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Porin Activity of the Native and Recombinant Outer Membrane Protein Oms28 of *Borrelia burgdorferi*

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The outer membrane-spanning (Oms) proteins of *Borrelia burgdorferi* have been visualized by freeze-fracture analysis but, until recently, not further characterized. We developed a method for the isolation of *B. burgdorferi* outer membrane vesicles and described porin activities with single-channel conductances of 0.6 and 12.6 nS in 1 M KCl. By using both nondenaturing isoelectric focusing gel electrophoresis and fast-performance liquid chromatography separation after detergent solubilization, we found that the 0.6-nS porin activity resided in a 28-kDa protein, designated Oms28. The *oms28* gene was cloned, and its nucleotide sequence was determined. The deduced amino acid sequence of Oms28 predicted a 257-amino-acid precursor protein with a putative 24-amino-acid leader peptidase I signal sequence. Processed Oms28 yielded a mature protein with a predicted molecular mass of 25,363 Da. When overproduced in *Escherichia coli*, the Oms28 porin fractionated in part to the outer membrane. Sodium dodecyl sulfate-polyacrylamide gel-purified recombinant Oms28 from *E. coli* retained functional activity as demonstrated by an average single-channel conductance of 1.1 nS in the planar lipid bilayer assay. These findings confirmed that Oms28 is a *B. burgdorferi* porin, the first to be described. As such, it is of potential relevance to the pathogenesis of Lyme borreliosis and to the physiology of the spirochete.

The etiologic agent of Lyme borreliosis, *Borrelia burgdorferi*, initially causes a flu-like illness that, if untreated, may develop into a systemic disease characterized by arthritic, cardiac, and neurological involvement (1, 24, 37, 43, 44, 45, 47). The molecular pathogenesis of Lyme disease is poorly understood because, until recently, basic characterization of the cell surface had been compromised by the lack of methods for outer membrane (OM) isolation (8, 25, 34, 42).

With isolation and purification of the OM of *B. burgdorferi*, we have been able to focus on characterization of its outer membrane-spanning (Oms) protein constituents (42). We reasoned that since porin proteins are indisputable markers of the OM in gram-negative bacteria, their identification in *B. burgdorferi* could provide a much-needed model of the membrane-spanning organization of other Oms proteins. Two porin activities were associated with the outer membrane vesicles (OMV) derived from *B. burgdorferi*, one having a single-channel conductance of approximately 0.6 nS and the other having a conductance of approximately 12.6 nS (42). These two porin proteins represented the first two functional Oms proteins characterized biochemically in *B. burgdorferi.*

In this report, we describe the fast-performance liquid chromatography (FPLC) purification of the 0.6-nS native porin protein from *B. burgdorferi* that we have designated Oms28 for outer membrane-spanning 28-kDa protein. In addition, we have cloned and determined the nucleotide sequence of the *oms28* gene. The 28-kDa Oms28 porin protein was overproduced in *Escherichia coli* and localized partially to the OM. Additionally, recombinant Oms28 (rOms28) isolated from the *E. coli* OM demonstrated porin activity, indicating that a portion of the exported rOms28 was assembled in the *E. coli* OM in a conformation compatible with porin activity. The results presented here confirm that Oms28 is an Oms protein, the first to be described for *B. burgdorferi.*

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MATERIALS AND METHODS

Bacterial strains and plasmids. *B. burgdorferi* sensu stricto strain B31 was used in most of the experiments presented in this study and will be referred to as *B. burgdorferi* B31. Virulent, low-passage *B. burgdorferi* was isolated and cultivated as described previously (42). The avirulent *B. burgdorferi* strain B31 (ATCC 35210) has been extensively passaged and is noninfectious for both mice and rabbits (42). *B. burgdorferi* bacteria were enumerated with a calibrated ausJena Laboval 4 dark-field microscope.

Additional *B. burgdorferi* strains used include 297 (46), ECM-86-NY (38), HB19 (46), N40 (4), and Sh-2-82 (38). These strains were isolated and cultivated as described previously (15). European *B. burgdorferi* low-passage isolates 2872-2, 2872-3, 2872-6, and 3251-5, as well as *Borrelia garinii*, were kindly provided by Vittorio Sambri, University of Bologna, Italy. *Borrelia hermsii* HS1 serotype 7 (low-passage isolate) and serotype 33 (high-passage isolate) were both generously provided by Alan Barbour, University of Texas Health Science Center, San Antonio. *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) was cultivated and obtained as described previously (7).

The *E. coli* strain BL21 DE3(pLysE) (Novagen, Madison, Wis.) was used to overproduce the *B. burgdorferi* Oms28 porin protein (see below). The *E. coli* strain DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used to subclone the *oms28* gene into the plasmid pBluescript KS^+ (Stratagene, Inc., San Diego, Calif.). The *oms28* locus was overexpressed by using the plasmid pET-17b (Novagen), which contains the T7 promoter upstream from a multicloning site. All *E. coli* cultures were grown with aeration at 37°C in Luria-Bertani (LB) liquid medium or on LB agar at 37°C (26). Ampicillin and chloramphenicol were used at concentrations of 100 and 25 μg/ml, respectively.

Isolation of *B. burgdorferi* genomic DNA. Linear and circular supercoiled plasmid DNA from virulent *B. burgdorferi* B31 passage 2 was obtained as described elsewhere (12). *B. burgdorferi* chromosomal DNA was purified as described previously for *T. pallidum* (6).

Isolation of OMV derived from *B. burgdorferi.* OMV derived from both *B. burgdorferi* B31 virulent and avirulent cells were obtained as described previously (42).

SDS-PAGE and immunoblotting. Protein samples were resolved by discontin-

uous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method outlined by Laemmli (23). Two-dimensional gel electrophoresis with nondenaturing isoelectric focusing (ND-IEF) gel electrophoresis in the first dimension and SDS-PAGE in the second dimension was conducted as described previously (5).

Western blotting (immunoblotting) was conducted as described previously (42). Rabbit serum specific for Oms28 (described below) was diluted 1:5,000, and rabbit serum specific for the *E. coli* proteins OmpA (kindly provided by W. Wickner, Dartmouth College) and F_1F_0 ATPase subunit c (kindly provided by J. Hermolin and R. Filingame, University of Wisconsin, Madison) were both diluted 1:10,000 for Western blot analyses. Donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase was diluted 1:5,000 and used as the secondary antibody (Amersham Corp., Arlington Heights, Ill.). Antigen-antibody complexes were detected with the enhanced chemiluminescence (ECL) system of Amersham as described previously (42).

Identification and polyacrylamide gel purification of native Oms28. Frozen aliquots of phosphate-buffered saline (pH 7.4; PBS)-washed *B. burgdorferi* B31 passage 2 corresponding to 3×10^9 whole cells or OMV derived from 5×10^9 *B. burgdorferi* B31 passage 7 were solubilized in Triton X-100 and analyzed by ND-IEF as described previously (5). After electrophoresis, the tube gel (0.2 by 12 cm) was cut into 24 equal 0.5-cm pieces, crushed with a sterile pestle in 0.1 M NaCl–0.1% Triton X-100, and assayed in the planar lipid bilayer assay as described below. After the location of the porin activity in the first-dimension gel was determined (see below), this region was further analyzed by SDS-PAGE in the second dimension. Two identical uncut tube gels were incubated in 0.1 M Tris HCl (pH 6.8)–0.1% SDS–10% glycerol–0.05% bromophenol blue for 30 min at 22°C and then separated in the second dimension by SDS-PAGE. One of the SDS-polyacrylamide gels was stained with Coomassie brilliant blue and destained (3). A 28-kDa protein common to the two-dimensional profiles from whole cells and the OMV preparations was cut out of an unfixed and unstained SDS-polyacrylamide gel and crushed with a sterile pestle in a 200- to 300-μl 0.1 M NaCl–0.1% Triton X-100 suspension and assayed for porin activity.

