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Identification of conserved antigens for early serodiagnosis of relapsing fever *Borrelia*

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Borrelia hermsii is a blood-borne pathogen transmitted by the argasid tick *Ornithodoros hermsi*. Since spirochaete clearance in mice is associated with an IgM-mediated response, an immunoproteomic analysis was used to identify proteins reactive with IgM. We report that IgM from both mice and human patients infected with *B. hermsii* not only reacted with the previously identified variable membrane proteins but also identified candidate antigens including heat-shock proteins, an adhesin protein, ABC transporter proteins, flagellar proteins, housekeeping proteins, an immune evasion protein, and proteins with unknown function. Furthermore, IgM reactivity to recombinant glycerophosphodiester phosphodiesterase was detected during early spirochaete infection and prior to a detectable IgG response. Lastly, a conserved hypothetical protein was produced in *Escherichia coli* and tested with immune serum against *B. hermsii* and *Borrelia recurrentis*. These results identify a much larger set of immunoreactive proteins, and could help in the early serodiagnosis of this tick-borne infection.

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INTRODUCTION

Relapsing fever spirochaetes in the genus *Borrelia* are blood-borne pathogens distributed throughout much of the world (Barbour & Hayes, 1986; Felsenfeld, 1971; Southern & Sanford, 1969). The spirochaetes are transmitted by either ticks or the human body louse, and other than *Borrelia duttonii* and *Borrelia recurrentis*, which are transmitted between humans (Burgdorfer, 1951; Buxton, 1946), the most common vertebrate hosts for the spirochaetes are small rodents (Dworkin *et al.*, 2002; Schwan & Piesman, 2002). During mammalian infection, a family of immunogenic variable membrane proteins undergo antigenic variation, allowing for immune evasion, recurrent spirochaetaemia, and persistence in the blood (Barbour, 1990; Barbour *et al.*, 2006; Dai *et al.*, 2006; Hayes *et al.*, 1988; Restrepo *et al.*, 1992; Stoenner *et al.*, 1982). Interestingly, each spirochaetaemic episode is rapidly cleared from the blood by a robust T-cell-independent response involving expansion of IgM-secreting B1b and marginal zone B cells (Alugupalli *et al.*, 2003a, b, 2004; Alugupalli, 2008; Barbour, 1990; Barbour & Bundoc, 2001; Belperron *et al.*, 2005; Newman & Johnson, 1984; Stoenner *et al.*, 1982).

Relapsing fever *Borrelia* can reach upwards of 10^7 spirochaetes per ml of infected blood (Bryceson *et al.*, 1970; Coffey & Eveland, 1967), and can be detected by microscopy. However, detection by microscopy lacks sensitivity between spirochaetaemic episodes. Glycerophosphodiester phosphodiesterase (GlpQ) has been used as a diagnostic antigen for infection with relapsing fever spirochaetes in North America and Africa (Nordstrand *et al.*, 2007; Porcella *et al.*, 2000; Schwan *et al.*, 1996). However, in one report 79 % of Ethiopian patients infected with *B. recurrentis* did not have detectable IgG responses to recombinant GlpQ (rGlpQ) during early spirochaete infection as determined by an ELISA (Porcella *et al.*, 2000).

With variable membrane proteins facilitating immune evasion, and the robust IgM-mediated response during early infection, understanding the broader antigenic

Abbreviations: GlpQ, glycerophosphodiester phosphodiesterase; rGlpQ, recombinant glycerophosphodiester phosphodiesterase.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences for the genes located on *B. hermsii* plasmids, and whose sequences have not previously been deposited in the GenBank database, are FJ446702 (*oppAIV*) and FJ446703 (hypothetical protein).

repertoire against conserved proteins is important. However, little is known about proteins reactive with IgM in relapsing fever spirochaetes (Anda *et al.*, 1996; Colombo & Alugupalli, 2008; Connolly *et al.*, 2004). In this study, protein candidates reactive with IgM were identified using serum from mice and human patients infected with *Borrelia hermsii*. The amino acid identity of *Borrelia* orthologues was compared to *B. hermsii*, and early IgM responses to rGlpQ were determined using serum samples from Ethiopian patients that had previously tested negative to rGlpQ during early spirochaete infection (Porcella *et al.*, 2000). Furthermore, a hypothetical protein from *B. hermsii* was produced and the immunogenicity of this recombinant protein was tested as a possible diagnostic antigen.

