Sequence Analysis and Characterization of a 40-Kilodalton *Borrelia hermsii* Glycerophosphodiester Phosphodiesterase Homolog

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We report the purification, molecular cloning, and characterization of a 40-kDa glycerophosphodiester phosphodiesterase homolog from *Borrelia hermsii*. The 40-kDa protein was solubilized from whole organisms with 0.1% Triton X-100, phase partitioned into the Triton X-114 detergent phase, and purified by fast-performance liquid chromatography (FPLC). The gene encoding the 40-kDa protein was cloned from a *B. hermsii* chromosomal DNA lambda EXlox expression library and identified by using affinity antibodies generated against the purified native protein. The deduced amino acid sequence included a 20-amino-acid signal peptide encoding a putative leader peptidase II cleavage site, indicating that the 40-kDa protein was a lipoprotein. Based on significant homology (31 to 52% identity) of the 40-kDa protein to glycerophosphodiester phosphodiesterases of *Escherichia coli* (GlpQ), *Bacillus subtilis* (GlpQ), and *Haemophilus influenzae* (Hpd; protein D), we have designated this *B. hermsii* 40-kDa lipoprotein a glycerophosphodiester phosphodiesterase (Gpd) homolog, the first *B. hermsii* lipoprotein to have a putative functional assignment. A nonlipidated form of the Gpd homolog was overproduced as a fusion protein in *E. coli* BL21(DE3)(pLysE) and was used to immunize rabbits to generate specific antiserum. Immunoblot analysis with anti-Gpd serum recognized recombinant *H. influenzae* protein D, and conversely, antiserum to *H. influenzae* protein D recognized recombinant *B. hermsii* Gpd (rGpd), indicating antigenic conservation between these proteins. Antiserum to rGpd also identified native Gpd as a constituent of purified outer membrane vesicles prepared from *B. hermsii*. Screening of other pathogenic spirochetes with anti-rGpd serum revealed the presence of antigenically related proteins in *Borrelia burgdorferi*, *Treponema pallidum*, and *Leptospira kirschneri*. Further sequence analysis both upstream and downstream of the Gpd homolog showed additional homologs of glycerol metabolism, including a glycerol-3-phosphate transporter (GlpT), a glycerol-3-phosphate dehydrogenase (GlpD), and a thioredoxin reductase (TrxB).

The pathogenic spirochete *Borrelia hermsii* is one of the etiologic agents of tick-borne relapsing fever. Infection in humans and other vertebrates is commonly characterized by recurring episodes of high fever and bacteremia with intermittent afebrile periods (55). In humans, other clinical manifestations may include pronounced muscle and joint pains, splenomegaly, hepatomegaly, jaundice, and rash, as well as respiratory, cardiovascular, and central nervous system involvement (7). The well-documented relapse phenomenon is associated with the ability of these organisms to undergo multiphasic antigenic variation during the course of disease (56). A major surface-exposed lipoprotein designated the variable membrane protein (Vmp) has been correlated with this antigenic variation (4, 9, 11). This switching from an initial Vmp type to another prior to the establishment of a protective antibody response is thought to enable the escape of these organisms from antibody-mediated opsonization and complement killing.

Although the molecular basis of antigenic variation has been thoroughly described by Barbour and colleagues (4–6, 8, 9, 11, 18, 25, 33, 40, 44–46), relatively little is known about other

aspects of pathogenesis and immunity. *B. hermsii*, like other pathogenic spirochetes, has been shown by freeze fracture electron microscopy to have an extreme low density of outer membrane-spanning proteins (400 particles/mm²) (63), like that for *Treponema pallidum* subsp. *pallidum* (62). Although visualized by freeze fracture electron microscopy, these transmembrane outer membrane proteins have not been identified in *B. hermsii*. Indeed, relatively few subsurface and surface proteins have been identified in *B. hermsii* (2, 10, 32, 39, 48). Since outer membrane proteins have been shown to serve as protective immunogens, we have attempted to identify and characterize these proteins from *B. hermsii* in order to relate ultrastructure to mechanisms of virulence, physiology, and immunity.

In this study, we report on the cloning of the gene encoding a 40-kDa outer membrane-associated *B. hermsii* glycerophosphodiester phosphodiesterase (Gpd) homolog. Amino acid sequence analysis of the cloned *gpd* gene revealed that the Gpd homolog was 50% identical to the *Haemophilus influenzae* glycerophosphodiester phosphodiesterase (Hpd; protein D) (23). The evidence presented also suggests that Gpd, like Hpd, is an outer membrane-associated lipoprotein.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *B. hermsii* HS-1 (ATCC 35209) serotypes 33 (4, 9, 33) and 7 (18) are avirulent and virulent *Ornithodoros* tick isolates, respectively. All *B. hermsii* strains were grown in BSK H medium (Sigma Chemical Co.) (41) supplemented with 6% normal rabbit serum (Sigma) at 34°C. Virulent (21) and avirulent (ATCC 35210; American Type Culture Collection, Rockville, Md.) *Borrelia burgdorferi* B31 isolates were grown in BSK II medium supplemented with 6% normal rabbit serum (3). *T. pallidum* subsp. *pallidum* was isolated from rabbits as previously described (15, 34), and *Leptospira kirschneri* was grown in Bovuminar medium (Intergen) as previously described (52). *Escherichia coli* BL21(DE3)(pLysE) [*F⁻ ompT r_B m_B⁻* (ADE3) pLysE Cm^r] (Novagen, Madison, Wis.) was used as a host strain for infection with lambda EXlox *B. hermsii* DNA phage libraries and expression of plasmid EXlox vectors. *E. coli* BM25.8 [*F⁻ traD36 lac⁺lacZ8M13 proAB/supE thi δ (lac-proAB) limm434(P1) Cm^r Kan^r*] (Novagen) was used as the host strain for in vivo excision and automatic subcloning into plasmid EXlox vectors. All *E. coli* strains were grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics. pES15 is the in vivo-excised pEXlox plasmid containing *gpd* and three open reading frames (ORFs) on a 5-kb *EcoRI* fragment.