FPLC purification of native Oms28. OMV derived from 5×10^{10} *B. burgdorferi* B31 passage 2 or B31 avirulent ATCC 35210 bacteria were solubilized in 50 mM Tris HCl (pH 8.0)–1% hydrogenated Triton X-100 (Calbiochem Corp., San Diego, Calif.). Residual particulate material was removed by two successive centrifugations at $13,000 \times g$. The supernatants were kept on ice, and the protein samples, ranging from 1 to 5 mg, were separated by FPLC with the Pharmacia model LCC-500 controller. The proteins were applied to a 1-ml Mono Q column and, when the optical density at 280 nm (OD_{280}) reached baseline, were eluted from the anion exchanger in a 30-ml volume with a 0 to 600 mM NaCl linear gradient buffered in 50 mM Tris HCl (pH 8.0)–0.5% hydrogenated Triton X-100. All fractions were collected in 0.5-ml volumes. The resulting fractions were screened for the presence of Oms28 by spotting 2 μl per fraction onto nitrocellulose and then incubating with Oms28-specific antiserum (see below) and ECL immunoblotting. Fractions containing Oms28 were pooled and repurified by FPLC with the 1-ml Mono Q column as described above. Fractions containing Oms28 were supplemented with SDS to a final concentration of 0.1% and glycerol to a final concentration of 10%, and the pH was adjusted to 6.8. The sample was resolved by SDS-PAGE, and the 28-kDa region was excised from the gel. Oms28 was eluted in 0.1 M NaCl–0.5% hydrogenated Triton X-100 and tested for porin activity as described below or rerun on SDS-PAGE, immunoblotted to a polyvinylidene difluoride (PVDF; Millipore Corp., Bedford, Mass.), and stained with either colloidal gold (AuroDye forte, Amersham) or amido black to determine the purity of the Oms28 porin.

Planar membrane assays of purified Oms28. Porin activity was assessed as described previously (42). FPLC- and gel-purified native Oms28 was diluted to a final concentration of 1:10,000 or 1:30,000 in 1 M KCl buffered in 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4) prior to its addition to the bilayer. Gel-purified recombinant Oms28 was diluted to a final concentration of 1:500 or 1:2,000 in the same buffer as that listed above prior to its addition to the bilayer.

Amino acid sequencing of Oms28. Triton X-100 detergent solubilizations, ND-IEF gel electrophoresis, and SDS-PAGE (i.e., nondenaturing two-dimensional analysis) were conducted essentially as described above for the gel purification of native Oms28 except that *B. burgdorferi* B31 passage 13 was used as the source of Oms28. After ND-IEF gel electrophoresis and SDS-PAGE, the Triton X-100-solubilized proteins were immunoblotted onto nitrocellulose (Scheicher & Schuell, Inc., Keene, N.H.) as described previously (49) and stained with 1% amido black. The blot was destained, and the spot corresponding to the 28-kDa Oms28 porin (approximately 15 μg) was excised from the membrane, placed in sterile water, and frozen at −20°C. The Oms28 protein was then processed for internal amino acid sequencing as described elsewhere (5, 48).

Cloning and nucleotide sequence of *oms28*. The Oms28 porin was digested with trypsin, and the resulting peptides were separated by reverse-phase high-performance liquid chromatography as described previously (5, 48). The sequences of five peptides were obtained (shown underlined in Fig. 3). Two peptides, designated A and B, were used to create degenerate oligonucleotides, and their sequences are as follows: peptide A, DSNNANILKPQSNVLEHS DQKDNK; peptide B, ALDETVQEAQK. The underlined amino acids correspond to the residues utilized to design the degenerate oligonucleotides. These oligonucleotides, designated 28A2 (with a 192-fold degeneracy) and 28B1 (with a 128-fold degeneracy), were end-labeled with [γ-^{32}P]ATP (Amersham) and used in Southern blot analysis (26) to probe *Hin*dIII-digested *B. burgdorferi* B31 passage 2 chromosomal, linear plasmid, and circular, supercoiled plasmid DNA to identify the gene encoding Oms28 (data not shown). The 28A2 and 28B1 oligonucleotides recognized a 1.6- and a 3-kb fragment, respectively, in the *Hin*dIII-digested linear plasmid DNA. This suggested that a *Hin*dIII restriction site split the *oms28* gene into two fragments and that the degenerate oligonucleotides recognized sequences both upstream (5′) and downstream (3′) of the *Hin*dIII site. These two fragments were cloned into the *Hin*dIII site of pBluescript KS$^+$ previously treated with shrimp alkaline phosphatase (United States Biochemicals, Cleveland, Ohio). Following transformation into DH5α, clones containing the 1.6- and 3.0-kb inserts were identified separately by colony hybridization with probes 28A2 and 28B1, respectively. Open reading frames were identified in the clones that confirmed both the presence of a single *Hin*dIII site in *oms28* and the amino acid sequence of the A and B tryptic peptides derived from Oms28. The *oms28* gene was sequenced to completion by primer walking on both strands by the dideoxynucleotide method of Sanger et al. (35) with [α-^{35}S]dATP (Amersham).

DNA and protein sequence analysis. The nucleotide sequence of *oms28* was analyzed by use of the DNA Strider version 1.0 program (27). Homology searches with either full-length Oms28 or tryptic peptides derived from Oms28 were conducted by use of a BLASTP search of the National Center for Biotechnology Information database (2).

Oligonucleotides. Oligonucleotides were synthesized with the Applied Biosystems model 470B automated DNA synthesizer as described previously (5).

PCR. PCR was conducted essentially as described previously (5). The amplimers were resolved by agarose gel electrophoresis buffered in 40 mM Tris acetate (pH 8.7)–1 mM EDTA and purified with Geneclean II (Bio 101, La Jolla, Calif.).

Tests of protein association with OMV. OMV preparations derived from 1.25×10^9 *B. burgdorferi* strain B31 passage 3 bacteria (in 10-μl volumes) were diluted to 100 μl with the following salt solutions: PBS (pH 7.4), 1 M NaCl, and 0.1 M Na_2CO_3 (pH 11.5). A control sample was presolubilized with 1% Triton X-100 and then incubated with 1 M NaCl. The samples were incubated on ice for 5 min, diluted to 1 ml with PBS (pH 7.4), and pelleted at $40,000 \times g$ for 1 h at 4°C. The supernatant was removed, and the protein was concentrated by precipitation with trichloroacetic acid. The pelleted and supernatant materials were resuspended in Laemmli sample buffer, and the proteins were resolved by SDS-PAGE (23). The proteins were then electroblotted onto a PVDF membrane and immunoblotted with antiserum specific for Oms28.

Triton X-114 phase extraction. *B. burgdorferi* B31 passage 6 whole cells (10^9) were subjected to Triton X-114 phase partitioning as described previously (42), analyzed by SDS-PAGE, and immunoblotted with Oms28-specific antiserum as described above.