METHODS

Animal inoculation and immune sera collection. Low-passage *B. hermsii* DAH isolate was grown in BSK medium containing 12 % rabbit serum (Barbour, 1984; Battisti *et al.*, 2008) to 1.0×10^8 spirochaetes ml^{-1}, and used to needle inoculate three mice intraperitoneally with 1.0×10^5 spirochaetes per mouse. Prior to, and for the first 4 days and the seventh day after inoculation, blood was obtained, and the spirochaetaemias were quantified by bright-field microscopy (Schwan *et al.*, 2003). Immune serum collected from the mice was pooled. All animal handling was in compliance with the Rocky Mountain Laboratories Animal Care and Use Committee.

Human serum was available from two patients who were infected with *B. hermsii*. Serum samples from both patients were collected within 1 month of the initial spirochaetaemic episode. Serum samples from patients infected with *B. recurrentis* were available from a hospital in Addis Ababa, Ethiopia, as previously described (Porcella *et al.*, 2000). The acute-phase serum samples were collected shortly after hospitalization; the convalescent-phase serum samples were collected 8–13 days later. Acute-phase serum samples used in this study had tested IgG negative to rGlpQ while convalescent-phase samples were positive (Porcella *et al.*, 2000).

Sample preparation, 2D electrophoresis and 2D immunoblotting. Low-passage (passage seven) *B. hermsii* DAH was grown in mBSK medium containing 12 % rabbit serum (Barbour, 1984; Battisti *et al.*, 2008) and harvested when the spirochaetes were at approximately 1.0×10^8 spirochaetes ml^{-1}. For all sample preparations, spirochaete concentrations were standardized by adjusting each preparation to OD_{600} 0.2 in 1 × PBS containing 5 mM $MgCl_2$, which is approximately 7.22×10^7 spirochaetes ml^{-1}. Samples were then centrifuged at 9000 ***g*** for 15 min at 4 °C, concentrated eightfold with 1 × PBS containing 5 mM $MgCl_2$ and Complete Mini EDTA-free protease inhibitors (following manufacturer's instructions) (Roche Diagnostics) to approximately 5.8×10^8 spirochaetes ml^{-1}.

The 5.8×10^8 spirochaetes were centrifuged at 13 000 ***g*** for 5 min and processed through the ReadyPrep Protein Extraction kit (Soluble/Insoluble) (Bio-Rad), following the manufacturer's instructions. After protein extraction, samples were resuspended in 100 µl 2D Rehydration Sample Buffer 1 (Bio-Rad) containing 2 mM tributylphosphine (TBP) (Bio-Rad) and 0.2 % ampholytes (Bio-Rad). Samples were precipitated using the ReadyPrep 2-D Cleanup kit (Bio-Rad), following the manufacturer's instructions.

Soluble and insoluble fractions were resuspended in 200 µl 2D Rehydration Sample Buffer 1 containing 2 mM TBP and 0.2 % ampholytes, and used to rehydrate 11 cm pH 4–7, pH 5–8, or pH 7–10 ReadyStrip IPG Strips (Bio-Rad). Rehydration was performed under active conditions, and IPG strips were focused for a total of 25 000 V h using the PROTEAN IEF (Bio-Rad).

IPG strips were equilibrated for 20 min as specified by the manufacturer (Bio-Rad) with equilibration buffer 1 containing 5 mM TBP. IPG strips were transferred onto 10.5–14 % Criterion Precast Gels (Bio-Rad), electrophoresed, and stained with the colloidal Coomassie SimplyBlue SafeStain (Invitrogen) or transferred onto a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen).

Mouse serum samples collected prior to and 7 days after infection, and immune serum from human patients infected with *B. hermsii*, were used to probe 2D immunoblots at a 1 : 100 dilution in I-Block blocking reagent (TROPIX). Secondary mAbs were goat anti-mouse IgM-HRP (Invitrogen) at 1 : 4000 or goat-anti-human IgM-peroxidase (Sigma-Aldrich) at 1 : 20 000. Membranes were developed by chemiluminescence with the ECL Western Blotting Detection Reagent (GE Healthcare), following the manufacturer's instructions. Images from 2D gels and 2D immunoblots were acquired with the GelDoc (Bio-Rad), and overlaid in order to select IgM-reactive protein spots.