TX-100 and TX-114 extraction of *B. hermsii*. *B. hermsii* HS-1 serotype 7 (10^{10} cells) was centrifuged at $5,800 \times g$ for 20 min, washed twice with 150 ml of 50 mM Tris-HCl (pH 8.0), and resuspended in 10 ml of 50 mM Tris-HCl (pH 8.0)–0.1% Triton X-100 (TX-100; Calbiochem, La Jolla, Calif.), and the organisms were rotated overnight at 4°C. Protoplasmic cylinders were pelleted twice at $13,000 \times g$ for 10 min at 4°C. After centrifugation, Triton X-114 (TX-114; Calbiochem) was added to the supernatant to a final concentration of 1%. The solution was phase partitioned, and the detergent phase was washed three times with cold 50 mM Tris-HCl (pH 8.0) as described previously (20, 43, 53). The detergent phase was then diluted to 10 ml with 50 mM Tris-HCl (pH 8.0)–0.1% hydrogenated TX-100 (hTX-100) (Calbiochem Corp., San Diego, Calif.).

FPLC purification of native Gpd. The TX-100/TX-114-extracted proteins (5 ml) were applied to a 1-ml Mono Q 5/5 anion-exchange column (Pharmacia Biotech, Piscataway, N.J.), using the Pharmacia fast-performance liquid chromatography (FPLC) system (51, 54). The column was washed with 50 mM Tris-HCl (pH 8.0)–0.1% hTX-100 until the optical density at 280 nm (OD_{280}) reached baseline. Proteins bound to the column were eluted with a 0 to 600 mM NaCl linear gradient in 50 mM Tris-HCl (pH 8.0)–0.1% hTX-100 and collected in 0.5-ml fractions. The fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and colloidal gold staining. Fractions containing the 40-kDa protein were pooled, dialyzed in 50 mM Tris-HCl (pH 8.0), reappplied to the Mono Q column, and eluted with a linear gradient of 10 to 450 mM NaCl containing 50 mM Tris (pH 8.0) and 0.1% hTX-100. Purified fractions containing the 40-kDa species were precipitated with 10% trichloroacetic acid and further analyzed by two-dimensional (2-D) non-equilibrium (NEPH) gel electrophoresis (NEPHGE) as described below.

2-D NEPHGE. Samples were solubilized in 1× NEPHGE sample buffer and separated in the first dimension at a constant voltage of 400 V for 4 h as previously described (37, 38, 53). Following electrophoresis, the NEPH gels were incubated in 2× SDS-PAGE final sample buffer (FSB) for 15 min, were layered onto a second SDS-polyacrylamide gel, and were separated by electrophoresis. The gel was then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, Mass.) and subjected to Western immunoblot analysis or colloidal gold staining.

SDS-PAGE, colloidal gold staining, and Western immunoblot analysis. Protein samples were precipitated with either 10% trichloroacetic acid or a 10-fold excess of ice-cold acetone. The precipitated samples were pelleted by centrifugation at $16,000 \times g$ for 15 min, washed once in acetone, resuspended in FSB and subjected to SDS-PAGE as previously described (27, 53). The separated proteins were transferred to a PVDF membrane (60) and stained with either 1% amido black or colloidal gold (Aurodyne forte; Amersham Corp., Arlington Heights, Ill.). For Western immunoblot analysis, the membranes were incubated with either serum from a mouse infected with *B. hermsii* (see below) or rabbit anti-Gpd (see below) at a dilution of 1:1,000. For other immunoblots, monoclonal antibodies H4825 to Vmp33 (8) and H10022 to Vmp 21 (11) (kindly provided by Alan Barbour, University of Texas) were used at 1:500 and 1:100 dilutions, respectively. Rabbit serum to *E. coli* OmpA (kindly provided by William Wickner, Dartmouth College) was diluted 1:10,000 for Western blot analysis. Polyclonal rabbit antibody to *H. influenzae* Hpd (kindly provided by Hakan Janson, Lund University, Malmö, Sweden) was used at a 1:1,000 dilution in Western immunoblots. Immobilized immune complexes were detected with either a 1:2,500 dilution of donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase (HRP), sheep anti-mouse immunoglobulin-HRP, or a 1:1,000 dilution of protein A-HRP. Immunoblots were developed by using an enhanced chemiluminescence (ECL) system (Amersham) and exposed to Kodak XAR-5 film (Eastman Kodak Co.).

Protein sequencing. The FPLC-purified 40-kDa protein was separated by SDS-PAGE, immobilized onto Transblot PVDF (Bio-Rad), and submitted to the UCLA Core Sequencing Facility for amino-terminal sequence analysis as previously described (52). Additionally, the FPLC-purified 40-kDa protein was immobilized onto nitrocellulose (Schleicher & Schuell, Keene, N.H.) and processed for internal amino acid sequencing. Edman chemical sequencing (13, 58) was

performed on three selected trypsin-digested peptides, designated T24A, T24B, and T58.

Affinity-purified antibody to the 40-kDa protein. C₃H/HeJ mice were inoculated intraperitoneally with 2×10^7 *B. hermsii* cells in 0.5 ml of BSK II medium and bled at 90 h and week 3 (21a). FPLC fractions containing the 40-kDa protein were separated by 2-D NEPHGE and transferred to a PVDF membrane, and the 40-kDa protein was excised and blocked with 5% milk–0.1% Tween 20–phosphate-buffered saline (PBS) for 2 h. To generate affinity-purified antibodies against the 40-kDa protein, the infection-derived mouse serum was diluted 1:5 in PBS and incubated at 4°C overnight with the PVDF membrane-bound purified 40-kDa protein. The membrane was washed twice in 10 mM Tris (pH 8.0)–0.85% NaCl–0.01% sodium azide, and the affinity-bound antibodies were eluted with 300 µl of 0.1 M glycine (pH 2.2) and neutralized to pH 7.4 with 60 µl of 1 M Tris-HCl (pH 9.0). The affinity-purified antibody was used at a 1:100 dilution in ECL Western immunoblot screening of the *B. hermsii* expression library.