Fractionation and localization of Oms28 in *E. coli*. The *oms28* gene, including the leader sequence, was cloned into pET-17b vector (Novagen) by using PCR primers with restriction enzyme sites engineered at their ends. The primer *oms28*N5′ (5′ GGAATTC**CATATG**ACTAAAATATTTAGTAAT 3′) contains a *Nde*I site (in bold) that encodes the codon for the initiating methionine (underlined) of *oms28* directly at the 5′ end. A primer corresponding to the carboxy terminus, designated *oms28*E3′ (5′ CGCGGATCC**GAATTC**CTATCTCATGTA TAAAGAAAT 3′), contains an *Eco*RI site (in bold) immediately 3′ from the stop codon of *oms28* (underlined; corresponds to the stop codon sequence from the noncoding strand). A PCR with 10 ng of *B. burgdorferi* B31 passage 2 linear plasmid DNA as the template and the primers *oms28*N5′ and *oms28*E3′ yielded a product of approximately 800 bp that was then digested with *Nde*I and *Eco*RI, as was the vector pET-17b, and all fragments were purified with Geneclean II. The PCR amplimer and pET-17b were ligated together and transformed into BL21 DE3(pLysE). Positive clones were grown in 50 ml of LB broth, and overproduction of rOms28 was conducted as outlined by Novagen. After overproduction, the OD_{600} of the culture was determined and the cells were harvested by centrifugation at $8,000 \times g$ for 10 min. The cells were then resuspended in PBS such that the density was between 5 and 10 OD_{600} equivalents per ml and were frozen at −20°C overnight. The sample was thawed the next day, and the cells were lysed with a French pressure cell set at 600 to 1,000 lb/in^2. Unlysed cells were pelleted at $4,000 \times g$ for 10 min. The supernatant was transferred to a new tube and centrifuged again at $10,000 \times g$ for 1 min. The supernatant was then recentrifuged at $40,000 \times g$ for 30 min at 4°C to pellet the total membrane. The supernatant represented the soluble protein fraction. Pelleted membrane was resuspended in PBS–2% Triton X-100 and rocked at 4°C for 1 h and then at room temperature for 1 h. OM was pelleted by centrifugation at $40,000 \times g$ for 30 min. The supernatant was saved as the Triton X-100-soluble inner membrane (IM) fraction. IM protein was concentrated by trichloroacetic acid precipitation. The OM pellet was washed with PBS and recentrifuged at $40,000 \times g$ for 30 min at 4°C. The final OM pellet was resuspended in PBS at a concentration equivalent to 1 OD_{600}(ml)/μl. Fractions were then analyzed by SDS-PAGE and either stained with Coomassie brilliant blue or immunoblotted with antiserum specific for Oms28 that was adsorbed with BL21 DE3(pLysE, pET-17b) as described previously (17). Additionally, OM fractions were tested for porin activity by excising the 28-kDa regions from an SDS-PAGE, separating OM protein derived from induced BL21 DE3(pLysE) cells with or without *oms28* cloned into pET-17b as described above.

Antiserum. Antiserum specific for Oms28 was obtained by overproducing rOms28 by using the T7 regulated plasmid vector pET-17b (Novagen) as follows. Oligonucleotides specific for the sequence corresponding to the amino terminus (N) and carboxy terminus (*oms28*E3′) of mature Oms28 were synthesized with *Bam*HI and *Eco*RI restriction sites at the 5′ ends, respectively. The N oligonucleotide (5′ CGC**GGATCC**AGATTCTAACAATGCAAATATT 3′; *Bam*HI site is in bold) and *oms28*E3′ oligonucleotide (5′ CGCGGATCC**GAATTC**CTATCT CATGTATAAAGAAAT 3′; *Eco*RI site is in bold) were processed for PCR as described above. The amplified DNA fragment, approximately 700 bp, was digested with *Eco*RI and *Bam*HI, purified, and cloned into the plasmid pET-17b previously digested with *Bam*HI and *Eco*RI. The ligated construct was transformed into *E. coli* BL21 DE3(pLysE). The resulting construct encoded a fusion protein containing 22 residues from the T7 gene 10 protein fused to the processed or mature Oms28 protein. Overproduction of the Oms28 fusion protein was performed as described in the manufacturer's instructions (Novagen) and further purified by FPLC as described above for native rOms28. Fractions containing rOms28 fusion protein were separated by preparative SDS-PAGE and visualized by staining with 0.05% Coomassie brilliant blue in distilled H_2O for 10 min. rOms28 was used to immunize and boost rabbits as described previously (5). Serum was obtained 17 days postboost and was adsorbed with *E. coli* BL21 DE3(pLysE, pET-17b) as described previously (17).

Nucleotide sequence accession number. The DNA sequence of *oms28* was deposited in the GenBank database under the accession number U61142.

RESULTS

Identification of a porin activity associated with the OM of *B. burgdorferi*. To determine which OM protein had the 0.6-nS porin activity we had previously observed in our OMV preparation (42), whole *B. burgdorferi* strain B31 cells and OMV derived from *B. burgdorferi* were incubated in 1% Triton X-100 and the solubilized proteins were separated by ND-IEF gel electrophoresis. After the ND-IEF gel was cut into separate pieces and the protein was eluted and assayed in the planar lipid bilayer assay system, a single-channel conductance of 0.6 nS was observed in a fraction containing several proteins, of which one with an apparent molecular mass of 28 kDa was the most abundant (Fig. 1). Similar ND-IEF analyses were conducted with OMV derived from both virulent *B. burgdorferi* B31 passage 7 and avirulent *B. burgdorferi* B31 ATCC 35210, and a similar 0.6-nS conductance was observed for the solubilized OMV material (data not shown). Comparison of the solubilized whole cells and the solubilized OMV material indicated that the 28-kDa species was the only protein in the ND-IEF eluted sample that was common between these different preparations, suggesting that the 28-kDa protein was the 0.6-nS porin.

FPLC purification of the native Oms28 porin protein. To determine whether the 28-kDa protein encoded the 0.6-nS porin activity, we separated Triton X-100 detergent-solubilized OMV proteins by FPLC. A 28-kDa protein was observed in fractions that eluted from the Mono Q column at a NaCl concentration ranging between 80 and 90 mM. These fractions were pooled and separated again by FPLC, and the 80 to 90 mM NaCl eluates were tested for porin activity. The FPLC fractions containing the 28-kDa protein also contained a *B. burgdorferi* 10-nS channel-forming activity (42). Fractions containing Oms28 were separated by SDS-PAGE, the 28-kDa region was excised, and the protein was eluted from the gel and tested for purity and porin activity (Fig. 2). Under these conditions, the contaminating large channel was completely eliminated, on the basis of differences in molecular mass (data not shown), and a 0.6-nS channel was observed in the gel-eluted material that corresponded to the 28-kDa region of the SDS-polyacrylamide gel. The stepwise channel conductance observed (Fig. 2C) and the large number of individual insertional events into the bilayer (n = 181; Fig. 2D) suggested that the 28-kDa protein was in a native conformation. Porin activity was observed at final concentrations of Oms28 ranging from 1 ng/ml to 333 pg/ml. Unlike some porin proteins, no higher-molecular-weight or oligomeric forms of purified, native Oms28 were detected when the samples were incubated in conventional SDS-PAGE sample buffer either with or without boiling (data not shown). When whole *B. burgdorferi* cells or OMV derived from *B. burgdorferi* were incubated in modified sample buffer at room temperature containing 0.2% SDS but lacking β-mercaptoethanol, oligomeric forms of Oms28 were observed at low levels (data not shown). These results implied that if Oms28 formed an oligomeric structure, it was sensitive to the concentration of SDS or β-mercaptoethanol used in the

