Elimination of Channel-Forming Activity by Insertional Inactivation of the *p13* Gene in *Borrelia burgdorferi*

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P13 is a chromosomally encoded 13-kDa integral outer membrane protein of the Lyme disease agent, *Borrelia burgdorferi*. The aim of this study was to investigate the function of the P13 protein. Here, we inactivated the *p13* gene by targeted mutagenesis and investigated the porin activities of outer membrane proteins by using lipid bilayer experiments. Channel-forming activity was lost in the *p13* mutant compared to wild-type *B. burgdorferi*, indicating that P13 may function as a porin. We purified native P13 to homogeneity by fast performance liquid chromatography and demonstrated that pure P13 has channel-forming activity with a single-channel conductance in 1 M KCl of 3.5 nS, the same as the porin activity that was lost in the *p13* mutant. Further characterization of the channel formed by P13 suggested that it is cation selective and voltage independent. In addition, no major physiological effects of the inactivated *p13* gene could be detected under normal growth conditions. The inactivation of *p13* is the first reported inactivation of a gene encoding an integral outer membrane protein in *B. burgdorferi*. Here, we describe both genetic and biophysical experiments indicating that P13 in *B. burgdorferi* is an outer membrane protein with porin activity.

Lyme disease is caused by infection with the spirochete *Borrelia burgdorferi* sensu lato and is transmitted by *Ixodes* ticks (18, 58). Like gram-negative bacteria, *B. burgdorferi* contains an inner membrane, an outer membrane, and a periplasmic space including the murein sacculus (4). However, a number of studies have revealed major differences between the *B. burgdorferi* cell envelope structure and membrane composition and those of gram-negative bacteria (7, 23, 24, 47, 63). The flagella are localized to the periplasmic space (4), and *B. burgdorferi* has been shown to lack lipopolysaccharide (59). Proteins associated with the *Borrelia* outer membrane are mostly lipoprotein (46, 54). Another important difference is that a large portion of the membrane lipids in *B. burgdorferi* consist of glycolipids, which comprises only galactose as a monosaccharide constituent (13, 32). These glycolipids are absent in gram-negative bacteria such as *Escherichia coli* (13). Only a few integral membrane proteins have been identified and characterized for *B. burgdorferi* (17, 43, 45, 56).

Proteins embedded in bacterial membranes fulfill a number of tasks that are crucial for bacterial cells, such as solute and protein transport, signal transduction, and interaction with other cells (1). Substances can be taken into bacteria by diffusion through the outer membrane via porin proteins (9) or via receptor-mediated uptake (41). Porins are located in the outer membrane, where they often form trimers of three identical subunits, allowing the diffusion of molecules across lipid bilayer membranes (8, 34). Their structure, location, and diversity confer on them multiple functions, including those of general diffusion pores and substrate-specific channels (1, 8).

Porin loops are potential targets for adhesion to other cells (14), to bacteriophages (64), and to bactericidal compounds (52). In addition, porins can also activate complement (2, 40) and insert into eukaryotic host cellular membranes; an example of the latter is PorB of pathogenic *Neisseria* strains (50).

Variation of the loop structure as a mechanism to escape immune pressure (33) or modulation of porin expression in response to the pressure of antibiotics (48) are survival strategies used by some pathogenic bacteria. The presence of porins in other spirochetes has been investigated. Tromp1 of *Treponema pallidum* and OmpL1 from pathogenic *Leptospira kirschneri* are reported to have porin activity (15, 53). The major surface protein (Msp) of the oral spirochete *Treponema denticola* has been shown to form very large conductance ion channels and to form oligomers on the bacterial surface (27). This protein also binds to cell surface receptors and forms channels in the cytoplasmic membranes of epithelial cells (39). *Borrelia* spirochetes have an extremely limited metabolic capacity but a complex lifestyle, allowing them to persist in warm-blooded animals and arthropod hosts. Therefore, they need to efficiently use nutrients in these different environments. Hence, the few integral membrane proteins present in the *B. burgdoferi* outer membrane could act as channel-forming proteins. Two porins have been characterized for *B. burgdorferi*, Oms28 (56) and Oms66 (P66) (57). The Oms66 (P66) porin has been further shown to act as an adhesin and to bind to β$_3$-integrin (22, 25). Porin activities have also been found in *Borrelia hermsii* (54).

In an earlier study, a 13-kDa integral outer membrane protein, P13, of *B. burgdorferi* sensu lato was characterized (43). P13 was first identified when monoclonal antibodies (MAbs) binding to a 13-kDa protein inhibited the growth of a *B. burgdorferi* strain lacking several of the major outer surface lipoproteins (51). P13 is processed at the amino (N) terminus, where

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TABLE 1. Oligonucleotides (5'-3') used in this study

Oligo-nucleotide	Sequence[a]	Location
z1-*Pst*I	ATC**CTGCAG**TTTATACTATTAAAGATC	Upstream of *p13*
z2-*Xba*I	CCTT**CTAGAA**TATCTCCTTGAGCAAAG	*p13*
z3-*Xba*I	TTT**CTAGA**TCAAAGCGCTTGATGGTATTAC	*p13*
z4-*Bam*HI	ATGAAA**GGATCC**ATATAAAATTACCTGG	Downstream of *p13*
z12b	GGGAGAAAATGATGAATTTGTCG	*p13*
z43b	TTTAAAGAAAAGAGGGATATAC	*p13*
KNa1	**TCTAGA**TGTCTGTCGCCTCTTGTGGCT	P$_{flaB}$ upstream of *kan*
KNa1rev	**TCTAGA**GGCGAATGAGCTAGCGCCGTC	Downstream of *kan*
KNaB	CCATGTTGGAATTTAATCGCGG	*kan*

[a] Restriction sites are in boldface type.

the first 19 amino acids are cleaved off, most likely by signal peptidase I, and at the carboxyl (C) terminus by the removal of 28 amino acids (42, 43). Furthermore, it was recently shown that the N terminus of P13 was blocked for Edman degradation by pyroglutamylation (42).