Cloning and sequencing of *gpd*. Chromosomal DNA was isolated from approximately 10^{11} *B. hermsii* serotype 7 passage 10 organisms (14), and linear plasmid and supercoiled plasmid DNAs were isolated from CsCl preparations as previously described (19). *B. hermsii* genomic, linear, and circular plasmid DNA libraries were constructed by using the lambda EXlox vector (Novagen) as follows. *B. hermsii* genomic and linear plasmid DNAs were digested with *ApoI*, and fragments with a size range of 0.5 to 6 kb were cloned into the *EcoRI* site of λ EXlox. Partial digests of supercoiled circular plasmid DNA obtained by using *DraI* and *RsaI*, each in the size range of 0.5 to 4 kb, were ligated to *EcoRI* adapters (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and cloned into *EcoRI* arms of λ EXlox. The ligated DNA was packaged with Gigapack II Gold (Stratagene). *E. coli* BL21(DE3)(pLysE) was infected with λ EXlox bacteriophage containing *B. hermsii* clones and plated as outlined by the manufacturer. Approximately 10,000 plaques were screened, and nitrocellulose filters (Schleicher & Schuell) impregnated with 10 mM isopropylthiogalactopyranoside (IPTG) were placed over the surface of the plates and incubated for 4 h at 37°C. Filters were removed and washed in 0.2% Tween–PBS and blocked in 10% nonfat dried milk (Carnation Co.)–0.1% Tween 20–PBS (T-PBS) for 1 h. Filters were then incubated with the affinity-purified antibody to the 40-kDa *B. hermsii* protein at a 1:100 dilution for 3 h, washed three times in T-PBS for 5 min each, and incubated in sheep anti-mouse HRP-conjugated serum for 1 h. Filters were then washed five times for 5 min each, incubated in ECL reagents, and exposed to film. The positive plaque was purified and was converted to pEXlox plasmid DNA (pES15) by in vivo excision in *E. coli* BM25.8 according to manufacturer's instructions (Novagen). Both strands of insert recombinant DNA were sequenced by the dideoxynucleotide chain termination method by Joan Strange at the University of Montana Molecular Biology Sequencing Facility. The in vivo excised plasmid (pES15) was subjected to restriction enzyme mapping. PCR analysis of the insert was performed as previously described with the T7 gene 10 and SP6 primers (Novagen). Degenerate oligonucleotides to part of internal peptide T24A (PEHTLE) were synthesized and used to screen plasmid pES15 digested with *EcoRI*, *BamHI*, and *PstI* by Southern hybridization as previously described (52).

DNA and protein sequence analysis. DNA sequences were analyzed by the DNA Strider version 1.0 program (31). Homology searches were performed with the BLASTP program from the National Center for Biotechnology Information database (1), and alignment of DNA sequences was performed with the Pileup program from the University of Wisconsin Genetics Computer Group, Inc. (1). The hydrophobicity of the protein was analyzed by the Kyte-Doolittle algorithm (26).

Cellular localization of Gpd in *E. coli*. *E. coli* BL21(DE3)(pLysE) containing pES15 with the entire gene sequence was grown to an OD_{600} of 0.3 and incubated with or without 100 mM IPTG for 1 h and with rifampin (50 mg/ml) for an additional hour. The cells were pelleted, and the proteins were separated by SDS-PAGE and subjected to Western immunoblot analysis using affinity-purified antibodies to the 40-kDa protein. In other studies, *E. coli* BL21(DE3) (pLysE) harboring pES15 was grown to an OD_{600} of 2.1. The 50-ml culture was centrifuged, and the cells were adjusted to a density of 10 OD_{600} equivalents/ml in PBS (pH 7.4) and frozen. The sample was thawed, and the cells were disrupted by passage through a French pressure cell ($\sim 12,000$ lb/in²) three times. Unbroken cells were removed by centrifugation at $3,000 \times g$ for 5 min, and the total membrane was pelleted at $40,000 \times g$ for 30 min at 4°C. The supernatant containing the cytoplasmic and periplasmic proteins was acetone precipitated. The total membrane pellet was resuspended in PBS with 2% TX-100 and extracted for 1 h at 4°C and an additional hour at room temperature to solubilize the inner membrane. The sample was centrifuged at $13,000 \times g$ for 30 min, and the inner membrane supernatant was acetone precipitated. The outer membrane pellet was washed in 1 ml of PBS, recentrifuged, and resuspended in 2× FSB. Each fraction, at a density equivalent to an OD_{600} of 0.2, was subjected to SDS-PAGE and Western immunoblot analysis with anti-Gpd serum or anti-OmpA serum.

Expression and purification of rGpd in *E. coli*. The *gpd* gene lacking its leader sequence was cloned into the pET-17b expression vector (Novagen) by using two PCR primers with restriction enzymes engineered at their ends. The primer PD-N (5'-CGCGGATCCACAGGGCGAAAAATGAGTCAA3'), containing a *BamHI* site (underlined) and the *gpd* initiating methionine codon (in boldface), and primer PD-C (5'-CCGGAATTCCTATGGTTTATTTTGTGAT3'), con-

FIG. 1. Purification of the native 40-kDa protein (Gpd). Shown are 2-D NEPHGE of 5×10^8 whole *B. hermsii* HS-1 cells stained with colloidal gold (A), colloidal gold-stained 0.1% TX-100-extracted proteins from 5×10^8 organisms which were subsequently phase partitioned into the TX-114 detergent phase (B), colloidal gold stain of the 40-kDa protein purified by Mono Q anion-exchange FPLC (C) and Western immunoblot analysis of FPLC-purified Gpd (40 kDa), using serum from mice infected with *B. hermsii* HS-1 (D). The higher-molecular-mass substances (~ 67 kDa) in panel C are unrelated gel contaminants normally observed during sensitive staining techniques. The acidic and basic ends of the first dimension are indicated at the top, and molecular mass markers of the second dimension are indicated at the left in kilodaltons.

taining an *Eco*RI site (underlined) and the stop codon (in boldface; on the opposite strand), were used to amplify *gpd*, using 10 ng of pES15 as the template. The resulting 961-bp PCR product was excised from an agarose gel and purified by using Gene Clean II (Bio 101, La Jolla, Calif.). The 961-bp PCR product and plasmid pET-17b (Novagen) were digested with *Eco*RI and *Bam*HI, ligated together with T4 ligase, and transformed into *E. coli* BL21(DE3)(pLysE). The positive clones were grown in LB broth in the presence of ampicillin (100 μ g/ml) to an OD_{600} of 0.6 and induced for 1 h with 1 mM IPTG and for an additional hour with rifampin (150 μ g/ml). The 35-ml culture was centrifuged at $3,000 \times g$ for 15 min, and the cells were resuspended in 5 ml of 50 mM Tris (pH 8.0)–0.1% TX-100 and sonicated three times for 15 s each. The disrupted cells were centrifuged for 20 min at $12,000 \times g$ at 4°C , and the proteins in the supernatant were separated by SDS-PAGE. A 40-kDa recombinant Gpd (rGpd) band was excised from the gel, electroeluted overnight at 35 V, and dialyzed against PBS. Purified rGpd was then used as antigen for Western immunoblots or for immunization of rabbits to generate antiserum specific for Gpd.

