al.*, 1996, 1998a, b, 2002), *mlp* (Porcella *et al.*, 2000; Yang *et al.*, 1999, 2000), family 95 (*bapA* and *eppA*) (Miller & Stevenson, 2003) and *vls* (Wang *et al.*, 2001; Zhang *et al.*, 1997; Zhang & Norris, 1998a, b). In *B. burgdorferi* B31, there are nine members of gene family 48 with the sequence identity to *p13* ranging from 28·6 % for *bbj03* to 40·9 % for *bba01* (Casjens *et al.*, 2000; Fraser *et al.*, 1997). One member, the chromosomally encoded, channel-forming outer-membrane protein P13 has been thoroughly investigated (Nilsson *et al.*, 2002; Noppa *et al.*, 2001; Östberg *et al.*, 2002), and is among only a few integral membrane proteins characterized in *B. burgdorferi* (Bunikis *et al.*, 1995; Carroll *et al.*, 2001; Noppa *et al.*, 2001; Skare *et al.*, 1996). Since this gene family has not been identified in any other bacteria beside the genus *Borrelia*, the genes might encode proteins important for the infectious cycle of Lyme borreliosis spirochaetes.

In this study we investigated the heterogeneity of the *p13* gene from all species causing Lyme borreliosis. Significantly, the most heterogeneous, surface-exposed region of

The GenBank accession numbers for the *p13* nucleotide sequences determined for the Lyme disease *Borrelia* strains described herein are AY330920 to AY330935.

P13 appeared to be the natural epitope. Furthermore, we combined epitope mapping with computer-based predictions to determine the membrane-spanning architecture of P13 in the outer membrane. The presence of the other paralogous genes of family 48 in a number of different *Borrelia* strains was also examined, and transcription of *p13* and three of its paralogues (*bba01*, *bbi31*, *bbh41*) was investigated both under normal culture conditions and during infection in mice. Expression of the paralogue BBA01 in Lyme disease *Borrelia* was also investigated *in vitro*.

METHODS

Bacterial strains and growth conditions. The *Borrelia* strains used are listed in Table 1. Spirochaetes were grown in either BSK-H (Sigma) or BSK-II (Barbour, 1984) medium supplemented with 6 % rabbit serum (Sigma) at 32 °C until cell density reached approximately 10^7–10^8 cells ml^{-1}, after which the cell culture was pelleted for protein, DNA or RNA preparation.

Escherichia coli strains TOP10 (Invitrogen), DH5α (Invitrogen) and Rosetta (DE3)pLysS (Novagen) were used for cloning and expression. These strains were grown in Luria–Bertani (LB) liquid medium or agar

Table 1. Detection of the *p13* gene and its paralogues

Borrelia strain	Reference	*p13*	*bba01*	*bbi31*	*bbh41*	*bbq06*
Lyme disease *Borrelia*						
B. burgdorferi						
B31HP*	ATCC 35210	+	+	−	−	−
B31LP*	ATCC 35210	+	+	+	+	+
HB19	Barbour (1984)	+	+	+	+	+
B31-A	Bono *et al.* (2000)	+	+	−	+	+
B313	Sadziene *et al.* (1995)	+	−	−	−	−
Sh2-82	Schwan *et al.* (1988b)	+	+	+	+	ND
N40	Barthold *et al.* (1988)	+	+	+	−	−
B. afzelii						
ACAI HP	Åsbrink *et al.* (1984)	+	+	−	−	ND
ACAI LP	Åsbrink *et al.* (1984)	+	+	−	−	ND
U01	Shoberg *et al.* (1994)	+	+	−	−	ND
F1	Åsbrink *et al.* (1984)	+	+	−	−	ND
ECM1	Xu & Johnson (1995)	+	+	−	−	ND
VS461	Peter & Bretz (1992)	+	+	−	−	ND
LU81	Ornstein *et al.* (2001)	+	+	−	−	ND
B. garinii						
Ip90 HP	Kriuchechnikov *et al.* (1988)	+	+	−	−	ND
Ip90 LP	Kriuchechnikov *et al.* (1988)	+	+	−	−	ND
Lab	Bunikis *et al.* (1996)	+	+	−	−	ND
NBS16a	Bergström *et al.* (1992)	+	+	−	−	ND
NBS23a	Bergström *et al.* (1992)	+	+	−	−	ND
Fis01	Bunikis *et al.* (1996)	+	+	−	−	ND
Far01	Bunikis *et al.* (1996)	+	+	−	−	ND
Far02	Bunikis *et al.* (1996)	+	+	−	−	ND
Mal01	Bunikis *et al.* (1996)	+	+	−	−	ND
Mal02	Bunikis *et al.* (1996)	+	+	−	−	ND
LU190	Ornstein *et al.* (2001)	+	+	−	−	ND
LU59	Ornstein *et al.* (2001)	+	+	−	−	ND
Lyme disease related						
B. valaisiana vs116	Wang *et al.* (1997)	+	−	−	−	ND
B. bissettii CA128	Postic *et al.* (1998)	+	+	−	−	ND
B. tanukii Hk501	Fukunaga *et al.* (1996)	+	−	−	−	ND
B. japonica HO14	Kawabata *et al.* (1993)	+	+	−	−	ND
B. lusitaniae PotiB2	Le Fleche *et al.* (1997)	+	−	−	−	ND
B. turdi Ya 501	Fukunaga *et al.* (1996)	+	+	−	−	ND
Avian borreliosis						
B. anserina†	Noppa *et al.* (1995)	−	−	−	−	ND

ND, Not determined.

*LP, low passage (less than six passages); HP, high passage (more than 20 passages).