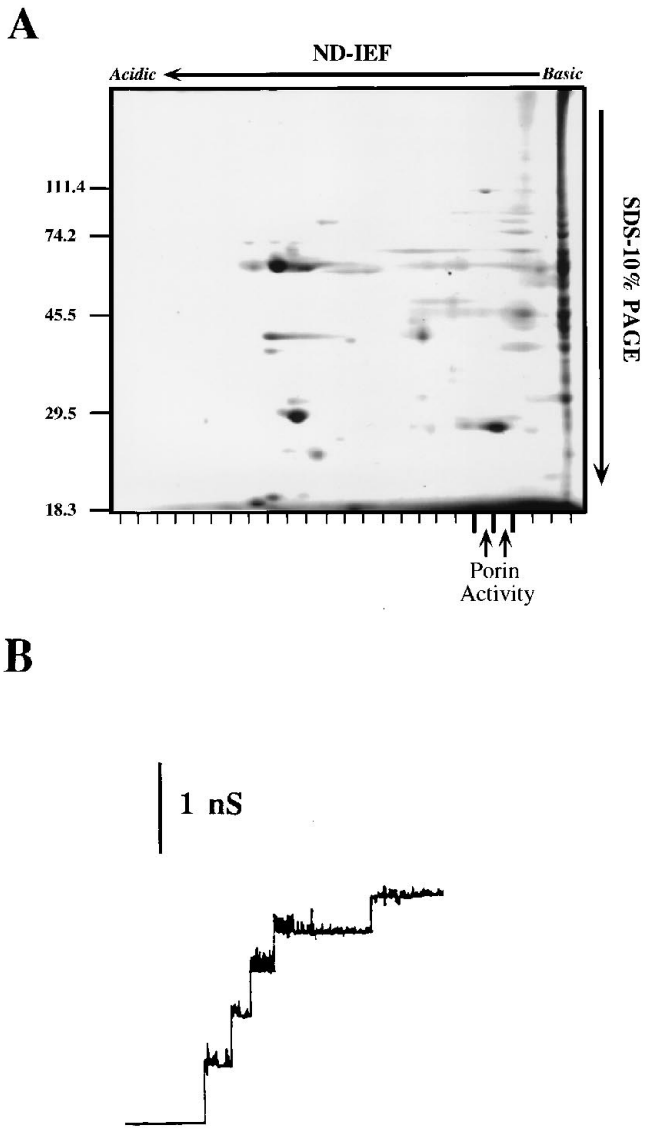

FIG. 1. Identification of a 0.6-nS porin activity from *B. burgdorferi* B31. (A) Triton X-100-solubilized protein from 3×10^9 *B. burgdorferi* B31 passage 7 whole cells separated by two-dimensional gel electrophoresis. The proteins were resolved by ND-IEF gel electrophoresis in the first dimension and by SDS–10% PAGE in the second dimension. Vertical lines along the bottom of the figure represent each of the 24 separate pieces that were tested for porin activity following the elution of protein from the ND-IEF gel. Arrows denote gel fragments that demonstrated porin activity. The numbers on the left represent the molecular masses of protein standards (in kilodaltons). (B) Conductance profile of protein extracted from active ND-IEF gel fractions when added to a planar lipid bilayer (fractions used are specified by arrows in panel A). It is important to note the uniformity of the single-channel conductances observed. Each stepwise increase of conductance represents the insertion of a single ion channel.

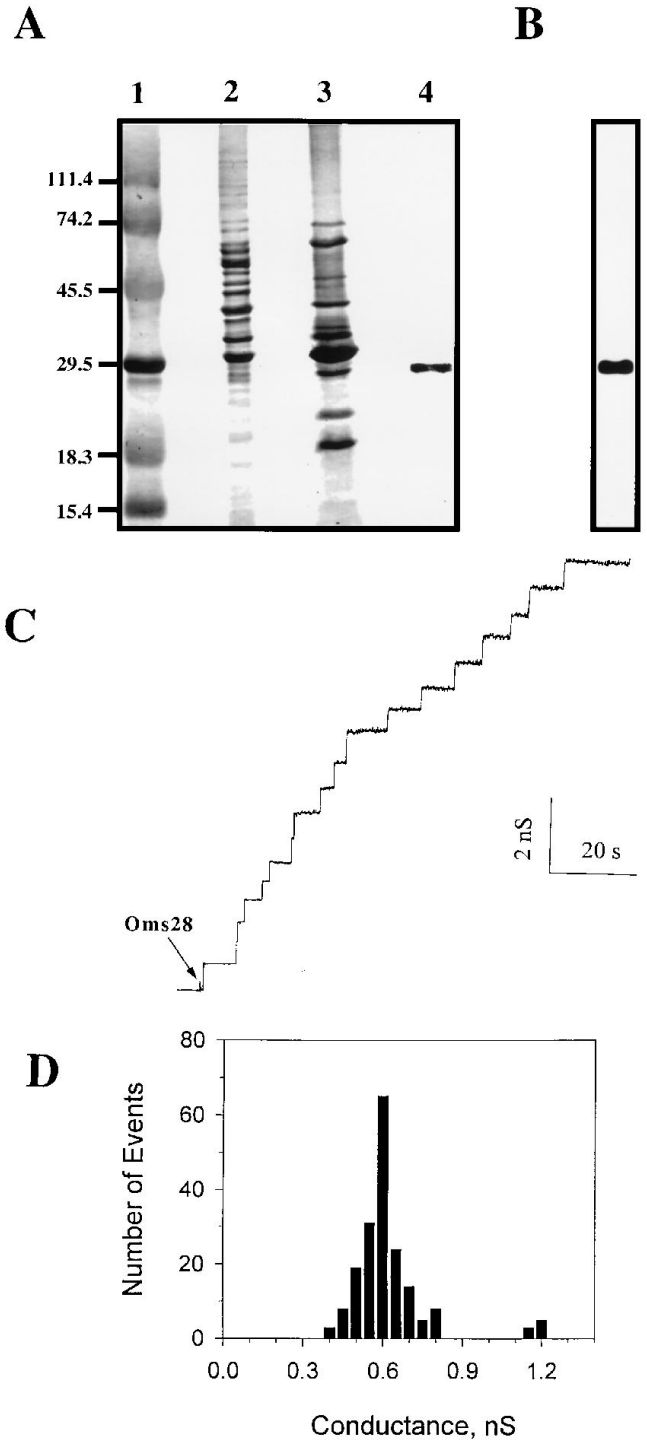

FIG. 2. FPLC purification of native Oms28 and porin activity of purified Oms28. (A) Amido black-stained blot from an SDS–12.5% polyacrylamide gel showing the purification of Oms28. Lanes: 1, protein molecular mass standards in kilodaltons; 2, 10^8 *B. burgdorferi* B31 passage 3 whole cells; 3, OMV derived from 2.5×10^9 *B. burgdorferi* B31 passage 3 whole cells; 4, Oms28 purified from OMV derived from 2.5×10^{10} *B. burgdorferi* B31 passage 3 whole cells. (B) Immunoblot of FPLC- and gel-purified Oms28 probed with Oms28 antiserum. The Oms28 shown was purified from OMV derived from 8×10^9 *B. burgdorferi* B31 passage 3 whole cells. (C) Single-channel conductance steps of purified native Oms28. Purified Oms28, at a final concentration of 0.4 ng/ml, was added to a planar lipid bilayer with a diameter of 500 μm bathed in 1 M KCl and buffered in 5 mM HEPES (pH 7.4). The arrow indicates when native Oms28 was added to the planar lipid bilayer. (D) Histogram of the single-channel conductance events observed for purified native Oms28 (n = 181).

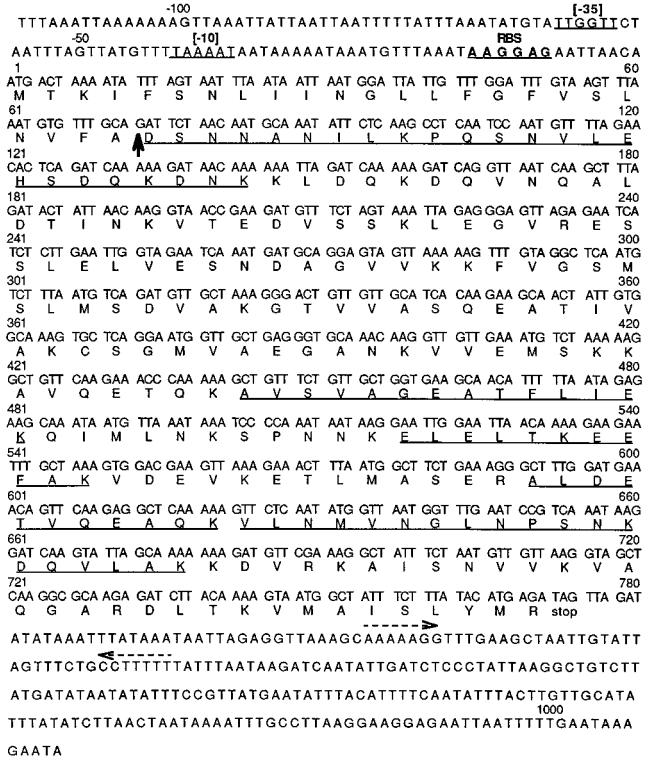

FIG. 3. Nucleotide sequence of the *oms28* gene and deduced amino acid sequence of Oms28 from *B. burgdorferi* B31. The numbers shown are relative to the start of the *oms28* open reading frame. The *oms28* locus encodes a 257-amino-acid protein with a putative 24-amino-acid leader peptidase I signal sequence. The predicted cleavage site is denoted by a vertical arrow between residues 24 and 25. The putative ribosome binding site (RBS) is underlined in bold, and putative −35 and −10 σ^{70} promoter regions are underlined. Dotted lines with arrows represent a potential rho-independent transcription termination signal. Underlined amino acids correspond exactly to sequences obtained following partial proteolytic digestion of purified Oms28 with trypsin.