Immunization of rabbits with rGpd. Two New Zealand rabbits were immunized with 150 μ g of purified rGpd in complete Freund's adjuvant as previously described (13). The rabbits were boosted with 150 μ g of rGpd in incomplete Freund's adjuvant at 1, 2, and 3 months following the primary immunization and bled 2 weeks after each boost.

Cross-reactivity of antibodies to *H. influenzae* Hpd and Gpd to rGpd and rHpd. Approximately 1 μ g of rGpd and rHpd (kindly provided by Hakan Janson) were separated by SDS-PAGE, transferred to PVDF, and subjected to immunoblot analysis with anti-*B. hermsii* rGpd serum or anti-*H. influenzae* Hpd serum at a 1:1,000 dilution.

Detection of Gpd in *B. hermsii* OMV. *B. hermsii* outer membrane vesicles (OMV) were generated by the method previously described for the isolation of the *B. burgdorferi* OMV (15, 50, 53). Briefly, 10^{11} *B. hermsii* cells were washed twice in PBS supplemented with 0.1% bovine serum albumin (BSA) and centrifuged at $6,000 \times g$ for 20 min. The pelleted cells were resuspended in 90 ml of ice-cold 25 mM citrate buffer (pH 3.2) containing 0.1% BSA. The suspension was vigorously shaken for 2 h at room temperature and vortexed for 1 min every 15 min. The suspension was centrifuged at $20,000 \times g$ for 30 min, and the pelleted protoplasmic cylinders and OMV were resuspended in 12 ml of 25 mM citrate buffer (pH 3.2) with 0.1% BSA. The samples (6 ml) were layered onto discontinuous sucrose gradients consisting of 25, 42, and 56% (wt/wt) sucrose as previously described (53) and centrifuged at $100,000 \times g$ for 16 h at 4°C . The OMV band (upper band) and protoplasmic cylinder band (lower band) were removed by needle aspiration and diluted fivefold with PBS. The protoplasmic cylinders were pelleted at $15,000 \times g$ for 20 min at 4°C , resuspended in 1 ml of PBS, and frozen. The outer membrane vesicle was pelleted at $141,000 \times g$,

resuspended in 1 ml of 25 mM citrate (pH 3.2), applied to a 10 to 42% (wt/wt) continuous sucrose gradient, and centrifuged at $100,000 \times g$ for 16 h at 4°C . The OMV band was needle aspirated as described above, diluted sevenfold with PBS, and pelleted at $141,000 \times g$. The OMV preparation was resuspended in 200 μ l of PBS and frozen. The outer membrane was visualized via electron microscopy as previously described (15, 50, 53) and shown to be free of inner membrane contamination by the absence of β -NADH oxidase activity as previously described (50, 53).

Identification of Gpd homologs in other spirochetes. In an attempt to identify Gpd homologs in other spirochetes, whole-cell lysates from 10^8 *B. hermsii* HS-1 serotype 7, avirulent *B. burgdorferi* B31 (ATCC 35210), *B. burgdorferi* B31 passage 6 (virulent isolate), *T. pallidum* subsp. *pallidum*, and *L. kirschneri* cells were separated by SDS-PAGE. Proteins were transferred to PVDF and subjected to immunoblot analysis using anti-rGpd serum at a 1:1,000 dilution.

Nucleotide sequence accession number. The DNA sequence for *gpd* reported here has been deposited in the GenBank database under accession number U65980.

RESULTS

Purification of Gpd. To enrich for membrane proteins of *B. hermsii*, whole cells were solubilized with 0.1% TX-100. The solubilization of the *B. hermsii* outer membrane was observed by dark-field microscopy as a decrease in the diameter of the organisms (20). To select for hydrophobic proteins, we further treated the TX-100 supernatant with 1% TX-114 followed by phase partitioning into hydrophobic detergent and hydrophilic aqueous phases. The detergent phase contained approximately 25 hydrophobic proteins, with Vmp7 being the most prominent protein observed in a colloidal gold stain of a 2-D NEPH gel (Fig. 1B). The hydrophobic proteins were further separated over a Mono Q anion-exchange FPLC column in which Vmp7 did not bind to the column and was observed in the flow-through fractions (data not shown). Similar results were observed with the OspA and OspB lipoproteins of *B. burgdorferi* (54). A 40-kDa protein was FPLC purified (Fig. 1C) and was recognized by serum generated in mice infected with *B. hermsii*.

sii, indicating that it was an infection-derived antigen (Fig. 1D). The purified protein did not cross-react with monoclonal antibodies to Vmp33 and Vmp21 (data not shown).

Cloning and sequence analysis of *gpd*. Affinity-purified antibody generated against the 40-kDa FPLC-purified protein was used to screen 10,000 plaques from the *B. hermsii* chromosomal, linear, and supercoiled plasmid DNA λ EXlox expression libraries. One positively reacting plaque was identified from the chromosomal library, plaque purified, and in vivo excised to form plasmid pES15. Restriction digestion and PCR analysis of the insert demonstrated that it contained a 5-kb fragment (data not shown). Sequence analysis of the insert revealed a cluster of four ORFs encoding homologs of glycerol-3-phosphate (G3P) metabolism (Fig. 2A). ORF1 encoded a G3P transporter protein homolog (GlpT), ORF2 encoded a glycerophosphodiester phosphodiesterase homolog (Gpd), ORF3 encoded a G3P dehydrogenase homolog (GlpD), and ORF4 encoded a thioredoxin reductase homolog (TrxB).