†Obtained from the strain collection of Alan G. Barbour, University of California, Irvine, CA, USA.

plates supplemented with carbenicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or chloramphenicol (34 μg ml^{-1}) when required.

Identification and sequencing of *p13* and its paralogous genes from different *Borrelia* species. Genes of interest were amplified by PCR using *Taq* polymerase (Roche) under the following conditions: 6 cycles at 94 °C for 30 s, 40 °C for 1 min, 72 °C for 1 min followed by 35 cycles at 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min.

Primers Y12 and Y18 (Noppa *et al.*, 2001) were used for amplification of the *p13* gene. For amplification of *bba01*, *bbi31* and *bbh41*, the primer combinations of A01-M2 with A01-M2Rev, I31-M2 with I31-M2Rev, and H41-M2 with H41-M2Rev were used respectively (Table 2). Every PCR reaction was repeated twice to ensure the validity of the results. For sequencing, the *p13* and *bba01* PCR products were cloned into pGEM-T Easy vector (Promega) and maintained in DH5α. Plasmids were prepared using the Qiaprep Spin miniprep kit (Qiagen) and sequencing was performed using the BigDye Kit (Perkin Elmer) and an Applied Biosystems ABI 377 Sequencer. The sequences were analysed using GCG [Wisconsin Package Version 9.1, Genetics Computer Group (GCG)] software and alignment of the consensus sequences was obtained using the BioEdit program (Tom Hall, Department of Microbiology, North Carolina State University).

Detection of plasmids lp28-3 and lp28-4. *B. burgdorferi* strains B31, N40 and Sh2-82 were initially grown from low passage (approx. passage 4) and then subsequently cultivated by adding 1 ml of the culture in exponential growth phase ($\sim 10^7$ cells ml^{-1}) to 12 ml of BSK-II medium. The presence of the paralogous gene sequences and corresponding plasmids was determined by PCR in each passage using PCR conditions as described above. The primers used for amplification of the lp28-3 and lp28-4 plasmids were H-M4 with H-M4Rev and I-M4 with I-M4Rev respectively (Table 2). PCR reaction using the same reaction conditions as described above. PCR products were analysed on a 1 % TBE-agarose gel stained with ethidium bromide (5 μg ml^{-1}).

Mouse infection. *B. burgdorferi* strain B31 was used for mouse infections. Prior to infection, the presence of the query genes in B31 was confirmed by PCR. Four-week-old C3H/HeN mice (Bomholt Gård Breeding, Denmark) were subcutaneously injected with 10^5 spirochaetes in 0·1 ml culture medium. The number of bacteria was determined microscopically in a Petroff–Hausser chamber. Negative controls were injected with 0·1 ml BSK-II medium. Three weeks post-infection, the mice were anaesthetized by injection of a mixture of Dormicum (Roche), Hypnorm (Janssen Pharmaceutica) and water (1 : 1 : 2), and killed by cardiac puncture. Ears, heart and bladder were aseptically removed and one ear and heart were immediately frozen in liquid nitrogen. The other ear and bladder were added to BSK-II medium supplemented with sulfamethoxazole (1·25 μl ml^{-1}) and phosphomycin (4 μl ml^{-1}) for detection of *Borrelia* spirochaetes.

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ to 3′)*	Target	Position†
P13-RT1‡	AAATGGTGCGTTTGGGATGAGTG	*p13*	32015–31993
P13-RT2‡	AATCCTCCTAAAGCTACATTAAGGC	*p13*	31626–31650
A01-RT1‡	AATTCAAATCTTTGCCACAAAAGACACAC	*bba01*	629–657
A01-RT2‡	AGCCTGCAAGTTCAGCGCTGAGTC	*bba01*	1011–988
A01-M2	AAAAGACACACACAAAATAGAATTGAAAAAGGC	*bba01*	645–677
A01-M2Rev	CTACCCCTATTAATATGTATCCGGTTAAT	*bba01*	900–872
H41-RT1‡	AAGAGAAAAGGAGTTCATTCAAAACAG	*bbh41*	28099–28075
H41-RT2‡	AAGTAGGATGATCCAAACGTGAGC	*bbh41*	27770–27793
H41-M2	GCTTTAAATTTCACTTCAAAATATAAAATTCAG	*bbh41*	28143–28111
H41-M2Rev	GTAGTATCGATCTTCAAATAGTACTGGCAA	*bbh41*	27727–27756
H-M4	CCTACATTTTCTGCAAGTCTTAAAAAG	lp28-3	11112–11138
H-M4Rev	GGGGGACATTTAAGATAGTGTT	lp28-3	11591–11570
I31-RT1‡	GAAAAAAGTGTTGGAAGTATTGAAACC	*bbi31*	20037–20011
I31-RT2‡	AGATTTGCATTGTGTTTATTTGCAAATG	*bbi31*	19709–19736
I31-M2	CAACACAAGATAAGCTTGAAAAAAGTGTTGG	*bbi31*	20054–20024
I31-M2Rev	GTTGCTCTTGTTTCATCATCTGTATGGCC	*bbi31*	19814–19842
I-M4	GGACTTTGCCACATGCTGTATTAACT	lp28-4	15413–15438
I-M4Rev	CAAAATAGATAAGTTATAAAGCCTTC	lp28-4	16599–16574
Q06-RT1‡	AAAGGTGTTGGAGATATTGCAACCG	*bbq06*	3653–3677
Q06-RT2‡	TCTGTAACGAATCCAATATCCCCGG	*bbq06*	3867–3843
P13M1F	CTTTGCCATGGCTAATGATTCTAAAAATGG	*p13*	32039–32010
P13M1R	TCAAGGTACCCTTAAGCTACATTAAGGCTA	*p13*	31623–31652
Y54MR	CTTTGGTACCTTAATAAGCCCCCGCAAG	*p13*	31804–31831
Y55MF	ATAGTCATGATACTTGCGGGGGCTTATT	*p13*	31843–31816
Y56MR	TCACGGTACCTCAAACCACACCTGCTAA	*p13*	31658–31686
Y57MF	AAGGTCATGATGTTAGCAGGTGTGGTTAC	*p13*	31756–31728

*Restriction sites are underlined.