In the present investigation, we studied the function of integral outer membrane protein P13. The *p13* gene was inactivated by allelic exchange; when the mutant was compared to an isogenic wild-type strain by use of a lipid bilayer assay, porin activity was found to be lost from the mutant. A fast performance liquid chromatography (FPLC) system (Pharmacia Biotech, Piscataway, N.J.) with a MonoQ column was used to purify P13 from *B. burgdorferi*, and the pure protein formed pores in a planar bilayer assay. Thus, we have shown by use of both genetic and biochemical analyses that the P13 protein of *B. burgdorferi* has channel-forming activity. The results presented in this study suggest that this protein forms an unusual porin structure compared to those of other known outer membrane porins. Furthermore, the structure and the substrate specificity of the P13 channel remain to be determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study were as follows. *B. burgdorferi* B31 (ATCC 35210), the prototype strain of *B. burgdorferi* sensu stricto, was originally isolated from a tick collected on Shelter Island, N.Y. (18). Strain B31-A, a high-passage, noninfectious clone of B31, was used for gene inactivation experiments (16). *B. burgdorferi* N40 is an infectious strain isolated from an *Ixodes* tick (5, 6). Strain B313 was derived from B31 by cultivation in the presence of antibodies against the major outer surface proteins (51). Bacteria were grown in BSK-H (Sigma, St. Louis, Mo.) or BSK-II (3a) supplemented with 6% rabbit serum at 32 or 34°C. Single colonies were obtained as described elsewhere (49).

Construction of plasmid pYMP13. A recombinant plasmid was constructed to inactivate the *B. burgdorferi p13* gene. A 1.3-kb fragment containing 215 bp of the *p13* gene and upstream DNA was amplified by PCR with an Expand high-fidelity PCR system (Roche, Mannheim, Germany) from the chromosomal DNA of strain B31 by using primers z1-*Pst*I and z2-*Xba*I (Table 1 and Fig. 1). After restriction enzyme digestion of the PCR product, the fragment was cloned into vector pUC18 (New England Biolabs, Beverly, Mass.). A 1.4-kb fragment containing 265 bp of the *p13* gene and downstream sequence was amplified by using primers z3-*Xba*I and z4-*Bam*HI (Table 1 and Fig. 1) and ligated downstream of the first fragment in the same vector. The following reaction conditions were used: 94°C for 30 s, 45°C for 1 min, and 72°C for 1 min for 5 cycles and 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min for 35 cycles. The 1.3-kb P$_{flaB}$-kanamycin resistance fragment from plasmid pJLB12a was used to inactivate the *p13* gene (16). This fragment was amplified by using primers KNa1 and KNa1rev containing *Xba*I sites (Table 1 and Fig. 1) and 35 cycles of 94°C for 30 s, 60°C for 1 min,

and 72°C for 1 min. After *Xba*I digestion, the kanamycin resistance fragment was ligated into the *p13* gene. To prevent the introduction of an ampicillin resistance gene into *B. burgdorferi*, the *p13*-containing fragment was recloned into pOK12, a 2.1-kb low-copy-number plasmid containing a kanamycin resistance gene (62). An *Xho*I site was identified 921 bp upstream of the *p13* gene, and the *p13::kan* fragment was excised from vector pUC18 by restriction with *Xho*I and *Bam*HI and ligated into pOK12. The resulting plasmid was called pYMP13.

Allelic exchange mutagenesis and screening of *B. burgdorferi* transformants. Preparation of competent *B. burgdorferi*, electroporation, and plating of transformants were done as described previously (60). Briefly, for each transformation, 20 μg of plasmid DNA in 5 μl of water was used. After electroporation, the bacteria were resuspended in 5 ml of BSK-H and incubated overnight at 34°C. The transformed bacteria were plated in solid BSK-II medium containing 200 μg of kanamycin per ml. Plates were incubated at 34°C with 1% CO_2 until colonies were visible.

Kanamycin-resistant colonies were screened for allelic exchange at the *p13* locus by PCR (Fig. 1). Individual colonies were picked with sterile toothpicks and added to tubes containing a PCR mixture, and oligonucleotides 1 and 9 (kan5'+*Nde*I and pOK.7+*Nhe*I) described by Bono et al. (16) were used to detect the *kan* gene. A number of potential transformant clones were chosen for further analysis by using primer z12b, primer z43b spanning the *p13* gene, and primer pair KNaB-z43b amplifying a portion of the *kan* gene and a portion of the *p13* gene (Fig. 1). PCR was carried out for 35 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min. To obtain clonal mutants, individual colonies were aspirated with a sterile Pasteur pipette and grown in 5 ml of BSK-H with 200 μg of kanamycin per ml.

Determination of the plasmid profile. Total DNAs from B31-A and a *p13* knockout mutant were prepared by using a Wizard genomic DNA purification kit (Promega, Madison, Wis.), and plasmid contents were determined by PCR as previously described by Elias et al. (28).

Protein electrophoresis, immunoblotting, and antiserum. For gel electrophoresis, proteins were heated to 85°C for 5 min in Novex 2× Tricine-sodium dodecyl sulfate (SDS) sample buffer (Invitrogen, Carlsbad, Calif.) or to 70°C for 10 min in Novex 4× NuPAGE sample buffer and separated through Tricine–10 to 20% polyacrylamide gradient gels or through 4 to 12% NuPAGE bis-Tris gels by using a Novex XCell Sure Lock electrophoresis cell (Invitrogen). Total *B. burgdorferi* proteins were prepared by growing cells to stationary phase, harvesting cells by centrifugation, and washing cells twice in phosphate-buffered saline. Proteins in peak FPLC fractions were concentrated by precipitation with a 2.5-fold excess of ice-cold ethanol. Precipitated proteins were pelleted by centrifugation at 13,000 × *g* for 40 min at 4°C and resuspended in sample buffer. Proteins were stained with Coomassie blue R-250 (Sigma). For immunoblotting, proteins were transferred to a polyvinylidene difluoride membrane (PALL Corporation, Ann Arbor, Mich.) and probed with antibodies. Polyclonal antibodies used against P13 were described before (43). MAb 15G6, which recognizes P13, was previously described by Sadziene et al. (51). Bound antibodies were detected by using peroxidase-conjugated anti-rabbit or anti-mouse antibodies (DAKO A/S, Glostrup, Denmark) and enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, England).