Although attempts to obtain an N-terminal amino acid sequence with 400 pmol of the 40-kDa protein yielded no sequence, Edman degradation sequencing of three selected trypsin-digested peptides, designated T24A, T24B, and T58, yielded the sequences GASGYLPEHTLEAK, ITDLTSLA GK, and YSDGIGPQIIIEG, respectively. The three peptides were generated immediately after lysine or arginine residues, consistent with trypsin digestion. All three peptide sequences were found in the deduced amino acid sequence of ORF2, confirming that the DNA sequence obtained was that encoding the purified 40-kDa protein (Fig. 2B). In addition, Southern blot analysis of pES15 by using a degenerate probe to one of the internal peptides (T24A) further confirmed that the cloned DNA encoded the 40-kDa protein. The entire ORF2 consisted of 1,020 bp and encoded a 340-amino-acid protein with a predicted mass of 38,900 Da. *E. coli*-like -35 (TTAA TA) and -10 (TATAAT) σ^{70} promoter regions and a putative ribosome binding site (AGGT) were identified upstream from the ATG start codon. The deduced amino acid sequence predicted that the protein contained a 20-amino-acid signal peptide typical of lipoproteins (61) with amino-terminal basic residues (positions 2, 5, and 7) and a hydrophobic core (positions 9 to 20), ending in a putative Leu-Thr-Ile-Ser-Cys leader peptidase II cleavage site, suggesting that the encoded protein is acylated (Fig. 2B). Kyte-Doolittle hydropathy plot analysis of the entire amino acid sequence showed that Gpd is a hydrophilic protein, lacking any hydrophobic regions with the exception of the putative signal peptide (Fig. 2C). The processed mature protein of 320 residues would have a calculated mass of 36,773 Da and a calculated pI of 5.7. The predicted mass of the protein differs from the apparent 40-kDa mass observed by SDS-PAGE, which may be due to the lipid modification of the processed mature protein. The calculated pI is consistent with the migration of Gpd at the more acidic end of NEPH gels (Fig. 1C). No rho-independent terminator sequence was found after the stop codon.

Analysis of the deduced amino acid sequence revealed significant homology with the *E. coli* (72% similarity) and *B. subtilis* (55% similarity) periplasmic glycerophosphodiester phosphodiesterase (GlpQ) (36, 59) as well as the *H. influenzae* membrane-bound glycerophosphodiester phosphodiesterase lipoprotein (68% similarity) (23, 35) (Fig. 3 and Table 1). Pileup analysis of the homologs indicated several highly conserved regions. We have therefore designated the 40-kDa protein the glycerophosphodiester phosphodiesterase homolog (Gpd) of *B. hermsii*.

Cellular location of recombinant Gpd in *E. coli*. Preliminary induction studies with IPTG and rifampin showed no increase

FIG. 2. Genetic organization and nucleotide sequence of *gpd*. (A) Partial restriction map of the 5-kb *Apol* fragment containing four ORFs encoding the G3P transporter (*glpT*), glycerophosphodiester phosphodiesterase (*gpd*), G3P dehydrogenase (*glpD*), and thioredoxin reductase (*trxB*). (B) Nucleotide sequence and deduced amino acid sequence of the *B. hermsii gpd* gene. Putative -35 and -10 promoter regions are indicated. The putative ribosome binding site is indicated by the double-underlined sequence. The putative signal peptidase II cleavage site is indicated by a vertical arrow. Sequences obtained from the tryptic peptides are indicated by the single-underlined residues. (C) Kyte-Doolittle hydropathy plot of the derived amino acid sequence of Gpd. The amino-terminal peak corresponds to the hydrophobic signal peptide.

in Gpd expression compared to uninduced *E. coli* BL21(DE3) (pLysE) harboring pES15, suggesting that the T7 promoter, which is 946 bp upstream of Gpd, does not have an effect on the expression of Gpd. Standard fractionation of uninduced cells into soluble, inner membrane, and outer membrane fractions showed that the majority of Gpd was localized to the inner membrane of *E. coli*, with a small amount present in the

1	50
Ecoli	MKLTLLKNSM AIMMSTIV.. MGSSAMAAD.SNEKI VIAHRGASGY
Hinfl	..MKLKTLLAL SLLAAGVL.. AGCSSHSSNM ANTMKMSDKI IIAHRGASGY
Bherm	.MKSIFAKSL .IFIINIF.. LTISCCQGEKM SONQKSP..L VIAHRGASGY
Bsubt	.MRKNRILAL FVLSLGLLSF MVTPTSAAASK GNLLSPDRIL TVAHRGASGY
51	100
Ecoli	LPEHTLPKMA MAYAQGADYL EQDLVMTKDD NLVVLHDHYL DRVTDVADRF
Hinfl	LPEHTLESKA LAFAQHSYDL EQDLAMTKDG RLVIHHDHFL DGLTDVAKKF
Bherm	LPEHTLEAKA YAYALGADYL EQDIVLTKDN IPVIMHDPPEL DTTTNVAKLF
Bsubt	VPEHTLTSYE TAQRMKADF I ELDLQMTKDG KLIVMHDEKL DRTT.....
101	150
Ecoli	PDRARKDGRY YAIIDFTLDEI KSLKFTGEGD IENGKKVQTY PGRFFPMGKSD
Hinfl	PYRHRKDGRI YVIDFTLKEI QSLMENTENFE TKDGKQAVY PNRFLPWKSH
Bherm	PERARENGRY YSVDFTLDEL KSLSLSERFD ..PASRNFTY PNRFLNEHD
Bsubt	NGMG WKDHTLADI KKLDAGSWFNEAY PEKAKPQVVG
151	200
Ecoli	FRVHTFEEEI EFVQGLNHST GKNIGIYPEI KAP.WFHHQE GKDIAAKTLE
Hinfl	FRIHTFEDEI EFIQGLEKST GKKVGIYPEI KAP.WFHHQN GKDIATETLK
Bherm	FKIPTLEEEI QFIQGLNKST GRNVGIYPEI KKP.LWHKQQ GKDISKIVIE
Bsubt	LKVPTLEEVL DRF..... GKHNYYIET KSPDTPYGM EKLIAS..LQ
201	250
Ecoli	VLKKYGYTGK DDKVYLQCFD ADELKRIKNE LEPKMGMEIN LVQLIAYTDW
Hinfl	VLKKYGYDKK TDMVYLQTFD FNELKRIKTE LLPQMGMDLK LVQLIAYTDW
Bherm	ILNKYGYKSK EDKIYLTQFD FDELKRIREE L....GYQK LIMLVGENDW
Bsubt	KHKLLGKHSK PGQVITQSFS RESLVKVH.. ...QLQPNLP TVQLEAKQM
251	300
Ecoli	NETQKQPDG SWVNNYNDWM FKPGAMQVA EYADGIGEDY HMLIE.ETSQ
Hinfl	KETQEKDPKG YWVNNYNDWM FKPGAMAEV KYADGVGPWG YMLVNKESK
Bherm	DEAPT.....DYEYI KSEEGMAEVA KYSDGIGPWI PQIIEG...
Bsubt	AS..... MTDAALEEIK TYAVGAGPDY KALNQEN...
301	350
Ecoli	PGNIKLTGMV QDAQNKLIV HPYTVRSKDL PEYTPDVNQL YDALYNKAGV
Hinfl	PDNIVYTPLV KELAQYNVEV HPYTVRKDAL PEFFTDVNQM YDALLNKSGA
Bherm	KITDLT SLAHKHNMEV HPYTFRIDAL PSYVKDADEL LDLLFNKAKV
Bsubt	V RMIRSHGLL HPYTVNNEA.DMHRL LDW.....GV
351	372
Ecoli	NGLFTDFPDK AVKFLNKE.. ..
Hinfl	TGVFTDFPDT GVEFLKGIK. ..
Bherm	DGIFTDFDT VVNFITKIKP ..
Bsubt	TGVFTNYPD. ...LFHKVKK GY