†Nucleotide position in the genome.

‡Primers used for RT-PCR.

Isolation of RNA and RT-PCR. All reagents were prepared with diethylpyrocarbonate (DEPC)-treated water. Total RNA was isolated from *in vitro*-cultured *Borrelia* using the Ultraspec-II RNA isolation system (Biotex Laboratories) or Trizol reagent (Invitrogen) and from mouse heart with Trizol reagent essentially according to the manufacturer's instructions. Aliquots (2 μg) of each RNA preparation were then treated with 3 units of RNase-free DNaseI (Roche) to remove contaminating DNA.

Both 2-step and 1-step RT-PCR were used in this study. First-strand cDNA reactions were primed with 5 pmol gene-specific primer (Table 2) and synthesized using the AMV reverse transcriptase (Roche). For gene-specific PCR amplification from cDNA, 5 μl of first-strand cDNA reaction was amplified in a total volume of 50 μl, containing 20 nM of each primer, 200 μM of each dNTP and 0·5 units of *Taq* DNA polymerase. For 1-step RT-PCR from the 1 μg of RNA, the Superscript One-Step RT-PCR with Platinum *Taq* kit (Invitrogen) was used following the manufacturer's instructions. In each RT-PCR, two negative controls were included: sterile water to verify the purity of the reagents, and RNA from uninfected mice to confirm the reaction specificity and the absence of cross-contamination during sample processing. Using DNA extracted from *B. burgdorferi* cultures we monitored amplification efficiency. Two additional positive controls were included: primers specific for the mouse housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) (Pinto do *et al.*, 1998) were used to monitor RT-PCR efficiency on RNA preparations from organs, and the flagella-specific *flaB* primers FB1 and FB2 (Noppa *et al.*, 2001) were used for *Borrelia* RNA detection. For all 1-step RT-PCR reactions the absence of DNA contamination was verified by PCR. Ten microlitres of each RT-PCR product was analysed on 1 % TBE-agarose gel stained with ethidium bromide (5 μg ml^{-1}).

Protein electrophoresis, immunoblotting and antibodies. Proteins were separated in Tricine 10–20 % polyacrylamide gradient gels or in Bistris 4–12 % polyacrylamide gradient NuPage gels by using the Novex XCell Sure Lock electrophoresis cell (Invitrogen). Total *Borrelia* proteins were prepared by growing cells to stationary phase, harvesting by centrifugation at 8000 ***g*** at 4 °C, and washing twice in phosphate-buffered saline (PBS). The outer-membrane proteins (B-fraction) of *B. burgdorferi* strain B31 were prepared as described elsewhere (Magnarelli *et al.*, 1989). For immunoblotting, proteins were transferred to a PVDF membrane (PALL Corp.) and probed with antibodies. The polyclonal antibody recognizing P13 is described elsewhere (Noppa *et al.*, 2001). For production of serum against the BBA01 protein, a synthetic peptide with the sequence EKGIESFNKYDKEKC (amino acids 28–41 plus a terminal cysteine) was used (obtained from Innovagen). The peptide fragment was conjugated to a keyhole limpet haemocyanin (KLH) carrier via its terminal cysteine with maleimide cross-linker (Agrisera). The synthetic peptide was used for immunizing a rabbit and an immune serum was obtained (Agrisera). The monoclonal antibody (mAb) 15G6, recognizing P13, was previously described (Sadziene *et al.*, 1995). Bound antibodies were detected using peroxidase-conjugated anti-rabbit or anti-mouse antibodies (DAKO) and enhanced chemiluminescence (ECL) reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Overexpression of different P13 fragments. Three fragments (A, B and C) covering the sequence of mature P13 were obtained using the primer pairs P13M1F with Y54MR (A), Y55MF with Y56MR (B), and Y57MF with P13M1R (C). All primer sequences are given in Table 2; the restriction sites included were: *Acc*65I for primers P13M1R, Y54MR and Y56MR, *Bsp*HI for primers Y55MF and Y57MF, and *Nco*I for primer P13M1F. The PCR conditions used were as follows: 5 cycles at 94 °C for 30 s, 45 °C for 1 min, 72 °C for 1 min and 35 cycles at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min. The PCR products were further purified using High Pure PCR Product Purification Kit (Roche) columns; fragments B and C were digested with *Acc*65I and *Bsp*HI restriction enzymes, whereas fragment A was digested with *Acc*65I and *Nco*I restriction enzymes (New England Biolabs). Thereafter, the PCR products were cloned in pETM-20 vector (EMBL, Heidelberg, Germany) digested with *Acc*65I and *Bsp*HI and transformed into *E. coli* TOP10. Colonies were screened for the correct inserts and plasmids containing the three different fragments of the *p13* gene (A, B and C) were purified using the Qiaprep plasmid miniprep kit (Qiagen) and transformed into Rosetta (DE3)pLysS cells for protein expression. After 3 h induction, the cultures were harvested by centrifugation at 8000 ***g*** for 5 min, resuspended in 200 μl PBS, and the protein concentration was estimated using Bio-Rad Protein Assay kit. Protein samples (10 μg) were then analysed on 4–12 % Bistris acrylamide gels and stained with Coomassie G-250. Epitope mapping was performed using Western blotting and mAb 15G6.