Separation of the outer membrane proteins of *B. burgdorferi* and purification of P13. Outer membrane fractions of *B. burgdorferi* strains B313, B31-A, P13-18 (a clone with an inactivated *p13* gene), and N40 were prepared as described elsewhere (37). P13 was purified by preparative SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (43). For purification of native P13, the outer membrane proteins of strain B313 were applied to a MonoQ column in conjunction with an FPLC system. The column was washed with 15 ml of 0.4% lauryl-dimethyl-amine-oxide (LDAO; Sigma) buffered in 10 mM Tris HCl (pH 8.0), and 12 separate 0.5-ml flowthrough fractions were collected. Proteins bound to the column were eluted by using a 0 to 1 M linear NaCl gradient containing 0.4% LDAO buffered in 10 mM Tris HCl (pH 8.0).

Chemical cross-linking. Proteins of intact high-passage *B. burgdorferi* strain B313 were cross-linked by a modification of a procedure described elsewhere (55). Approximately 1.3×10^8 freshly harvested and washed spirochetes were suspended in 350 ml of 0.1 M sodium phosphate buffer (pH 6.8). Formaldehyde (Riedel-de Haën, Seelze, Germany) was added to a final concentration of 1%, and the samples were incubated for 30 min at room temperature. The cells were washed once in cold 0.1 M sodium phosphate buffer (pH 6.8) and centrifuged, and the cell pellets were solubilized in 4× NuPAGE sample buffer and subjected to electrophoresis and then immunoblot analysis.

Planar lipid bilayer assay. The methods used for black lipid bilayer experiments have been described elsewhere (10). The instrumentation consisted of a Teflon chamber with two compartments separated by a thin wall and connected
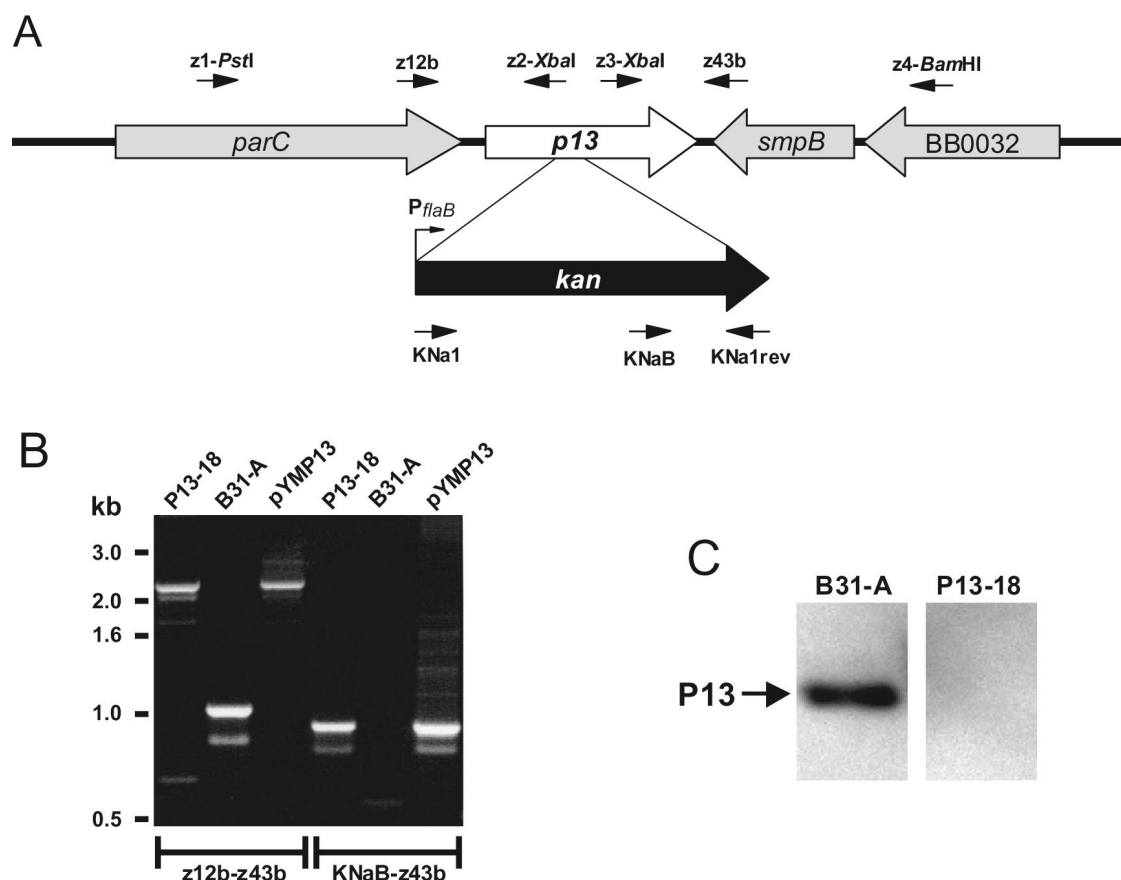
FIG. 1. Insertional inactivation of P13. (A) Organization of the *p13* gene and flanking genes on the *B. burgdorferi* B31 chromosome and insertion of the *kan* gene cassette. Arrows indicate relative positions of oligonucleotides used. The diagram is not drawn to scale. (B) PCR analysis of strain B31-A and kanamycin-resistant mutant P13-18 with primer pairs z12b-z43b and KNaB-z43b (Table 1). (C) Western blot analysis of P13-18 (mutant) and B31-A (wild type) with polyclonal antiserum against P13.

by a small circular hole with an area of 0.2 mm². The membranes were formed from a 1% (wt/vol) solution of diphytanoyl phosphatidylcholine (PC) (Avanti Polar Lipids, Alabaster, Ala.) in *n*-decane. The porin-containing protein solutions were highly diluted in 1% Genapol (Fluka, Buchs, Switzerland) and added to the aqueous phase either immediately before membrane formation or after the membrane had turned black. All salt solutions were used unbuffered and had a pH of about 6. The membrane current was measured with a pair of Ag-AgCl electrodes with salt bridges switched in series with a voltage source and a highly sensitive current amplifier (Keithley 427). The voltage dependence of the porin channels was checked as described elsewhere (36) by using membrane potentials as high as −150 to +150 V. Zero-current membrane potential measurements were performed by establishing a salt gradient across membranes containing 100 to 1,000 channels as described earlier (11). Zero-current membrane potentials were measured with a high-impedance electrometer (Keithley 617).