SDS-PAGE sample buffer. The 28-kDa porin was designated Oms28 for outer membrane-spanning 28-kDa protein.

Sequence analysis of *oms28*. The nucleotide sequence of *oms28* revealed an open reading frame of 771 bp encoding a 257-amino-acid protein with a calculated molecular mass of 28,002 Da (Fig. 3). Upstream sequences resembling a conventional gram-negative −35 (TTGGTT) and −10 (TAAAAT) σ^{70} promoter as well as a putative ribosome binding site (AAGGAG) were identified (Fig. 3). A putative rho-independent transcriptional termination sequence was also identified. The predicted amino-terminal end of the full-length Oms28 protein contained a 24-amino-acid leader peptide sequence typical of exported proteins with a basic residue followed by a hydrophobic core (amino acids 4 to 20) and a potential leader peptidase I cleavage site (50), Val-Phe-Ala. The cleavage of the 24-amino-acid leader sequence would yield a mature Oms28 protein composed of 233 amino acids with a molecular mass of 25,363 Da. Comparison of the deduced amino acid sequence of Oms28 with the tryptic peptide A sequence (see above) derived from native Oms28 indicated that the amino terminus of peptide A was preceded by an alanine residue instead of an arginine or lysine residue required for cleavage by trypsin (see above and Fig. 3). This indicated that peptide A represented the amino-terminal end of the cleaved, mature Oms28 protein.

Proteins homologous to Oms28 were not identified from a

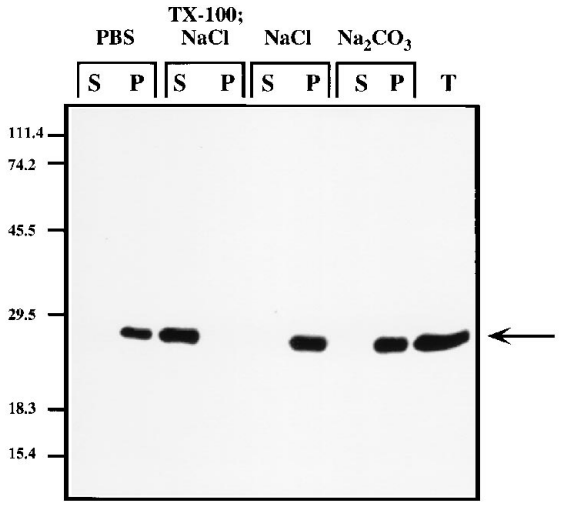

FIG. 4. Association of Oms28 with the OMV preparations. OMV derived from 1.25×10^9 *B. burgdorferi* B31 passage 3 cells were washed for 5 min with salt solutions to determine if Oms28 was a membrane-spanning protein. P and S, pellet and supernatant, respectively, following a 1-h centrifugation at $40,000 \times g$. Protein samples corresponding to the P and S samples were separated by SDS–12.5% PAGE, immunoblotted onto a PVDF membrane, and probed with antiserum specific for Oms28. The numbers on the left represent the molecular masses of protein standards (in kilodaltons). The arrow on the right denotes the location of Oms28. Abbreviations: PBS, OMV incubated with PBS (pH 7.4); TX-100, NaCl, OMV solubilized with 1% Triton X-100 and then incubated with 1 M NaCl; NaCl, OMV incubated in 1 M NaCl; Na_2CO_3, OMV incubated in 0.1 M Na_2CO_3 (pH 11.5); T, total untreated, unpelleted OMV.

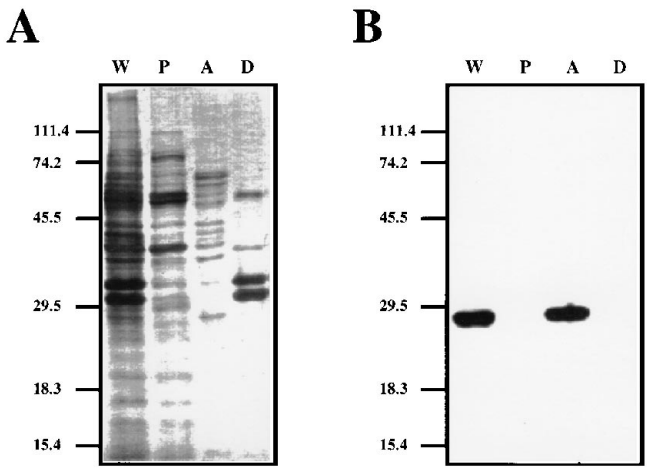

FIG. 5. Triton X-114-extracted and phase-partitioned material from 10^8 *B. burgdorferi* B31 passage 6 whole cells. (A) Amido black-stained immunoblot of an SDS–12.5% polyacrylamide gel prior to ECL analysis. (B) Immunoblot shown in panel A probed with antiserum specific for Oms28. The numbers on the left represent the molecular masses of protein standards (in kilodaltons). W, whole organisms; P, protoplasmic cylinders; A, Triton X-114 aqueous-phase proteins; D, Triton X-114 detergent-phase proteins.

search of the National Center for Biotechnology Information database using the full-length amino acid sequence of Oms28 (2). Taken together with the porin activity described above, these observations indicated that Oms28 was a *B. burgdorferi* Oms protein, the first to be functionally characterized. Additionally, *oms28* represented the first gene to be cloned and sequenced that encoded a functional Oms protein.

OM localization of Oms28 in *B. burgdorferi*. To confirm that native Oms28 was an Oms protein, we utilized harsh salt solutions which are known to release soluble proteins yet retain integral membrane proteins (16). As shown in Fig. 4, Oms28 remained exclusively with the pelleted membrane material, as detected with recombinant Oms28 antisera and ECL immunoblotting, after incubation in either 1 M NaCl or 0.1 M Na_2CO_3 (pH 11.5). Under identical conditions, contaminating bovine serum albumin was detected only in the supernatant (data not shown). By comparison, OMV presolubilized with Triton X-100 released Oms28, which was found in the supernatant following centrifugation. These results suggested that Oms28 was an integral membrane protein, consistent with its porin activity.

B. burgdorferi B31 passage 6 whole cells were subjected to Triton X-114 phase partitioning to determine if Oms28 was a detergent-phase protein as one would predict for a spirochetal Oms protein. Surprisingly, Western blot analysis showed that Oms28 partitioned exclusively into the aqueous phase (Fig. 5B), suggesting that Oms28 was no longer folded into a membrane-spanning conformation and therefore fractionated anomalously.

OM localization and functional activity of rOms28 in *E. coli*. rOms28 was overproduced in *E. coli*, and the cells were fractionated to determine its localization. When *oms28* was overexpressed, boiled in SDS-PAGE sample buffer, and resolved by SDS-PAGE, rOms28 was distributed in the soluble IM, and OM fractions, with the majority in the soluble fraction (Fig. 6A, lanes 4 through 6, respectively). Residual rOms28 was detected in the IM fraction by Coomassie brilliant blue staining (Fig. 6A, lane 5), and approximately 30% was observed in the IM fraction following ECL immunoblotting with specific antisera generated against rOms28 (Fig. 6B, lane 5). This suggested that either the IM fraction was contaminated with OM or residual steady-state levels of rOms28 were being processed across the IM at the time of cell harvesting or were overproduced such that the rOms28 may have saturated the processing system. Antibodies to known *E. coli* IM and OM proteins, F_1F_0 ATPase subunit c and OmpA, respectively, were used to determine the degree of purity of the IM and OM fractions. The antibody to the F_1F_0 ATPase subunit c predominantly recognized a 10-kDa protein (and several higher-molecular-weight proteins as a result of the boiling of the sample prior to SDS-PAGE) in the IM only (data not shown). No such proteins were observed in the OM fraction. Conversely, antiserum specific for OmpA recognized a single 35-kDa species in the boiled OM fraction but did not react with the IM fraction (data not shown). Therefore, the presence of rOms28 in the IM fraction may be an artifact of its overproduction.