FIG. 3. Amino acid similarities between *B. hermsii* Gpd (Bherm) and glycerophosphodiester phosphodiesterases of *E. coli* (Ecoli), *H. influenzae* (Hinfl), and *B. subtilis* (Bsubt). Dots represent sequence gaps introduced in order to optimize alignment.

TABLE 1. Homology of the *B. hermsii* glycerophosphodiester phosphodiesterase homolog to other species

Species	Protein	Gene	Size (kDa)	% Similarity	% Identity
<i>E. coli</i>	Glycerophosphodiester phosphodiesterase	<i>glpQ</i>	38	71.69	51.81
<i>H. influenzae</i>	Protein D; glycerophosphodiester phosphodiesterase	<i>hpd</i>	42	67.86	50.29
<i>B. subtilis</i>	Glycerophosphodiester phosphodiesterase	<i>glpQ</i>	32	55.16	31.32

soluble fraction (Fig. 4). The detection of Gpd was specific, since antiserum to Gpd did not cross-react with the *E. coli* GlpQ (data not shown). No detectable levels of Gpd were found in the *E. coli* outer membrane. Antiserum to the *E. coli* outer membrane protein A (OmpA) reacted with a 35-kDa protein in the outer membrane fraction but did not react with the inner membrane fraction, demonstrating the purity of the inner membrane fraction (data not shown). The membrane localization of recombinant Gpd is similar to that of recombinant *H. influenzae* Hpd (23, 35) and differs from the exclusive periplasmic location of the *E. coli* GlpQ (28, 30). It has been found, however, that nonlipidated rHpd (due to a mutation in the cysteine residue) fractionates exclusively to the periplasm

FIG. 4. Cellular location of lipidated Gpd expressed in *E. coli*. Western immunoblotting was performed with affinity-purified antibody to Gpd. French press total-cell lysate (lane T), cytoplasmic and periplasmic soluble (lane S), inner membrane (lane I), and outer membrane (lane O) fractions of *E. coli* BL21(DE3)(pLysE) harboring pES15. Each lane contains proteins derived from 2×10^8 organisms. Markers are indicated at the left in kilodaltons.

of *E. coli* (22). Likewise, acylation of rGpd is most likely responsible for its membrane localization.

Expression and purification of rGpd. To produce large amounts of soluble rGpd, *gpd* lacking its leader sequence was cloned into pET-17b in the form of a T7 gene 10 fusion protein, which when overproduced would yield a protein with an additional 2.3 kDa relative to native Gpd. Induction with IPTG and rifampin resulted in the overproduction of a 42-kDa fusion protein. Sonication of the cells and subsequent centrifugation resulted in the release of rGpd into the sonicate supernatant (Fig. 5, lane 5). Separation of the supernatant by SDS-PAGE followed by gel excision and electroelution of the overproduced 42-kDa protein resulted in the purification of rGpd (Fig. 5, lane 6).

Cross-reactive antisera to rGpd and rHpd. Based on the sequence similarities and the putative lipoprotein nature of Gpd and the *H. influenzae* Hpd, we attempted to determine if these proteins shared common epitopes. Antiserum to rHpd and antiserum to rGpd were used in Western immunoblots to probe rGpd and rHpd. Antiserum to rHpd recognized both recombinant and native Gpd (Fig. 6A). Although rGpd migrated more slowly due to the 2.3-kDa fusion protein, native Gpd from *B. hermsii* had a molecular weight similar to that of the *H. influenzae* Hpd. Conversely, antiserum to rGpd recognized rHpd (Fig. 6B). Their lipoprotein nature and their cross-reactive epitopes suggested that Gpd was more similar to the *H. influenzae* Hpd than to the periplasmic *E. coli* GlpQ.

Localization of Gpd in *B. hermsii*. In an attempt to address whether Gpd is outer membrane associated, we isolated *B. hermsii* OMV by using the protocol reported for the isolation of the *B. burgdorferi* and *T. pallidum* OMV (15, 50, 53). The outer membrane nature of the *B. burgdorferi* and *T. pallidum* outer membrane preparations was demonstrated previously by visualization of membrane vesicles by using electron microscopy and by the detection of porin proteins with the planar lipid bilayer assay (15, 50, 53). The OMV preparations were also shown to be free of inner membrane contamination by the absence of penicillin binding proteins in the case of *T. pallidum* (15) and the absence of NADH oxidase activity in *B. burgdorferi* (53). Similarly, for *B. hermsii*, the outer membrane nature of this preparation was shown by the visualization of membrane vesicles by using electron microscopy and by the demonstration of porin activity by using the planar lipid bilayer assay (50). In addition, antiserum to a *B. burgdorferi* surface-exposed porin protein, p66/Oms66 (17, 42), was also found to react with a 66-kDa outer membrane-associated protein of *B.*