RESULTS

Construction of the *p13* mutant. In order to investigate the function of P13, we inactivated the *p13* gene by targeted insertion of a kanamycin resistance gene cassette (16) (Fig. 1A). P13 maps at positions 32089 to 31553 on the linear chromosome of *B. burgdorferi* B31 and is transcribed as a monocistronic mRNA (29, 43). To confirm that the *p13* gene was inactivated by allelic exchange, we amplified a chromosomal region spanning the *kan* gene and flanking sequences (Fig. 1A). We also used primers binding inside the *kan* gene and downstream of the *kan* gene insertion site (Fig. 1A). The PCR results were compatible with allelic exchange occurring only at the *p13* locus (Fig. 1B). Using primers z12b and z43b, covering the *kan* gene and flanking sequences (Fig. 1A), we amplified a DNA fragment of the same size from both the *p13* mutant and control plasmid pYMP13 but amplified a smaller fragment from B31-A (Fig. 1B). To further confirm that the *kan* gene was inserted into the chromosome of the mutant, a primer (KNaB) that binds within the *kan* gene cassette and another primer (z43b) downstream of the *kan* gene insertion site were used for amplification (Fig. 1A). A PCR product was obtained from the mutant but not from B31-A, as expected (Fig. 1B). The PCR results also showed that the *kan* gene was inserted in the same direction as the inactivated gene (Fig. 1A and B).

Clone P13-18 was grown to late exponential phase and analyzed for P13 expression. Western blot analysis with polyclonal rabbit serum against P13 provided additional evidence for the inactivation of the *p13* gene (Fig. 1C). Total protein from B31-A was used as a positive control and, as expected, analysis of the total protein content of P13-18 revealed no P13 protein (Fig. 1C). Thus, we could successfully inactivate the gene for the integral outer membrane protein P13 in *B. burgdorferi*. Interestingly, no obvious defect of the mutant was found under normal growth conditions.
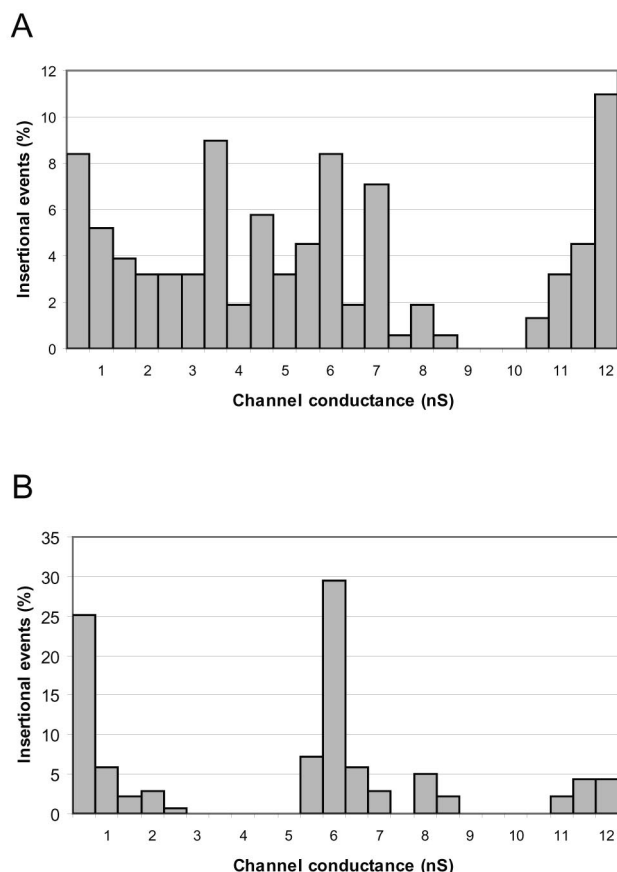
A

B

FIG. 2. Porin activities of outer membrane proteins. Outer membrane proteins from B31-A (wild type) (A) and P13-18 (mutant) (B) in PC–n-decane membranes were analyzed. The total numbers of insertional events were 143 for B31-A and 139 for P13-18. The aqueous phase contained 1 M KCl. The temperature was 20°C, and the applied voltage was 20 mV.

Porin activities in the outer membrane fraction. In a recent study, P13 was shown to be an integral membrane protein (43). To investigate whether P13 has porin activity, the channel-forming activities of outer membrane proteins from P13-18, B31-A, and infectious strain N40 were compared. Outer membrane proteins were enriched from *B. burgdorferi* strain B313 as previously described (37). Proteins were solubilized in 1% Genapol to a concentration of 0.05 μg/μl, diluted 100,000-fold, and investigated for channel-forming activities in planar lipid bilayer experiments. Strain P13-18, with an inactivated *p13* gene, exhibited the same pore-forming activities as B31-A, with the exception of an activity of about 3.5 nS that was lost in the mutant (Fig. 2). The same numbers of insertional events were measured for both the *p13* mutant and B31-A. However, the smaller number of events observed for the 12-nS pore in the total outer membrane extract of the *p13* mutant than in that of B31-A may be due to relative differences in the amounts of certain proteins present in the outer membrane fractions. Another possible explanation for the reduction in the 12-nS channel-forming activity is that the inactivation of *p13* lowers the level of expression of the protein responsible for the 12-nS activity or that the lack of P13 in the membrane fraction lowers

the reconstitution of the other channel in the bilayer experiments. It is also noteworthy that the frequency of channel reconstitution in lipid bilayer membranes does not necessarily reflect the relative amounts of different porins expressed in the bacteria. Channel frequency in lipid bilayer measurements is also dependent on how efficiently a particular porin is reconstituted in the lipid bilayer; i.e., channel frequency is dependent on the porin's channel-forming activity. We therefore have provided genetic evidence that 13-kDa protein P13 is a *B. burgdorferi* porin. In addition, strains B31-A and N40 showed similar single-channel conductance activities, ranging from 0.3 to 12 nS, with main activities of approximately 0.6, 2, 3.5, 6, and 12 nS (data not shown).