2D gel protein sample preparation for mass spectrometry. Protein samples of interest were excised from colloidal Coomassie-stained (Invitrogen) gels using the 1.0, 2.0, or 3.0 mm Harris UniCore sampling punch tool (Sigma-Aldrich) and placed into a new Ultra Low Attachment 96-well Costar Plate (Corning Inc.). Gel plugs were destained, reduced, alkylated, and then rehydrated in 25 µl of 10 ng $µl^{-1}$ sequencing grade trypsin solution (Promega).

After an overnight incubation at 37 °C, supernatants containing eluted trypsinized peptides were removed and placed into a new Ultra Low Attachment 96-well Costar Plate. Peptides were further extracted by adding 5 % formic acid (Sigma-Aldrich) and 50 % acetonitrile (ACN) to the gel plugs for 30 min at 37 °C. The solution was removed and pooled with the supernatants from the overnight trypsinization and the extraction repeated. Trypsinized peptides were dried under vacuum (Jouan) and dissolved in 30 µl of 0.1 % trifluoroacetic acid (TFA) (JT Baker).

Peptide samples were desalted with the C18 ZipTips (Millipore), following the manufacturer's instructions with minor modifications. Briefly, ZipTips were conditioned with 90 % methanol and 0.1 % TFA, equilibrated with 0.1 % TFA, and peptides bound by pipetting. Peptides were washed with 0.5 % acetic acid (Sigma-Aldrich), eluted into a Thermo-Fast 96-well skirted plate (ABgene House) with 70 % ACN and 1 % acetic acid, and dried under vacuum centrifugation for mass spectrometric analysis.

Mass spectrometry, *B. hermsii* database search, and basic local alignment search tool (BLAST) analysis. Identification of proteins from 2D gels was performed by mass spectrometry, and this was repeated four separate times. Tryptic digests were analysed by coupling the Nanomate (Advion BioSciences), an automated chip-based nano-electrospray interface source, to a quadrupole–time of flight mass spectrometer, QStarXL MS/MS System (Applied Biosystems/Sciex). Peptide sequence information was provided by MS/MS. AnalystQS software (Applied Biosystems/Sciex) was used for data acquisition. Data processing and database searching were performed with the MASCOT software (Matrix Science). A protein database was generated from the genome sequence of *B. hermsii* DAH and submitted to MASCOT as a separate database for searching. Generally, MASCOT ion scores greater than 24 correspond to a probability of 95 % or greater that the peptide match is not a random event.

To determine the amino acid identity of non-variable membrane proteins, protein BLAST analysis was performed at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Also, individual variable small proteins (Vsps) and variable large protein (Vlp) 5 were named according to