FIG. 5. Overproduction and purification of rGpd. The sequence encoding the mature, nonacylated Gpd was cloned into pET-17b (Novagen) and expressed in *E. coli* BL21(DE3)(pLysE). Shown is a Coomassie blue-stained gel of uninduced (lane 1) and induced (lane 2) *E. coli* BL21(DE3)(pLysE) containing pET-17b alone, uninduced (lane 3) and induced (lane 4) BL21(DE3)(pLysE) containing recombinant *gpd*-pET-17b, sonicated supernatant of induced *E. coli* BL21(DE3)(pLysE) containing recombinant *gpd*-pET-17b (lane 5), and purified gel-excised rGpd (lane 6). Markers are indicated at the left in kilodaltons.

hermsii (50). To determine if Gpd was present in the outer membrane, antiserum to rGpd was used to probe an immunoblot containing a protein lysate of *B. hermsii* OMV. As shown in Fig. 7, lane O, rGpd antiserum specifically recognized a 40-kDa protein, indicating that the 40-kDa protein that we had originally observed in OMV of *B. hermsii* was indeed Gpd.

Solubilization of *B. hermsii* whole cells with 0.1% TX-114 resulted in the partial release of Gpd into the detergent phase, while the majority of Gpd remained with the protoplasmic cylinders (Fig. 7). Similar results were observed with the *H. influenzae* Hpd lipoprotein (22). The release of only small amounts of Gpd under these conditions may indicate that only outer membrane-associated Gpd was solubilized.

Presence of Gpd in other pathogenic spirochetes. To determine whether proteins antigenically related to Gpd were present in other pathogenic spirochetes, Western immunoblot analysis with 10^8 equivalents of organisms was performed with rGpd antiserum. A 40-kDa reactive species was observed in *B. burgdorferi* and *T. pallidum*, while a faster-migrating 35-kDa

FIG. 7. Presence of Gpd in *B. hermsii* outer membrane vesicles and 0.1% TX-114 detergent phase. Western immunoblot analysis using rGpd antiserum against 10^8 equivalents of whole *B. hermsii* (lane W) or *B. hermsii* separated into TX-114 protoplasmic cylinders (lane P), detergent (lane D), and aqueous (lane A) phases. Lane O, *B. hermsii* outer membrane (3.3×10^9 equivalents). Markers are indicated at the left in kilodaltons.

species was observed in *L. kirschneri* (Fig. 8). These results indicate that there is considerable antigenic conservation of Gpd among the pathogenic spirochetes.

DISCUSSION

Outer membrane proteins of pathogenic bacteria are known to play key roles in pathogenesis and host immunity. In this regard, we attempted to identify proteins associated with the outer membrane of *B. hermsii* in an effort to further understand the molecular basis of relapsing fever and the immunity which develops during infection. In this study, we report the isolation of a 40-kDa glycerophosphodiester phosphodiesterase homolog from *B. hermsii* HS-1. This 40-kDa protein was first observed as a rare constituent in outer membrane vesicle preparations of *B. hermsii* and again as a copurifying protein in porin active fractions from detergent-solubilized *B. hermsii* (50). These preliminary results led us to further investigate this protein.

Because of the low amounts of recovery with the outer membrane protocol, we attempted to increase the recovery of this 40-kDa protein by applying several different techniques.

FIG. 6. Cross-reactivities of anti-Hpd antibody with rGpd and of anti-Gpd antibody with rHpd. (A) Western immunoblot analysis using Hpd antiserum. Lane 1, rGpd (1 µg); lane 2, *B. hermsii* HS-1 (6×10^6); lane 3, 1 µg of rHpd. (B) Western immunoblot analysis using rGpd antiserum. Lane 1, rGpd; lane 2, rHpd. Markers are indicated at the left in kilodaltons.

FIG. 8. Cross-reactivities of the 40-kDa affinity-purified antibody with other spirochetes. Western immunoblot analysis using rGpd antiserum against *B. hermsii* HS-1 serotype 7 (lane Bh), avirulent (lane BbA) and virulent (lane BbV) *B. burgdorferi* B31, *T. pallidum* (lane Tp), and *L. kirschneri* (lane Lk). Each lane contains protein lysates of 10^8 organisms. Markers are indicated at the left in kilodaltons.

FIG. 9. Glycerol metabolism in *E. coli*. Glycerol, G3P, and glycerophosphodiester (G3POR) enter the periplasm, where G3POR is converted into G3P by G3POR phosphodiesterase (GlpQ). G3P is then transported into the cytoplasm by the inner membrane G3P transport protein (GlpT). G3P can either be used in phospholipid synthesis or be converted into dihydroxyacetone phosphate by G3P dehydrogenase (GlpD), where it is then utilized in the glycolytic pathway.

Solubilization of whole organisms with low concentrations of the detergent TX-100 (0.1% TX-100) allowed for the selective solubilization of the outer membrane while minimizing the solubilization of inner membrane anchored lipoproteins. We used the phase-partitioning characteristics of TX-114 in conjunction with the TX-100 solubilization to further enrich for hydrophobic membrane proteins. The additional use of FPLC allowed for the subsequent purification of the 40-kDa protein away from Vmp and resulted in a homogeneous population of purified 40-kDa protein.

Affinity antibodies generated from mouse infection-derived serum to the FPLC-purified Gpd homolog were used to isolate the *gpd* gene from an expression library made from the *B. hermsii* chromosome. This result indicated that the Gpd homolog was expressed during infection and was recognized by the host immune response. The isolated clone which had a 5-kb insert contained a total of four ORFs which encoded proteins involved in G3P metabolism. Based on homology, ORF1 encoded a G3P transporter homolog (*glpT*), ORF2 encoded the glycerophosphodiester phosphodiesterase homolog (*gpd*), ORF3 encoded a G3P dehydrogenase homolog (*glpD*), and ORF4 encoded a thioredoxin reductase homolog (*trxB*). In bacterial metabolism, the glycerol moiety of phospholipid and triglyceride breakdown products are utilized in the form of G3P, glycerol, or glycerophosphodiesters (Fig. 9). In *E. coli*, glycerophosphodiesters in the periplasmic space are hydrolyzed to G3P by periplasmic glycerophosphodiester phosphodiesterases (GlpQ) (28, 30). G3P is then actively transported into the cytoplasm by the transmembrane inner membrane protein, G3P transporter protein, or permease (GlpT) (29), where it is utilized in lipid biosynthesis or further processed by aerobic G3P dehydrogenase (GlpD) into dihydroxyacetone phosphate and used in the glycolytic pathway. The *glpT* and *glpQ* genes of *E. coli* and *Bacillus subtilis* and the *glpQ* gene of *H. influenzae* (Hpd) have been cloned and shown to be highly conserved (23, 36, 59).