Plasmid contents of B31-A and P13-18. In vitro propagation of *B. burgdorferi* strains can result in the loss of plasmids; therefore, monitoring of plasmid content is essential for studying the phenotype of a derived mutant. To determine whether the *p13* mutant and background strain B31-A had the same plasmid profiles, PCR was performed as described elsewhere (28). Both B31-A and P13-18 harbored linear plasmids lp17, lp28-2, lp28-3, lp38, lp54, and lp56 and circular plasmids cp26, cp32-1, cp32-3, cp32-4, cp32-7, cp32-8, and cp32-9 (data not shown). These results strongly indicated that the loss of channel-forming activity observed in the *p13* null mutant was due to the inactivation of the *p13* gene alone.

P13 purification and oligomer formation. To purify the P13 protein, a membrane fraction from *B. burgdorferi* B313 corresponding to approximately 100 μg of total protein was solubilized in 2% LDAO, and proteins were subsequently purified by FPLC (Fig. 3). The P13 protein was eluted from the MonoQ column in the flowthrough fractions (Fig. 3) and was confirmed to be P13 by SDS-PAGE, staining with Coomassie blue R-250, and immunoblotting with MAb 15G6 (Fig. 4). P13 was present in flowthrough fractions 2 to 5. Pure P13, without detected contamination with other outer membrane proteins, was eluted from the column in fraction 4 (Fig. 4A). The amount of protein in fraction 4 containing P13 corresponded to approximately 10% (approximately 10 μg) of the total protein in the outer membrane fraction. Immunoblotting with MAb 15G6, demonstrating reactivity to FPLC-purified P13, revealed the formation of a P13 oligomer of about 60 kDa (Fig. 4B). Additionally, in FPLC-purified P13, a dimer form was detected by use of polyclonal antibodies (Fig. 4B). This result could be explained by masking of the epitope recognized by MAb 15G6 in the dimer form. Furthermore, the P13 oligomer was not detected in the outer membrane fraction, a result which could be explained by the presence during outer membrane preparation of the detergent *n*-octyl-β-D-glucopyranoside (OGP), which could prevent P13 oligomer formation. A dimer form of P13 was also detected in chemical cross-linking experiment with formaldehyde (data not shown). P13 purified by preparative SDS-PAGE does not form oligomers, as indicated by immunoblotting with the same MAb (Fig. 4B). The discrepancy in the SDS sensitivity of the P13 oligomer between P13 purified by preparative SDS-PAGE and P13 purified by FPLC and separated by SDS-PAGE could be caused by less influence of SDS on the latter form.

The addition of approximately 60 ng of pure P13 protein to lipid bilayer membranes composed of PC–n-decane showed that it had channel-forming activity, and the membrane con-
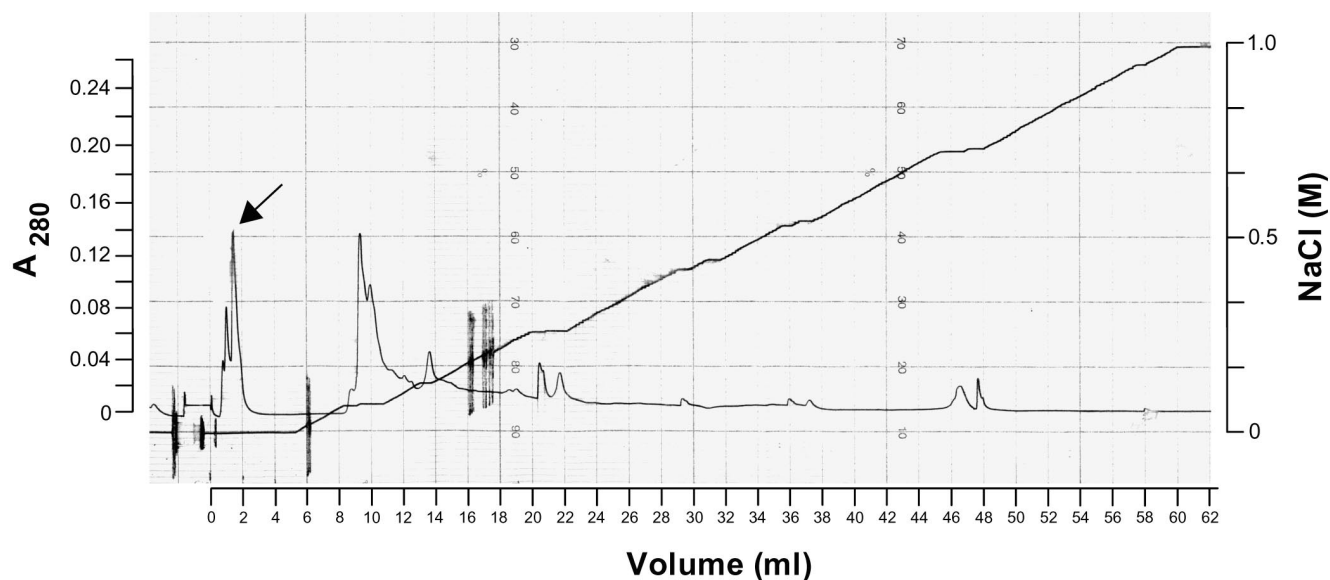
FIG. 3. FPLC MonoQ purification of P13. Shown is a chromatogram of FPLC-purified outer membrane proteins derived from *B. burgdorferi* B313. Detergent-solubilized membrane proteins were loaded on a MonoQ column and eluted as described in Materials and Methods. The arrow indicates the fraction containing pure P13. The solid line showing an increase indicates the concentration of NaCl for a given fraction volume (indicated on the *x* axis).