RESULTS

Analysis of the *p13* gene and its paralogues in different *Borrelia* species

The *p13* (*bb0034*) gene belongs to the paralogous gene family 48 with nine members (Table 3). Unlike *p13*, all the

Table 3. Gene family 48

This table is adapted from www.tigr.org.

Gene*	Nucleotide position	Location	Function
bb0034	32089–31553	Chromosome	Channel-forming protein P13
bba01	588–1070	lp54	Conserved hypothetical protein
bbg03	2104–2492	p28-2	Conserved hypothetical protein, authentic frameshift
bbh41	28197–27628	lp28-3	Conserved hypothetical protein
bbi31	20127–19618	lp28-4	Conserved hypothetical protein
bbj02.1	1475–2367	lp38	Conserved hypothetical protein, pseudogene
bbj03	1593–1742	lp38	Hypothetical protein (located inside BBJ02.1)
bbq06	3623–4105	lp56	Conserved hypothetical protein
bbq81	49246–49047	lp56	Conserved hypothetical protein, pseudogene

*Genes investigated in this study are highlighted in bold letters.

paralogues are plasmid encoded. Moreover, *bbg03* has an authentic frameshift, *bbj02.1* and *bbq81* are considered to be pseudogenes and *bbj03* is located inside the *bbj02.1* gene (Casjens *et al.*, 2000; Fraser *et al.*, 1997). We have earlier noted that four of the paralogous genes (*bba01*, *bbi31*, *bbh41* and *bbq06*) show more significant sequence similarities with the *p13* gene (Noppa *et al.*, 2001) and we therefore chose these for further investigation.

We analysed the presence of *p13* and its paralogous genes in different *Borrelia* species using PCR amplification of the *p13*, *bba01*, *bbi31*, *bbh41* and *bbq06* genes (Table 1). The *p13* gene was detected in all species investigated with the exception of the avian borreliosis agent *B. anserina* (Table 1). The *bba01* paralogue was detected in all Lyme disease species and all Lyme-disease-related species except *B. valaisiana*, *B. tanukii* and *B. lusitaniae* (Table 1). Interestingly, the *bbi31* and *bbh41* genes only appear to be present in *B. burgdorferi* strains. Within this strain collection, however, *bbi31* is not detected in strains B31-A and B313, while *bbh41* was absent in strain B313 and N40. These two paralogues are situated on the linear plasmids lp28-4 and lp28-3 respectively, which we estimated are lost after about 25 cultivation passages of *Borrelia* spirochaetes *in vitro* (data not shown).

Given the widespread distribution of the *p13* allele within Lyme disease *Borrelia* species and strains, the heterogeneity among the P13 protein was investigated. Amino acid sequence comparison of P13 revealed that it is considerably homogeneous and that most sequence heterogeneity is found outside the predicted transmembrane regions, especially between the transmembrane regions III and IV (Fig. 1). This observation is consistent with the probable surface exposure of these regions of P13, as is considered to be true also for the corresponding parts of the paralogue proteins within family 48 (Noppa *et al.*, 2001). Additionally, the most sequence heterogeneity of P13 is found between species. P13 of the *B. garinii* strains was found to be the least well conserved, as expected, since this genospecies is the most heterogeneous of all Lyme disease *Borrelia* spp. (Baranton *et al.*, 1998). We also analysed the