In *E. coli*, *glpT* and *glpQ* are located on an operon (29).

However, the presence of a putative promoter sequence upstream of the *B. hermsii* *gpd* and the absence of a termination stem-loop structure downstream of *gpd* suggest that in *B. hermsii*, the GlpT and Gpd homologs may not be arranged in an operon. Studies are currently in progress to determine the transcriptional organization of these genes.

Several lines of evidence indicate that the *B. hermsii* Gpd homolog is a lipoprotein. Native Gpd partitioned into the TX-114 hydrophobic detergent phase, suggesting that the protein has hydrophobic properties presumably due to an acyl modification. Attempts to obtain an amino-terminal amino acid sequence of native Gpd with as much as 400 pmol of purified material were unsuccessful, implying a blocked amino terminus by fatty acid modification. Analysis of the deduced amino acid sequence revealed a signal peptide followed by a Leu-Thr-Ile-Ser-Cys signal peptidase II cleavage site, indicative of a lipoprotein. Since the only hydrophobic domain of Gpd corresponded to the N-terminal signal peptide, the presence of Gpd in the TX-114 detergent phase must be due to its amphiphilic nature following lipid modification of the cysteine residue. This conclusion is supported by the evidence that nonlipidated Gpd is soluble in *E. coli* whereas fractionation of lipidated rGpd resulted in its localization to the *E. coli* inner membrane (Fig. 4 and 5). Similar results with nonlipidated and lipidated forms of the *H. influenzae* Hpd lipoprotein have been observed (22). The fractionation of *B. hermsii* Gpd to compartments other than the periplasm indicates that it differs from the *E. coli* periplasmic GlpQ.

A slight discrepancy was noted between the apparent molecular mass of native Gpd (40 kDa) and its deduced molecular mass (36.7 kDa). Other lipoproteins have also been observed to have different mobilities, which may be accounted for by the covalent addition of fatty acid to the protein (52, 57). In addition, the higher content of acidic amino acids (53 of 340 = 15%) noted for the Gpd homolog may have resulted in decreased SDS binding, which could alter or slow the electrophoretic mobility of the protein.

Homology alignment of glycerophosphodiester phosphodiesterase amino acid sequences from *E. coli* (GlpQ), *B. subtilis* (GlpQ), and *H. influenzae* (Hpd) demonstrated that the *B. hermsii* Gpd homolog was highly conserved among these phylogenetically divergent bacteria (31 to 52% identity) (Fig. 3 and Table 1). Gpd was found to be more similar to the gram-negative *E. coli* GlpQ (52% identity) and *H. influenzae* Hpd (50% identity) than to *B. subtilis* GlpQ (31% identity). The *H. influenzae* protein D (Hpd) was of particular interest due to similarities with the *B. hermsii* Gpd homolog. Hpd was demonstrated to be a membrane-anchored lipoprotein (22) with glycerophosphodiester phosphodiesterase activity (35), differing from the exclusive periplasmic location of the GlpQ homolog of *E. coli* (28). In addition, Hpd has been shown to be surface exposed, with some binding activity to immunoglobulin D myeloma 4490, but it is not an universal immunoglobulin D binding protein (47, 49). Interestingly, Hpd has been reported to be a virulence factor, since knockout mutants are 100-fold less virulent in animal models (24). These similarities were further extended by our data which showed that anti-Hpd serum cross-reacts with rGpd (Fig. 6A). Furthermore, we found that rGpd antiserum recognized Hpd and other spirochetal Gpd homologs (Fig. 6) but did not cross-react with GlpQ of *E. coli* (data not shown). This finding suggests that although these proteins are highly conserved on the amino acid level, there are differences in the conservation of epitopes and potentially in the function of these proteins. The glycerophosphodiester phosphodiesterase activity of Gpd is currently being analyzed. Of further interest was the finding that antiserum to rGpd specifically detected native Gpd in outer membrane vesicles prepared from *B. hermsii*. The exact location of Gpd in the outer membrane remains to be determined.

With respect to its lipoprotein nature and its homology with a periplasmic *E. coli* homolog, the *B. hermsii* Gpd is similar to the *T. pallidum* 38-kDa lipoprotein which is homologous to the periplasmic *E. coli* MglB glucose/galactose binding protein (12). Recent evidence suggesting that gram-positive bacteria possess high-affinity binding lipoproteins analogous to the periplasmic transporters of gram-negative bacteria has prompted researchers to hypothesize that the membrane structure of spirochetes may be similar in some instances to that of gram-positive bacteria (12). Further studies are required to elucidate the membrane structure of these spirochetes. The similarities of Gpd with the surface-exposed Hpd, in addition to the outer membrane association of Gpd, raise the question as to whether Gpd may also be surface exposed. It is interesting that Gpd may belong to a growing number of spirochetal lipoproteins that are found in both the inner and outer membranes rather than exclusively in one membrane like that of enteric lipoproteins (15, 53). These lipoproteins include the 45- and 17-kDa species of *T. pallidum* (15), the 41-kDa surface-exposed LipL41 of *L. kirschneri* (52), and OspA, -B, and -D of virulent *B. burgdorferi* B31 (16, 53). The significance of the dual-membrane location of these lipoproteins remains to be elucidated.

The identification of this Gpd lipoprotein homolog, present in part in the outer membrane of *B. hermsii*, certainly raises the issue of whether this molecule contributes to pathogenesis and host immunity during relapsing fever infection. The similarities between the conserved *B. hermsii* Gpd and *H. influenzae* protein D makes it tempting to speculate whether Gpd, like Hpd, may be a virulence factor or contribute to the development of host immunity. To this end, we are currently testing whether immunization with rGpd protects mice from challenge infection with virulent *B. hermsii*.

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