ductance increased in a step-like fashion (Fig. 5A). Control experiments with the detergent LDAO alone revealed that these conductance steps were specific for the presence of P13. Most of the conductance steps were directed upward, and closing steps were only rarely observed at a transmembrane potential of 20 mV. These results indicated a long life span of the channels formed by P13. Figure 5B shows a histogram of 211 conductance steps in 1 M KCl at a membrane potential of 20 mV. The most frequent value for the single-channel conductance of P13 was 3.5 nS, but we observed some other conductance steps of 7.0 to 7.5 nS that could be explained by the formation of more than one channel simultaneously. It is noteworthy that the P13-containing fraction had a high specific activity, since a 100,000-fold dilution of the protein yielded 211 individual stepwise insertion events over a time period of approximately 45 min in the lipid bilayer assay (Fig. 5A). P13 was found to be sensitive to SDS and therefore became inactive after purification by preparative SDS-PAGE. However, it was possible to regain some channel-forming activity when the protein was incubated in 1% Genapol at 4°C (data not shown). Together with the results of a previous computer analysis of the P13 protein (43), the results presented here suggest that the P13 protein forms an unusual porin structure that does not fit into the architecture shown for other outer membrane porins.

Single-channel analysis of P13. P13 was found to be permeable to a variety of different ions. Table 2 shows the single-channel conductance of the P13 channel in the presence of different salt solutions. Changing the cations by replacing potassium with lithium resulted in an alteration that had a substantial influence (3.9-fold) on the conductance of the P13 channel. The influence of the anions (Cl⁻ and CO₃COO⁻) on the single-channel conductance, on the other hand, was less pronounced (1.8-fold) (Table 2); this result is consistent with

the assumption that the P13 channel is cation selective. Alternatively, the single-channel conductance seen with different salts followed more or less the mobility sequence of the ions in the aqueous phase. This means that the P13 channel is wide and filled with water and has a small field strength on its interior face. Table 2 also shows the average single-channel conductance as a function of the KCl concentration in the aqueous phase. Despite the possible cation selectivity described above, we observed a 1:1 relationship between conductance and KCl concentration; this result is consistent with the absence of a binding site for ions within the channel and the absence of point charge effects.

Purified P13 is cation selective and voltage independent. For analysis of the ion selectivity of P13, we performed zero-current membrane potential experiments. After the incorporation of 100 to 1,000 porin channels into the PC membrane, the KCl concentration on one side of the membrane was raised from 0.1 to 0.5 M by the addition of 3 M KCl. The zero-current membrane potential was measured 3 min after the gradient was established. The more dilute side was always positive, indicating preferential movement of the cations through the channel; i.e., the channel was cation selective. The zero-current membrane potential was 14 mV under these conditions (from 0.1 to 0.5 M KCl). Analysis with the Hodgkin-Katz equation (11) suggested that anions could permeate the channel because the cation/anion permeability ratio was 2.1 for KCl. Together with the single-channel conductance data, this slight selectivity suggests that cations and anions can enter the channel and that the ion selectivity most likely is created by an excess of negatively charged residues within the channel, which can also create cation selectivity in a giant conductance porin channel (38). Additionally, the P13 channel exhibited no voltage dependence even at voltages as high as ±150 V
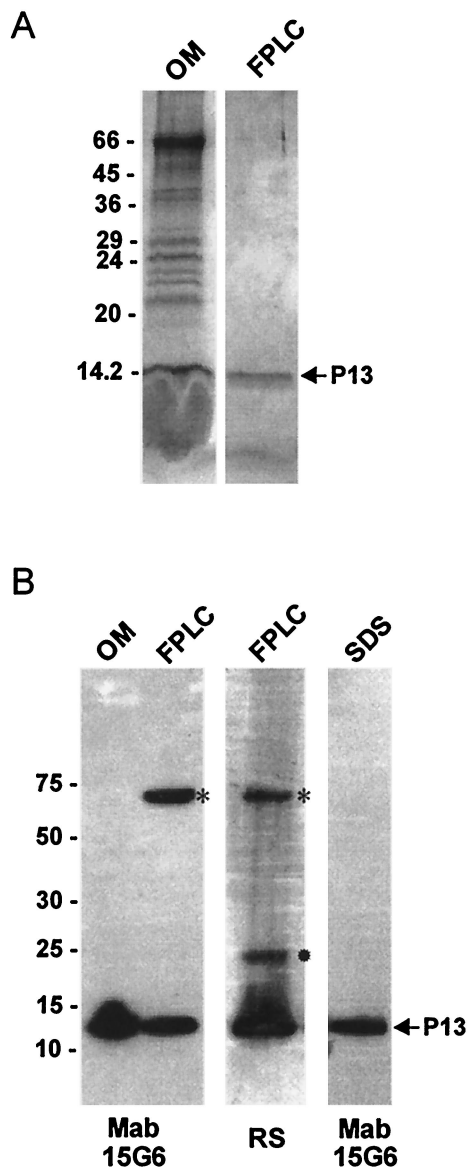
A

B

FIG. 4. Analysis of the P13 protein. (A) Analysis by SDS-PAGE and Coomassie blue R-250 staining of the membrane fraction (OM) of *B. burgdorferi* B313 and FPLC-purified P13 (FPLC). (B) Immunoblot analysis of the membrane fraction (OM) of *B. burgdorferi* B313, FPLC-purified P13 (FPLC), and preparative SDS-PAGE-purified P13 (SDS) probed with either MAb 15G6 or polyclonal rabbit serum (RS). ✱, possible dimer form; *, oligomer form. Molecular mass standards in kilodaltons are indicated at the left.

(data not shown). This means that P13 did not show voltage-dependent closure.