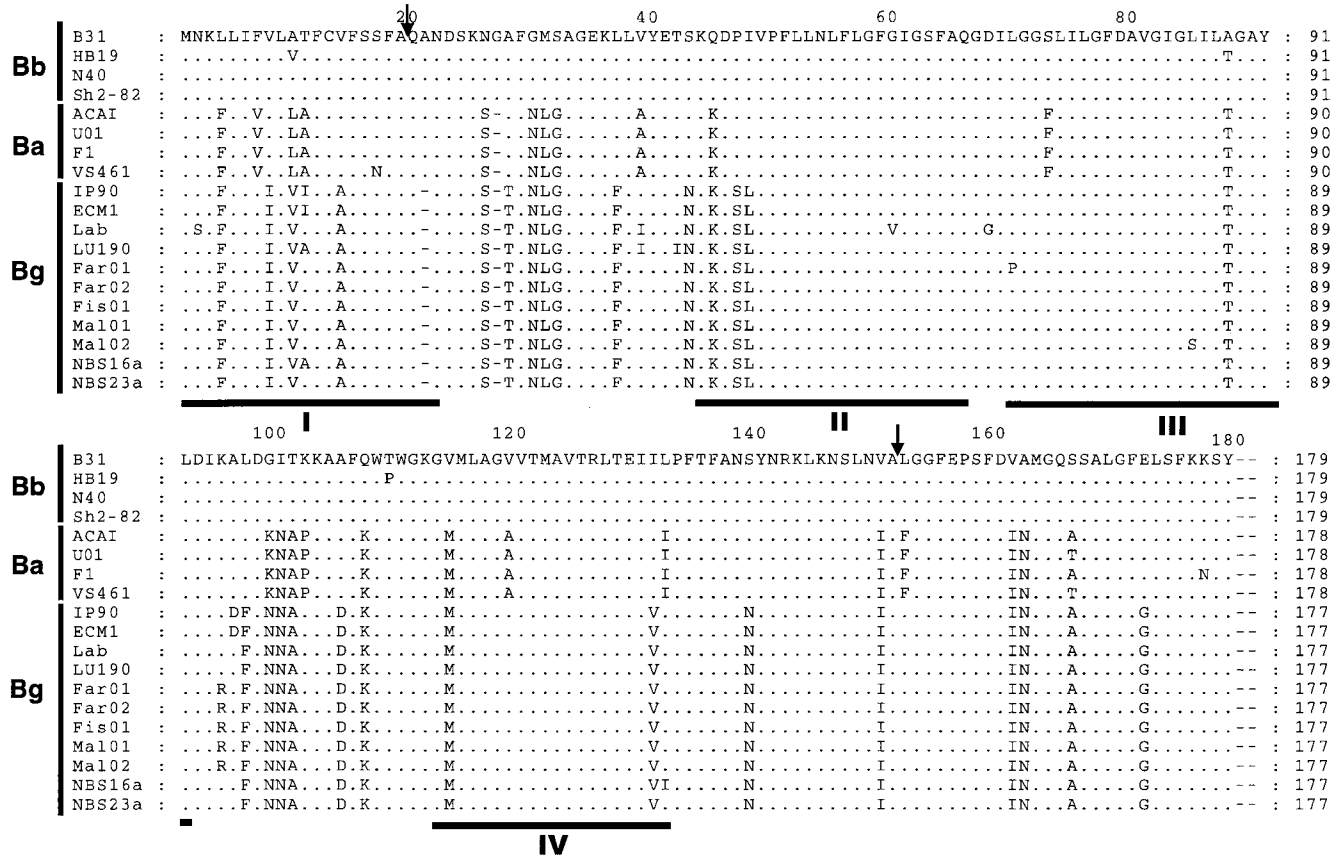

Fig. 1. Amino acid comparison of the full-length P13 protein from 19 different strains of Lyme disease *Borrelia*: Bb, *B. burgdorferi*; Ba, *B. afzelii*; Bg, *B. garinii*. The sequences were aligned against the P13 sequence of type strain B31 using the BioEdit program (Tom Hall, Department of Microbiology, North Carolina State University). Identical amino acids are represented by dots; letters represent amino acids that differ. The N- and C-terminal cleavage sites as determined by Noppa *et al.* (2001) are marked by arrows. The putative transmembrane domains (I–IV) predicted by the webserver SOSUI system (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) are highlighted by horizontal solid bars.

sequence heterogeneity of the *bba01* from different Lyme disease *Borrelia* strains by sequencing an internal region (located at nucleotide position 60–313) of the gene. The *bba01* gene was very homogeneous, with only minor heterogeneity between species (data not shown).

Epitope mapping of P13

To investigate the architecture of P13 and elucidate the natural epitope of the protein, we performed epitope mapping of the P13 protein derived from *B. burgdorferi* B31. Based upon the assumption that the most heterogeneous region of the protein is the outer-surface-exposed epitope, we expressed three fragments of mature P13 located at amino acid positions 20–92 (fragment A), 86–119 (fragment B) and 114–151 (fragment C). The expression vector of choice was pETM-20, which contains a His-tag, a recombinant tobacco etch virus (rTEV) protease cleavage site and a thioredoxin reductase (TrxA) fusion protein. Expressed proteins were detected on a Coomassie-stained gel (Fig. 2a), and by immunoblotting with mAb 15G6 (Fig. 2b). This antibody is supposed to recognize the natural epitope of the protein (Sadziene *et al.*, 1995). Indeed, mAb 15G6 recognized only the fragment of P13 which covers the predicted surface-exposed region, fragment B (Fig. 2b, lane 3). The other two fragments were not recognized, presumably because these correspond to transmembrane regions with only a short surface-exposed domain (fragment A) and a short domain that is directed into the periplasmic space (fragment C) (Fig. 2b, lanes 1 and 4). Interestingly, the polyclonal sera against P13 (Noppa *et al.*, 2001) recognized only fragments A and C, but not the surface-exposed epitope (fragment B) (data not shown). This indicates that the polyclonal antibody raised against purified P13 does not necessarily recognize the natural epitope of the protein. This result is consistent with our finding that this polyclonal rabbit antiserum does not recognize P13 of intact *B. burgdorferi*, but binds to P13 only when the outer membrane is permeabilized (data not shown). Based on these results we have proposed a model illustrating how P13 is located in the outer membrane of *Borrelia* (Fig. 3). This prediction also combines computer analysis of the transmembrane-spanning domains using the webserver SOSUI system (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), secondary structure prediction using the webservers TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and PSORT (http://psort.nibb.ac.jp/form.html), and our previous published results (Noppa *et al.*, 2001).

RT-PCR analysis of the *B. burgdorferi* gene family 48 in culture and mice

The presence of transcripts from *p13* and its paralogous genes (*bba01*, *bbi31*, *bbh41* and *bbq06*) was investigated by