Overproduced rOms28 fractionated partly to the OM in *E. coli* and, when the sample was not heated or exposed to reducing agents, formed, in addition to the 28-kDa species, an oligomeric species of approximately 75 kDa that reacted with antiserum specific for Oms28, as shown in Fig. 7. Approximately 3 μg of rOms28 was observed in OM derived from 10^9 induced *E. coli* cells (or approximately 1.5 OD_{600}[ml] of cells) expressing *oms28*. Neither the 28- nor 75-kDa form of rOms28 was observed in OM derived from induced *E. coli* cells harboring the vector plasmid alone. Since most of the porin proteins characterized have a trimeric stoichiometry (13), it is tempting to speculate that rOms28 has a similar organization. In support of this observation, oligomeric organization of the recombinant spirochetal porin proteins OmpL1 and Tromp1 has also been reported (11, 39). Additionally, oligomeric forms of native porin proteins have been reported for *Spirocheta aurantia* (22) and *Treponema denticola* (14, 51, 52).

To determine if rOms28 retained porin activity, unheated

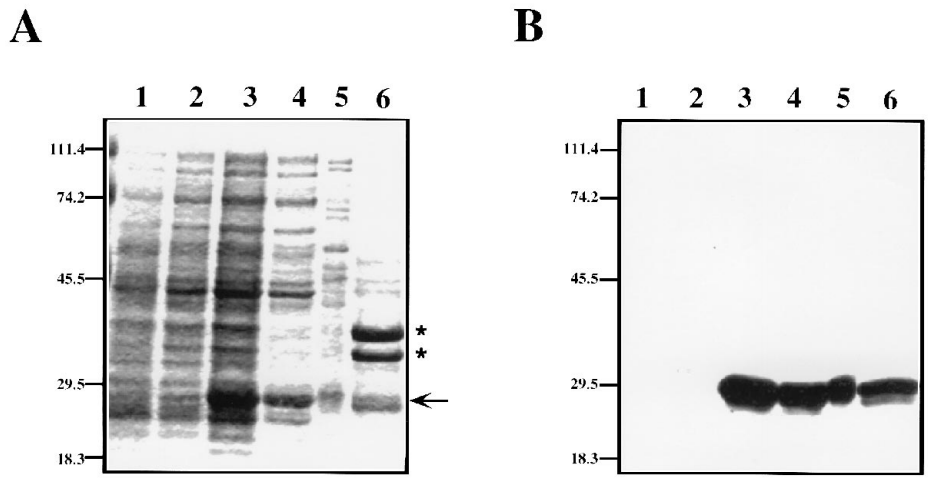

FIG. 6. Localization of rOms28 in fractionated *E. coli* BL21 DE3 pLysE expressing *oms28*. (A) Coomassie blue-stained SDS–10% polyacrylamide gel of the following: whole cells with the vector pET-17b alone induced with isopropyl-β-D-thiogalactopyranoside for 1 h and rifampin for an additional 2 h (lane 1); whole cells containing pET-17b *oms28*, uninduced (lane 2); whole cells expressing *oms28* induced as indicated above (lane 3); soluble protein from whole cells expressing *oms28* (lane 4); IM fraction from cells expressing *oms28* (lane 5); OM fraction from cells expressing *oms28* (lane 6). All samples were boiled for 5 min prior to electrophoresis. The arrow denotes the location of rOms28, and asterisks mark the locations of *E. coli* porin proteins. The numbers on the left represent the molecular masses of protein standards (in kilodaltons). (B) Immunoblot of samples identical to that shown in panel A probed with antiserum specific for Oms28.

OM samples from *E. coli* expressing either recombinant *oms28* or the vector alone were resolved by SDS-PAGE (as described in Materials and Methods), and the regions of the gel corresponding to the molecular mass of 28 kDa in both samples were tested for porin activity. Whereas the vector-only control showed no porin activity for the 28-kDa region (data not shown), the rOms28 sample exhibited a 1.1-nS channel-forming activity (Fig. 8A) similar to that observed at low levels for native Oms28 (compare Fig. 2D and Fig. 8B). The amounts of rOms28 required for detectable porin activity were between 10 and 15 ng/ml or approximately 15- to 45-fold greater than the amounts used to demonstrate porin activity for native Oms28 (Fig. 2C and D). The number of insertional events observed, 54 (Fig. 8B), and the similarity in conductance relative to that of native Oms28 further confirmed that Oms28 was one of the porin proteins previously observed in our OMV preparation (42).

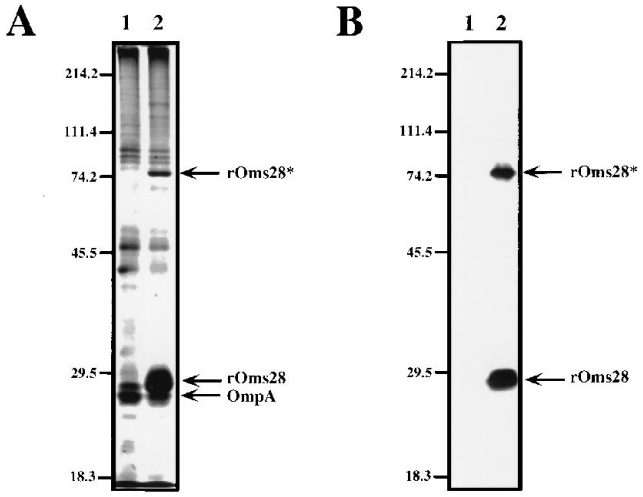

FIG. 7. Localization of rOms28 to the OM of *E. coli*. Note that the samples were not heated prior to electrophoresis. (A) Coomassie brilliant blue-stained SDS–10% polyacrylamide gel containing OM derived from 5 OD_{600}(ml) equivalents of BL21 DE3(pLysE, pET-17b) (lane 1) and BL21 DE3(pLysE, pET-17b) *oms28* overproducing rOms28 (lane 2). (B) Immunoblot of the identical samples shown in panel A probed with antiserum specific for Oms28. The numbers on the left represent the molecular masses of protein standards (in kilodaltons). Arrows indicate the locations of the *E. coli* OmpA protein and the monomeric and oligomeric forms of rOms28. An asterisk denotes the location of the oligomeric form of rOms28.

Presence of Oms28 in other American and European *B. burgdorferi* isolates. To determine whether proteins antigenically related to Oms28 were present in other virulent *B. burgdorferi* isolates, an immunoblot containing protein lysates from low-passage American and European isolates were probed with Oms28 antisera (Fig. 9). Additionally, we analyzed protein lysates from the *B. burgdorferi* sensu lato isolate, *B. garinii*, and in the etiologic agents of relapsing fever and syphilis, *B. hermsii* and *T. pallidum*, respectively (Fig. 9). Each of the American and European isolates tested contained an Oms28-like protein, although strain N40 and the European strain 2872-3 synthesized less Oms28 relative to the other *B. burgdorferi* isolates. A doublet was observed in strain 2872-3 that was not apparent in any other *B. burgdorferi* isolate tested. An Oms28 protein was not observed in the other spirochetal pathogens, *B. garinii*, *B. hermsii*, and *T. pallidum*, suggesting that Oms28 may be a protein specific to *B. burgdorferi* sensu stricto.