DISCUSSION

In the present study, we provide the first evidence via gene inactivation for the presence of a possible porin in the outer membrane of *B. burgdorferi*. Porins form water-filled channels through the outer membrane and allow the diffusion of hydrophilic substrates in and out of cells (8, 9). Porins may also function as virulence determinants (3, 14, 31). It was shown in earlier studies that a 13-kDa integral membrane protein (P13)

in *B. burgdorferi* is surface exposed, processed at both ends, and blocked for Edman degradation by a pyroglutamic acid modification (42, 43). In this study, we further investigated the function of the 13-kDa protein by inactivating the *p13* gene by targeted mutagenesis (16, 60) and determined the channel-forming activities of the outer membrane proteins of the mutant (Fig. 1 and 2). Since the *p13* knockout mutant, P13-18, did not display any porin activity of about 3.5 nS, we assumed that P13 is a channel-forming protein.

Due to the genetic instability of the *B. burgdorferi* genome during in vitro cultivation, we compared the plasmid contents of control strain B31-A and the *p13* knockout mutant. As expected, both strains contained the same plasmids. Thus, the
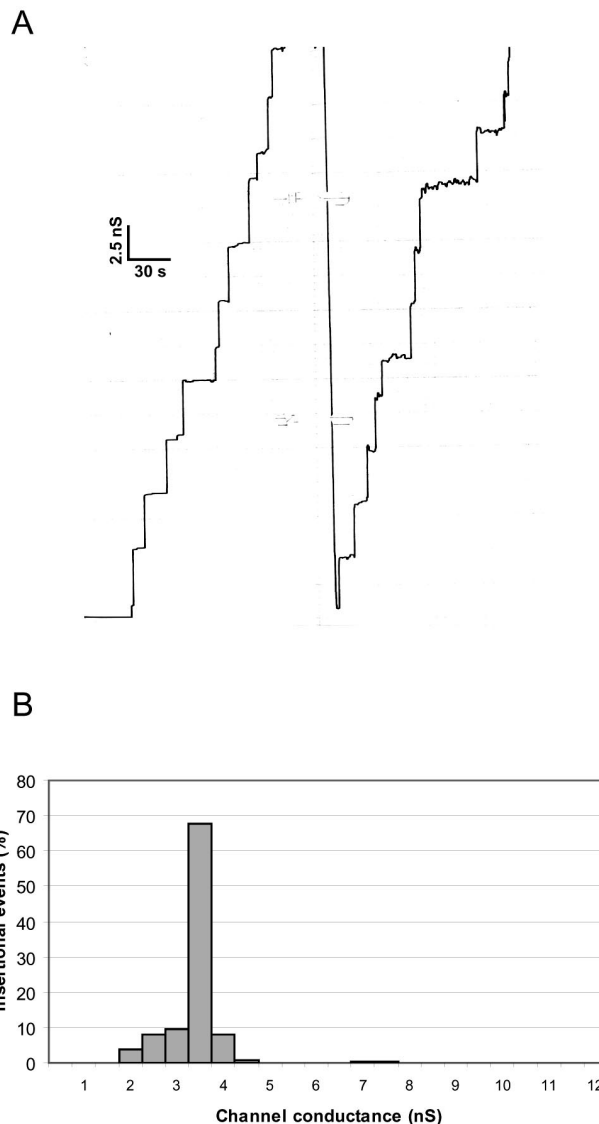
A

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FIG. 5. Porin activity associated with FPLC-purified P13. (A) Single-channel conductance observed for P13 in PC–*n*-decane membranes. The fraction containing FPLC-purified P13 was added to a lipid bilayer composed of PC bathed in 1 M KCl at a concentration of about 10 ng/ml. (B) Histogram of the individual single-channel conductance events observed for the purified P13 porin. The average single-channel conductance for 211 single-channel events was 3.5 nS.

TABLE 2. Average single-channel conductance of the
P13 channel in various salt solutions[a]

Salt	Salt concn (M)	Single-channel conductance (nS)	Hydrated cation radius (nm)
LiCl	1.0	0.9	0.216
KCl	0.1	0.35	0.110
	0.3	1.4	
	1.0	3.5	
	3.0	9.0	
KCO_3COO^-	1.0	2.0	0.110

[a] Membranes were formed from 1% PC dissolved in *n*-decane. The applied voltage was 20 mV, and the temperature was 20°C. The average single-channel conductance was calculated from at least 50 single events. The radii of the hydrated cations were calculated by using the Stokes equation (21) and the limiting molar conductivity of the various cations (61).

loss of porin activity in strain P13-18 is caused by insertional inactivation of the *p13* gene and not by differences in plasmid content.

Purifying P13 from detergent-extracted membrane proteins by FPLC with a MonoQ column (Fig. 3 and 4) and testing porin activity in a planar bilayer assay showed that the purified P13 protein displayed a channel-forming activity of 3.5 nS (Fig. 5). This channel-forming activity was the same as that lost in the *p13* knockout mutant. The only porins previously described for *B. burgdorferi* are Oms28 and Oms66 (P66), with single-channel conductance activities of 0.6 and 10.7 nS, respectively (56, 57). Our experiments showed that at least two or three additional channels may exist in the outer membrane fraction of *B. burgdorferi*, where P13 is responsible for the 3.5-nS activity (Fig. 2A and 5). The finding of additional porin activities that have not been discovered before could be explained by differences between the methods used to prepare outer membrane proteins or genetic differences in the strains investigated.

The *p13* gene is a member of paralogous gene family 48, which include seven additional members situated on different linear plasmids (20, 29). Two members of this family are regarded as pseudogenes, and one gene contains a frameshift. Sequence similarities between the deduced amino acid sequences for P13 and four other family 48 members (BBA01, BBH41, BBI31, and BBQ06) suggest that they also may function as porins (20, 29). The channel-forming activity of P13 may be different from the possible channel-forming activities of the other paralogous proteins. It is therefore possible that some of the additional porin activities seen in the membrane fraction of B31-A are caused by some of the family 48 members. However, one of the paralogous genes (*bbi31*) is situated on linear plasmid lp28-4, which is missing in B31-A; hence, the BBI31 protein cannot be responsible for any channel-forming activity in the experiments. Furthermore, the other members may not be expressed at all in cultured *B. burgdorferi* or may not be present in the membrane protein fraction used in the lipid bilayer experiments. Further investigations are needed to determine the role of the other members of family 48 in channel formation in *B. burgdorferi* membranes.