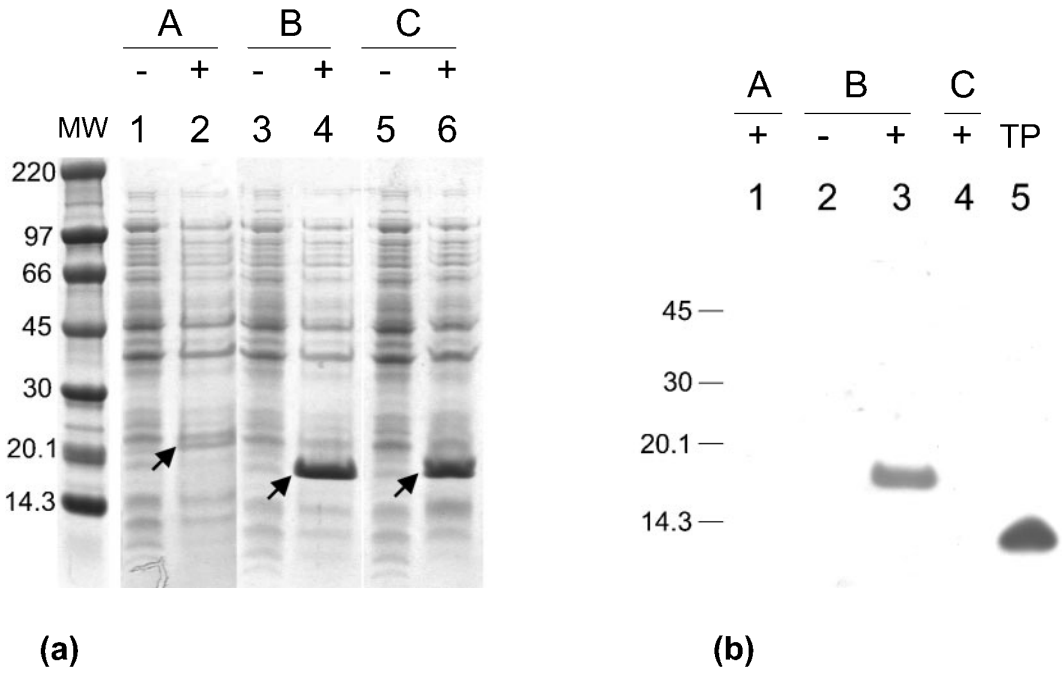

Fig. 2. Epitope mapping of P13: expression of the three different fragments, A, B and C, of P13 in vector pETM-20. (a) Ten micrograms of the total proteins from *E. coli* cultures grown in the presence (+) or absence (−) of the inducing agent IPTG were separated on Bistris 4–12 % polyacrylamide NuPage gel and stained with Coomassie G-250. Lanes: 1 and 2, fragment A; 3 and 4, fragment B; 5 and 6, fragment C. Expressed peptides are indicated by arrows. (b) Different fragments of P13 protein expressed in *E. coli* and investigated by immunoblotting using mAb 15G6 against P13. Lanes: 1, fragment A; 2 and 3, fragment B; 4, fragment C; 5, total protein (TP) of *B. burgdorferi* B31. Molecular mass markers in kDa are indicated to the left.

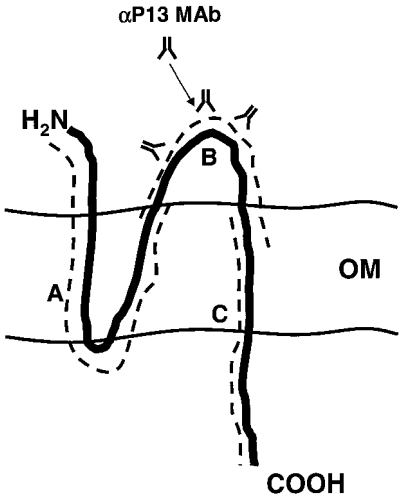

Fig. 3. Model of the P13 architecture in the outer membrane (OM) of *Borrelia*. Three fragments of P13 used as targets for epitope mapping are shown by dashed lines as A, B and C. The bold line shows the proteolytically cleaved, mature P13 in the outer membrane. mAb 15G6 is shown schematically to specifically recognize the surface-exposed epitope of P13.

RT-PCR from different *in vitro*-cultivated *B. burgdorferi* strains (Table 4). To study the expression of *p13* and its paralogues in mice, low-passage, infectious strain B31 was used. Three weeks after infection, the presence of *B. burgdorferi* in organs was analysed by *in vitro* culturing of tissue samples from ear and urinary bladder and subsequent microscopy screening (data not shown). Using PCR, we also investigated if the *p13* gene and its paralogous gene sequences were present in *B. burgdorferi* cultivated from mouse organs (data not shown). Two out of eight *B. burgdorferi* B31 infected mice were chosen for further experiments, and RNA from heart tissue was used for RNA preparation and subsequent RT-PCR analysis. The RT-PCR analysis revealed that *p13* and *bbi31* are expressed both in laboratory culture (Table 4) and in mice (Table 5), whereas *bba01* and *bbh41* were only expressed in culture and their expression was below the detectable level in mice (Tables 4 and 5).

Table 4. RT-PCR transcripts of genes from family 48 in *B. burgdorferi* strains grown *in vitro*

Strain*	*p13*	*bba01*	*bbi31*	*bbh41*	*bbq06*
B31 HP	ND	+	ND	ND	−
B31 LP	+	+	+	+	+
N40 LP	+	+	+	−	ND

ND, Not determined.
*HP, high passage; LP, low passage.

Table 5. RT-PCR transcripts of genes from family 48 in *B. burgdorferi* B31 isolated from mice

RNA source	*p13*	*bba01*	*bbi31*	*bbh41*	*flaB*	*hprt* (mouse)
B1-3 (B31-infected mouse)	+	−	+	−	+	+
B2-2 (B31-infected mouse)	+	−	+	−	+	+
C2 (non-infected mouse)	−	−	−	−	−	+
B31 (control from culture before infection)	+	+	+	+	+	−

Analysis of BBA01 expression in different strains of Lyme disease *Borrelia*

Since the *bba01* gene exhibits most sequence identity (40·9 %) to *p13* and was detected in all Lyme disease species, the expression of BBA01 protein in cultures of Lyme disease *Borrelia* was investigated using polyclonal rabbit antiserum raised against BBA01 (Fig. 4). For immunization, a synthetic peptide to a probable outer-surface-exposed region of BBA01 situated between the two computer-predicted transmembrane regions II and III was used (data not shown). The immunoblot reveals that the identified BBA01 protein is smaller than theoretically calculated (17·8 kDa) using the website http://www.expasy.org/tools/tagident.html (Appel *et al.*, 1994). Thus, BBA01 seems to be processed similarly to P13 (Noppa *et al.*, 2001). Our experiment shows that BBA01 seems to be produced only in low-passage strain B31, although the gene was detected in high-passage B31 (Table 4). As expected, BBA01 was not produced in strain B313, since this strain lacks lp54. Interestingly, we could not detect BBA01 in the outer-membrane fraction of *B. burgdorferi* B31 (Fig. 4,