DISCUSSION

The OM of gram-negative bacteria functions as a semipermeable barrier that protects the cell from the harsh molecules (i.e., proteases, immunoglobulins, and inhibitory peptides) present in the microenvironments where the bacteria reside. The OM is permeable by virtue of pores formed by proteins, designated porins. Porin proteins of gram-negative bacteria function as water-filled pores that allow for the passive diffusion of solutes through the OM (19, 29). The nutrients obtained in this manner are then actively transported across the IM and utilized for various metabolic processes. Porin proteins, like other Oms proteins, are characterized by stretches of amino acids that form amphipathic beta-pleated sheet struc-

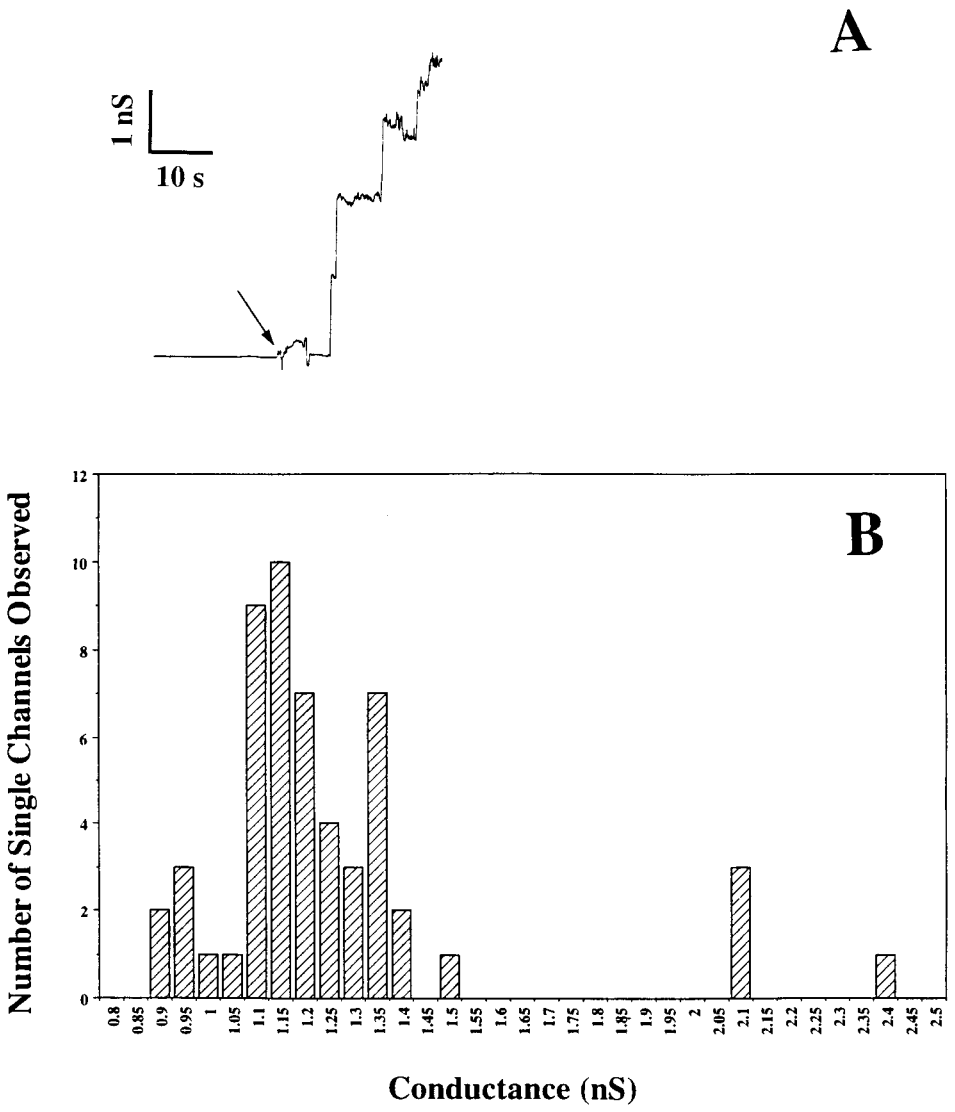

FIG. 8. Porin activity of rOms28. (A) Single-channel conductance steps of rOms28. Gel-eluted rOms28, at a final concentration of approximately 20 ng/ml, was incubated in a planar lipid bilayer system containing 1 M KCl buffered with 5 mM HEPES (pH 7.4). The arrow indicates when rOms28 was added to the planar lipid bilayer. (B) Histogram of the single-channel conductance events observed for purified rOms28 (n = 54).

tures that span the OM bilayer (13, 36, 53). *B. burgdorferi*, like other gram-negative bacteria, must encode for porin proteins within its OM to gain essential metabolites. Since no Oms proteins have been identified previously in *B. burgdorferi*, isolation and characterization of a porin protein would establish an important OM marker to aid in the identification of other Oms proteins in *B. burgdorferi* and may provide important information pertaining to the topological organization of Oms proteins in the OM of *B. burgdorferi.*

Until recently, the identification of OM proteins in *B. burgdorferi* has been impeded by the inability to separate its IMs and OMs, presumably because of the lack of lipopolysaccharide in *B. burgdorferi* that facilitates the separation of the IM and OM like that for enteric gram-negative bacteria (30). However, Bledsoe et al. have recently isolated the IM, OM, and hybrid membranes from *B. burgdorferi* by isopycnic centrifugation (8). Later, Radolf et al. reported the isolation of the *B. burgdorferi* OM by using hypertonic sucrose (34). More recently, we have independently reported the purification of OMV from *B. burgdorferi* and identified their constituent OM proteins (42). Additionally, we determined that two separate porin activities were associated with our OMV preparation with average single-channel conductances of 0.6 and 12.6 nS (42).

Our previous analysis focused on Triton X-114 detergent-phase proteins, since these proteins were the best candidates to begin studies designed to characterize functional Oms proteins (42). Although Oms28 demonstrated porin activity, it did not partition into the detergent phase and instead was found exclusively in the Triton X-114 aqueous phase (Fig. 5). The aqueous-phase character of Oms28 is in contrast to the other known spirochetal porin proteins, Tromp1 and OmpL1, which are exclusively detergent-phase proteins (5, 18). However, Oms28 is not the only membrane-spanning protein that has been associated with the aqueous phase. Recently, Probert et al. reported that a surface-exposed 66-kDa protein from *B. burgdorferi*, p66, also partitions into the Triton X-114 aqueous phase (31). We have recently determined that this same 66-

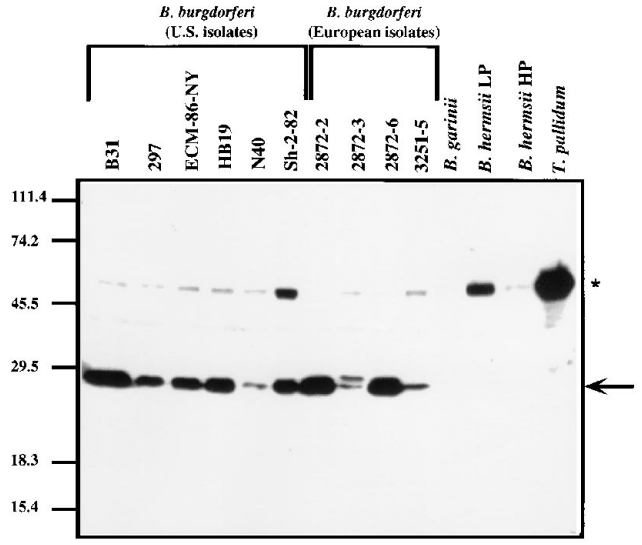

FIG. 9. Presence of Oms28 or Oms28 homologs in various international *B. burgdorferi* isolates and other pathogenic spirochetes. Protein derived from 5×10^7 whole cells was separated by SDS–12.5% PAGE, immunoblotted onto a PVDF membrane, and probed with antiserum specific for the strain B31 Oms28 protein. The brackets denote whether the *B. burgdorferi* sensu stricto isolate is associated with the United States or Europe. The American samples shown were all passage 1 virulent isolates. The European strains tested were virulent isolates that had been passaged no more than 15 times. Lanes containing protein from other pathogenic spirochetes are labeled accordingly. LP and HP, *B. hermsii* low-passage (serotype 7) and high-passage (serotype 33) isolates, respectively. The numbers on the left represent the molecular masses of protein standards (in kilodaltons). An arrow denotes the location of Oms28 observed in *B. burgdorferi* B31, and an asterisk marks the location of contaminating levels of rabbit immunoglobulin heavy chain.

kDa protein, which we have designated Oms66, is the source of the large channel activity associated with our OMV preparations (41, 42). Additionally, the gene encoding p66 has recently been cloned and sequenced (9). The association of Oms28 and Oms66 with the Triton X-114 aqueous phase suggests that these *B. burgdorferi* porin proteins behave differently as a result, perhaps, of a conformational alteration during the Triton X-114 extraction. In contrast, a recently identified *B. burgdorferi* 45-kDa porin protein, designated Oms45, is exclusively associated with the Triton X-114 detergent phase (40).