Our attempts to show porin activity of P13 purified by preparative SDS-PAGE failed because of the sensitivity of P13 to SDS. Although we could detect reconstitution of porin into the

lipid bilayer and regain some porin activity after incubation of SDS-PAGE-purified P13 in 1% Genapol at 4°C, we were not able to obtain clear single-channel activity (data not shown). Most enterobacterial porins are resistant to SDS; however, it was shown earlier that some porins can become inactive after treatment with SDS, such as Tsx of *E. coli* (12). It is noteworthy that after treatment of FPLC-purified P13 with SDS, a defective channel-forming activity pattern similar to that seen with P13 obtained from preparative SDS-PAGE and incubated in Genapol was observed (data not shown).

Secondary structure analysis indicates that the P13 monomer does not contain a sufficient number of β-barrel structures to form a monomeric channel. Therefore, it is likely that the protein functions as an oligomer. Cross-linking experiments with formaldehyde revealed that P13 could form dimers (data not shown). Oligomer formation was also observed in an immunoblot with FPLC-purified P13 and monoclonal antibodies directed against P13 (Fig. 4B). This presumably means that P13 does not fit the known porin structure, which forms trimers of three identical polypeptides containing three channels. The method used could not reveal the exact size of the P13 oligomer but suggested that the protein complex is probably formed by four or five monomers of P13. Another possibility is that P13 can form a heterodimer mixture together with another protein(s). This possibility has to be further investigated. In addition, we observed that the P13 oligomer was not detected by immunoblotting of outer membrane proteins with MAb 15G6. This finding could be explained by the presence during outer membrane preparation of the detergent OGP, which could prevent P13 oligomer formation. However, P13 channel-forming activity was detected in the outer membrane fraction, presumably because of drastic dilution of OGP and treatment of the proteins with 1% Genapol, which aids in the formation of the native porin structure during lipid bilayer experiments.

The P13 protein showed no voltage dependence and was cation selective, with a cation/anion permeability ratio of 2.1. This cation selectivity is not based on point charges at the channel mouth, since the single-channel conductance was a linear function of the cation concentration, as Table 2 clearly indicates. Although it was cation selective, the P13 channel also allowed anion diffusion because of some influence of the replacement of chloride by acetate on the single-channel conductance (Table 2). These results suggest that the P13 channel is wide and filled with water. A comparison with the single-channel conductance of *E. coli* porins suggests that it has at least the same permeability and the same diameter as the *E. coli* porins (9). However, further studies are required to elucidate the substrate for the 3.5-nS channel of *B. burgdorferi*. It is known that in addition to general diffusion pores that discriminate between solutes primarily on the basis of size and charge, the bacterial outer membrane may also contain channels with specificity for certain substrates, such as phosphate or carbohydrates (8, 9).

The specific role of P13 porin activity in *B. burgdorferi* metabolism or pathogenesis remains to be determined. However, we demonstrated that inactivation of the *p13* gene is not lethal for *B. burgdorferi* during in vitro cultivation, indicating that this gene is most likely required for the survival of *B. burgdorferi* inside the tick or mammalian host. Interestingly, inactivation

did not significantly affect the growth of the spirochetes in liquid medium. Since inactivation was done with the high-passage, noninfectious strain B31-A, the relevance for the pathogenesis of the P13 protein cannot be tested in an animal model. However, lipid bilayer experiments with detergent-solubilized outer membrane proteins from avirulent clone B31-A and virulent strain N40 revealed that these strains contain similar porin activities, including P13 porin activity (data not shown). These results indicate that genes coding for detected porins are present on the chromosome or on plasmids that are stably maintained during in vitro cultivation. This is the case for *oms66* (*p66*) and *p13*, which are present on the chromosome, and *oms28*, which is present on the 54-kb linear plasmid.

Structural analysis of the subunits of conventional *E. coli* outer membrane proteins, such as OmpA, OmpF, and LamB, reveal that these proteins contain between 8 and 18 β sheets that form a β-barrel structure spanning the outer membrane (26, 30, 44) and providing only for OmpF and LamB enough space for a transmembrane channel (8). Thus, the results of this study demonstrating channel formation by *B. burgdorferi* P13 suggest that this protein forms an unusual structure different from those of other known outer membrane porins because of its low molecular mass and unusual C-terminal processing. Gram-negative bacterial porins form trimers in the outer membrane with a minimum molecular mass of 30 kDa for the monomer (8, 34). A computer analysis of the P13 amino acid sequence shows that this protein does not contain pronounced or sufficient amounts of β sheets to form channels following conventional pore formation mechanisms; instead, three hydrophobic α helices are predicted to span the membrane (43). Additionally, crystallographic studies of TolC in *E. coli* have revealed that this channel-forming protein also lacks the common structural elements of channel-forming proteins. In the TolC protein, three subunits each contribute to four β strands that form a single 12-strand β barrel, and 12 α helices pack in an antiparallel fashion to form a hollow cylinder that spans the space between the outer and the inner membranes (19, 35). These findings show that unconventional structures for channel formation may exist; however, further structural analyses are needed to elucidate the novel channel structure formation of P13.

Borrelia spirochetes have an unusual outer membrane structure and have been shown to have a limited metabolic capacity, meaning that they need to efficiently take up nutrients from the environment. Presumably, a large number of transporters are needed to move essential compounds from extracellular sources across the inner membrane. However, it is still unknown how these compounds are transported to the periplasm. We suggest that porins, which form channels in the outer membrane, are very important for the survival strategy of *B. burgdorferi*, as this obligate parasite can inhabit both ticks and mammals. Thus, we believe that to obtain more complete knowledge of the biology of *Borrelia* spirochetes, it is important to understand and investigate the physical properties of outer membrane proteins, such as porins.

ACKNOWLEDGMENTS

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