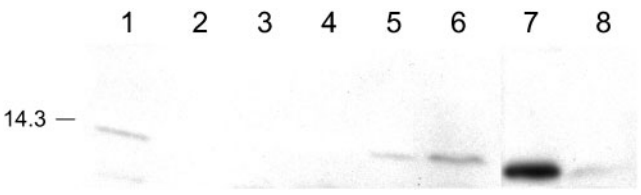

Fig. 4. Western blot analysis of BBA01 expressed in Lyme disease *Borrelia*. Ten micrograms of total protein was separated on 10–20 % Tricine gel, blotted onto a PVDF membrane and probed with rabbit immune serum against BBA01. Lanes: 1, total protein of low-passage B31 (8 passages); 2, outer-membrane fraction of low-passage B31 (9 passages); 3, total protein of high-passage B31 (>100 passages); 4, total protein of strain B313; 5, total protein of strain B31-A; 6, total protein of strain P13-18; 7, total protein of *B. afzelii* ACAI; 8, total protein of *B. garinii* lp90. Molecular size in kDa is indicated to the left.

lane 2). However, in the *p13* knockout mutant P13-18 (Östberg *et al.*, 2002), BBA01 production was significantly upregulated compared to the isogenic wild-type strain B31-A (Fig. 4, lanes 5 and 6). Moreover, the highest production of BBA01 was in the low-passage *B. afzelii* ACAI (Fig. 4, lane 7), but is also present in low-passage *B. garinii* Ip90 (Fig. 4, lane 8). This suggests that the BBA01 paralogue plays an important role in all three species of Lyme disease *Borrelia*.

DISCUSSION

Previous studies have revealed that the P13 protein is an integral outer-membrane protein with surface-exposed domains (Noppa *et al.*, 2001) and channel-forming activity (Östberg *et al.*, 2002). We therefore hypothesized that the computer-predicted outer-membrane-exposed domains should be the most heterogeneous regions of the protein. We analysed the heterogeneity of the P13 protein from several different Lyme disease *Borrelia* strains to confirm that the predicted outer-surface-exposed domains are the most variable regions (Fig. 1). We utilized an epitope mapping strategy to characterize the antigenic determinant of P13 and elucidate the possible membrane-spanning architecture of the protein. Consistent with *in silico* data, an expressed peptide encompassing the most diverse region of P13 (fragment B) was specifically detected with a monoclonal antibody (mAb 15G6) that apparently recognizes only the natural epitope of P13 protein (Fig. 2b, lane 3, and Sadziene *et al.*, 1995). Hence, we interpret this to indicate that the region composed of fragment B is surface exposed and serves as the natural epitope of the protein. Thus, a model was proposed for how P13 is situated in the outer membrane of *Borrelia* (Fig. 3). mAb 15G6 strongly inhibits growth of *B. burgdorferi* strain B313, deficient in the major outer-surface proteins (Osps), but has no effect on the growth of the Osps-bearing strain B31 (Sadziene *et al.*, 1995). Not only does this suggest that P13 performs an important function for the survival of *Borrelia*, but also that in the presence of Osps the P13 epitope accessibility is impaired. It is also noteworthy that in an immunoblot using our polyclonal antiserum against P13, only the two expressed peptide fragments (A and C, Fig. 3), which flanked surface-exposed fragment B, were recognized (data not shown). This discrepancy between the behaviour of the two antibodies could be explained by their different modes of development. Our P13 polyclonal antiserum was obtained by immunizing a rabbit with purified P13 (Noppa *et al.*, 2001) and could therefore recognize the entire P13 protein. In contrast, the mAb was raised against whole *Borrelia* cells, restricting recognition to the natural surface-exposed epitope of P13 (Sadziene *et al.*, 1995). Perhaps the polyclonal antiserum did not recognize the exposed epitope of P13 (fragment B) because of an altered conformation of the artificial peptide or simply because the serum is not specific enough to recognize this small region of the whole protein.

The loss of plasmids during *in vitro* cultivation is a well-known phenomenon for *Borrelia* spirochaetes (Barbour, 1989; Busch *et al.*, 1997; Grimm *et al.*, 2003; Labandeira-Rey & Skare, 2001; McDowell *et al.*, 2001; Norris *et al.*, 1995), although this is restricted to plasmids harbouring genes whose products are important for infectivity but not *in vitro* growth (Labandeira-Rey & Skare, 2001; Purser & Norris, 2000). Our results showed that paralogue *bba01*, situated on the 54 kb linear plasmid (lp54), is not lost during *in vitro* passage. Moreover, *bba01* is also the only paralogue detected in all Lyme disease *Borrelia* species. These data correlate with earlier studies and indicate that genes situated on plasmid lp54 are not needed for infectivity, but probably play a role during other stages of the *Borrelia* life cycle (Grimm *et al.*, 2003; Labandeira-Rey & Skare, 2001; Purser & Norris, 2000). On the other hand, we could show that plasmids lp28-3 and lp28-4 are lost during *in vitro* cultivation. This indicates that genes situated on these plasmids are not needed for growth of *Borrelia in vitro*, but could play a role in infectivity and survival of *Borrelia in vivo*. However, independent studies indicated that *B. burgdorferi* plasmids lp54 and lp28-3 are not lost during cultivation *in vitro* (Grimm *et al.*, 2003) and are always present in clones examined after murine infection (Purser & Norris, 2000). In addition, *B. burgdorferi* B31 lacking lp28-4 were modestly attenuated in a mouse infection model and carried an important antigen (VraA) for their infectivity in rabbits (Labandeira-Rey *et al.*, 2001; Labandeira-Rey & Skare, 2001). This correlates with our finding that the *bbi31* gene situated on this plasmid is transcribed in mice, while neither *bba01* nor *bbh41* was (Table 5). Taken together, however, these data contradict another study in which it was concluded that lp28-4 is not needed during infection of mice (Purser & Norris, 2000). Perhaps the different model systems used for the investigation of plasmid stability could explain these controversies. Nevertheless, the results also show that *Borrelia* is a complicated organism requiring a distinct plasmid content during different stages of its life cycle.