It is surprising that the OM of *B. burgdorferi* contains porins with single-channel conductances that are so disparate. This is in contrast to other spirochetes which appear to have porins that exhibit either a small single-channel conductance, as is the case for *T. pallidum* and *Leptospira kirschneri* (5, 39), or a large single-channel conductance, as observed for *S. aurantia* and *T. denticola* (14, 22, 51). The observation that *B. burgdorferi* is the only spirochete that contains porins of both classes suggests that both types of porins are necessary for survival within the different microenvironments in which *B. burgdorferi* is known to exist. That is, it is possible that one channel size is essential for survival within the tick midgut, whereas the other channel size may be required for persistence within an infected mammal. The importance of these different channels and the regulation of their gene expression, as well as their possible role in pathogenesis, remain to be determined.

Many previously identified porin proteins are organized as trimers (13, 19) whose proper conformation is essential for function. The OmpF and PhoE porins from *E. coli* have been crystallized, and their structures have been solved (13). Even though these two proteins have different primary structures, their secondary, tertiary, and quaternary structures are quite similar. It is therefore logical to assume that other gram-negative bacteria have porin proteins that are organized in a similar manner. Although no structural data are available for Oms28, the ability to visualize an oligomeric form of rOms28 in the OM of *E. coli* both by Coomassie blue staining and immunoblotting with anti-Oms28 serum (Fig. 7), coupled with the activity observed for rOms28 (Fig. 8), suggests that spirochetal porins conform to the structural paradigm established by the porin proteins of enteric gram-negative bacteria. In support of this contention, five additional spirochetal porins, a 36.5-kDa protein from *S. aurantia* (22), 53- and 64-kDa porins from *T. denticola* (14, 52), Tromp1 from *T. pallidum* (11) and OmpL1 from *L. kirschneri* (39), have all been observed as oligomers. Furthermore, the *S. aurantia*, *T. denticola*, and *L. kirschneri* porins are all heat modifiable (14, 22, 39, 51, 52).

The assignment of Oms28 as an OM protein is consistent with the evidence reported here. First, native Oms28 was present in our OMV preparations and was associated with the membrane fraction after treatment with salt washes (Fig. 4) which effectively release soluble proteins, including those trapped inside membrane vesicles (16). Second, the channel-forming activity associated with both native Oms28 and rOms28 indicated that Oms28 was an OM porin protein (Fig. 2 and 8). Consistent with these observations, purified native Oms28 exhibited a slight asymmetric voltage dependence indicative of OM porin proteins (19, 28, 29).

Expression of foreign porins in *E. coli* has been limited by the toxicity associated with the expression of membrane proteins from heterologous systems (10, 20, 21, 32, 33, 54). Cloning of the gene encoding the gonococcal porin in *E. coli* was possible only if the gene was split into two fragments; attempts to clone the intact gene were not successful unless the porin gene was placed under control of the T7 promoter (10). Overexpression of the meningococcal class 3 PorB porin by use of the T7 promoter-based pET-17b vector was not lethal to *E. coli*; however, the porin lacked its native leader and formed insoluble inclusion bodies (33). Inclusion bodies were also formed when the *Haemophilus influenzae* type b porin P2 was overproduced (32). To circumvent the potential lethality of *oms28* expression in *E. coli*, the entire *oms28* open reading frame was placed under control of an inducible T7 promoter. Overproduction of rOms28 was then facilitated by use of the T7 construct, and as observed for the *H. influenzae* and meningococcal porins, no lethality in *E. coli* was observed. However, in contrast to the *Haemophilus* and *Neisseria* class 3 recombinant porins, rOms28 was partially localized to the OM of *E. coli* and retained porin activity (Fig. 8), suggesting that the native *oms28* leader sequence is recognized by *E. coli* leader peptidase I and that a portion of the mature or processed form of rOms28 could be exported across the *E. coli* IM and assembled into the *E. coli* OM (Fig. 6 and 7). Similar results have also been observed with the class 1 meningococcal porin (54). When the gene encoding the class 1 porin was cloned and expressed in *E. coli* with its native leader sequence, it was also localized to the OM.

The differences in cellular localization between overproduced meningococcal class 3 porin and rOms28 may be due to the differences between these recombinant proteins at their amino termini. Whereas the meningococcal class 3 porin construct was engineered with 20 amino acids from the bacteriophage T7 gene 10 protein linked in frame to its amino terminus (33), our construct consisted of the entire *oms28* sequence, containing its own leader sequence, with no added T7 gene 10 sequence. It is possible that the additional amino acids from the T7 gene 10 protein inhibit the processing and/or export of

the meningococcal porin in *E. coli*, resulting in the accumulation of this protein in the cytoplasm as inclusion bodies (33). Interestingly, if a chimeric protein consisting of the *E. coli ompT* leader sequence is fused to the mature *oms28* sequence and overexpressed, this form of rOms28 does not localize to the *E. coli* OM even though the protein is synthesized in excess relative to the pET-17b native *oms28* construct reported here (data not shown). The inability of this construct to properly localize may be due to the additional four amino acids that are linked to the amino terminus of the processed rOms28 protein to link the *oms28* sequence in frame with the *ompT* leader sequence. These additional four amino acids may prevent this form of rOms28 from folding into a conformation that is either competent for export or recognized by leader peptidase I. The differences observed between the different rOms28 proteins, coupled with the meningococcal porin results and studies done with other recombinant OM porins, including the Tromp1 porin protein from *T. pallidum* (11), suggest that subtle changes or additions to recombinant porin proteins may dramatically affect their conformation, thereby changing the localization of these proteins within the cell.

There are two possible explanations for the different single-channel conductances observed for native Oms28 and rOms28. Since the conductance of rOms28 is approximately twofold greater than the native Oms28, it is possible that rOms28 incorporates preferentially as a dimer in the lipid bilayer. Alternatively, the difference in conductance observed between the two forms of Oms28 may reflect an alteration in the conformation of the rOms28 relative to that of native Oms28. By using the equation $\Lambda = \sigma\pi r^2/l$, where Λ is the single-channel conductance in nanosiemens, σ is the specific conductivity determined to be 11.2 nS/nm, r is the radius of the channel formed by the porin, and l is the length of the channel estimated to be 6 nm (19), the diameters of the native Oms28 and rOms28 channels are estimated to be 0.64 and 0.86 nm, respectively. The difference estimated for the internal diameter of these channels suggests that other structures, including surface-exposed epitopes, may also be altered; therefore, the use of rOms28 to simulate epitopes present in native Oms28 may not be possible. Along these lines, we have conducted preliminary experiments to determine if rOms28 present in the *E. coli* OM could serve as an immunogen to protect rabbits against challenge with infectious *B. burgdorferi* B31 (data not shown). rOms28, although capable of eliciting a significant humoral response, did not provide any protection against challenge. If one assumes that Oms28 can function as a protective immunogen, then these results suggest that either rOms28 does not retain a conformational epitope essential for protection or a protective antibody specific for a surface-exposed linear epitope of native Oms28 was not generated by using rOms28 as a vaccinogen.

In this study, we have demonstrated for the first time a functional role for an Oms protein, designated Oms28, in *B. burgdorferi*. Additionally, we have reported the nucleotide sequence of the gene encoding Oms28 and show that overproduced rOms28 is partially targeted to the OM in *E. coli*. These studies represent the first demonstration and molecular characterization of an Oms protein in *B. burgdorferi*. The identification of an Oms protein with demonstrable function should provide a foundation for the further characterization of other Oms proteins, most notably virulent strain-associated Oms proteins, which may be important in *B. burgdorferi* pathogenesis and protective immunity (42).

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