Since almost all plasmids (with exception of lp54) harbouring the investigated paralogous genes of family 48 are lost during cultivation, they are probably not needed for growth of *B. burgdorferi in vitro*, but could be needed for their pathogenicity. To investigate the possible function(s) of the paralogue proteins of family 48, we therefore analysed expression of *p13* and its paralogues in laboratory culture and during murine infection. Our results showed that the *p13* and *bbi31* are transcribed in both conditions, while all the other paralogous genes are only transcribed during *in vitro* culture and are below the detectable level in mice (Tables 4 and 5). Thus, our RT-PCR results indicate that the P13 and BBI31 proteins could be needed during the infection process and may constitute important virulence factors of *Borrelia*.

Our sequencing data for the *bba01* gene from *B. burgdorferi* strains B31, N40 and Sh2-82 have revealed sequence

homology with two earlier published *B. burgdorferi* N40 genes, *p11* and *p5*, located in an operon on lp54 (Feng *et al.*, 1996). The analysis of the genome sequence of *B. burgdorferi* B31 showed that the *p11* and *p5* genes were absent; instead the *bba01* gene was defined (Fraser *et al.*, 1997). In the study by Feng *et al.* (1996) a deletion of one nucleotide in the gene sequence had apparently led to the frameshift and a stop codon. Our sequencing data of the *bba01* gene from *B. burgdorferi* strains B31, N40 and Sh2-82 and the published *B. burgdorferi* B31 genome sequence have revealed that there is no frameshift in that particular region in any of these strains (data not shown). Therefore, the results presented in that study were confused by a single nucleotide deletion and are not due to a difference between the two *B. burgdorferi* strains B31 and N40.

The immune serum against the BBA01 paralogue was used to analyse BBA01 synthesis during *in vitro* culture of Lyme disease *Borrelia* (Fig. 4). Expression could not be detected in high-passage strain B31, although the plasmid and the gene are still present (Table 1). Interestingly, we could detect BBA01 in another high-passage strain, B31-A. This indicates that BBA01 synthesis is downregulated in continuously cultured *B. burgdorferi* B31, but not in B31-A. This could be explained by different plasmid content or maybe loss of some regulator for the BBA01 protein expression in *B. burgdorferi* B31 during *in vitro* propagation. This also supports the idea that P13 paralogues are not needed for growth of *Borrelia in vitro* but could be important during infection or survival *in vivo*. Surprisingly, we could not detect BBA01 in outer-membrane preparations of low-passage B31, although we expected that it could be an integral outer-membrane protein like P13 (Noppa *et al.*, 2001). This might suggest different subcellular localizations of BBA01 and P13 or that BBA01 is rapidly degraded during preparation of the outer-membrane proteins. We also observed that the highest levels of BBA01 are found in *B. afzelii* strain ACAI, perhaps reflecting an important role for this protein in this Lyme-disease-species. However, we can not rule out that the epitope of BBA01 from ACAI is recognized by the polyclonal antiserum more efficiently. Finally, we have shown that the BBA01 paralogue is expressed in all three Lyme-disease-causing species. Interestingly, BBA01 synthesis is upregulated in the P13 knockout compared to the wild-type (Fig. 4, lanes 5 and 6), which suggests that BBA01 could possibly compensate for the function of P13. The results also showed that polyclonal antibodies raised against the synthetic peptide of BBA01 are specific, such that the region used to synthesize the peptide could be the epitope of BBA01. Further studies are needed to elucidate this possibility.

It has been postulated that *B. burgdorferi* has evolved many plasmids and paralogous gene families to be capable of adaptation to different environments (Ojaimi *et al.*, 2003). Within this theme, we have shown that all investigated paralogues of family 48 can be expressed, but under different conditions, some of which are difficult to mimic *in vitro*. We also present a model of the P13 architecture in the outer membrane of *B. burgdorferi*, although more studies on the structure of this protein are required. Taking the results together, we have shown that the paralogue family 48 has at least two members (P13 and BBA01) that are synthesized during laboratory cultivation. Conversely, *p13* and *bbi01* are transcribed in mice and could therefore be important during establishment of infection in animals. In this context, we believe that altered expression of the various paralogues is important during adaptation to different environments. Upregulation of the BBA01 protein in the *p13* knockout strain indicates that paralogues not only have sequence homology, but also could exhibit some functional redundancy. Since *Borrelia* has no advanced metabolic capacity and obtains all the essential nutrients from the haemolymph of ticks or the blood of mammals, an efficient and selective uptake mechanism, such as porins, is required. Earlier, we established that P13 is a channel-forming protein (Östberg *et al.*, 2002). It is also evident that BBA01 is expressed *in vitro* and is apparently processed in a similar manner to P13. In addition, the gene is present in all strains of Lyme disease *Borrelia* investigated. Therefore, we propose that the BBA01 paralogue could also be a channel-forming protein, needed at certain stages of the *Borrelia* life cycle. Further studies are needed to investigate this possibility. Moreover, expression of the paralogues in different environments, in both ticks and different animals, may give a better understanding of the biology of *Borrelia* spirochaetes and the pathogenesis during Lyme disease infection.

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