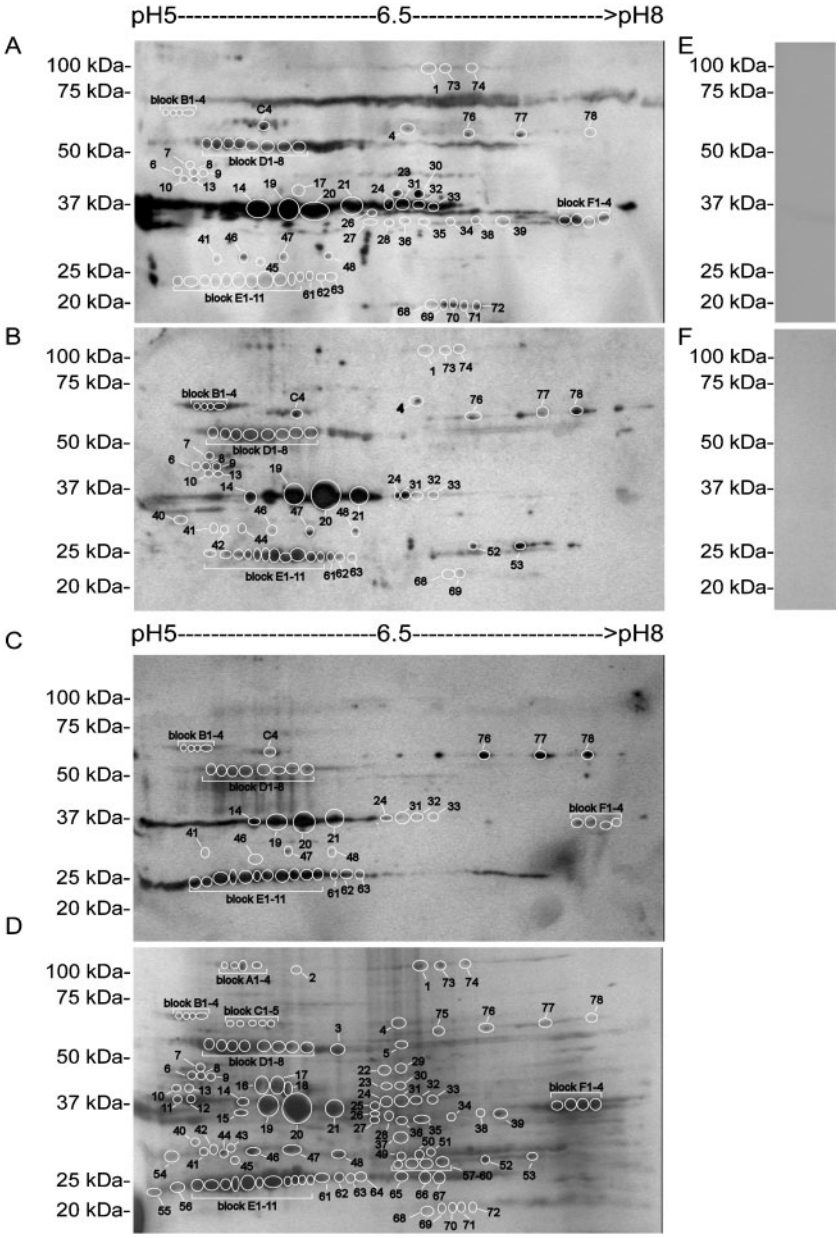

Fig. 1. 2D immunoblots (A–C) and a Coomassie stained gel (D) of *B. hermsii* lysate. Serum samples from three mice infected with *B. hermsii* were collected, pooled, and used to probe immunoblots (A). Serum from patient 1 (B) and patient 2 (C) was collected after *B. hermsii* infection. Proteins identified by mass spectrometry from 2D gels are numbered on 2D immunoblots (A–C). 1D immunoblots confirmed that murine pre-immune sera (E) and human serum from an uninfected donor were negative to *B. hermsii* whole-cell lysate (F). Molecular mass standards are shown on the left.

BLAST analysis. A BLAST threshold *E*-value of 1.0×10^{-4} was used to identify borrelia proteins.

Production of rGlpQ and recombinant BH0238 (rBH0238). *B. hermsii* and *B. recurrentis glpQ* were expressed as previously described (Porcella *et al.*, 2000; Schwan *et al.*, 2003). Genomic DNA from *B. recurrentis* 115 and *B. hermsii* DAH was used to amplify *glpQ*. *glpQ* was expressed as a His-tagged fusion protein in the pET-15b (Novagen) and pET-32a (Stratagene) expression vectors for *B. recurrentis* and *B. hermsii*, respectively, following the manufacturer's instructions.

bh0238 was expressed using the pBAD/TOPO ThioFusion expression system following the manufacturer's instructions (Invitrogen). Forward and reverse primers used to amplify *bh0238* from *B. hermsii* DAH genomic DNA were 5′-ATGACTAGATTTTTAGTGGAGG-TTAGCATGAG-3′ and 5′-TTTTATTGAAAAGAGTACCCATTTA-TCATCC-3′, respectively. PCR amplification consisted of 34 cycles with a denaturing temperature of 94 °C for 30 s, annealing temperature of 55 °C for 30 s, and an extension temperature of 72 °C for 2.5 min. After confirming the correct orientation and nucleotide sequence within the pBAD/TOPO ThioFusion vector using the Vector NTI software package (Invitrogen), *bh0238* was induced with a final concentration of 0.2 % arabinose and purified using a His-Bind Quick Column (Novagen) following the manufacturer's instructions.

1D SDS-PAGE and 1D immunoblotting. For detecting anti-GlpQ Abs during early mouse infection, whole-cell lysates of *B. hermsii* DAH, 6 μg His-tagged rGlpQ (Schwan *et al.*, 2003), and *Escherichia coli* containing the empty shuttle vector were separated by 1D SDS-PAGE as previously described (Schwan *et al.*, 1989) and transferred to a nitrocellulose membrane as stated above. Murine immune sera collected for the first 4 days post-inoculation were used at a dilution

Table 1. IgM-reactive proteins identified by mass spectrometry

Protein annotation	Spot	NCBI ID	Pred MM*	MASCOT ion score†
Heat-shock proteins				
DnaK	B1–B4	BH0518	68.7	274–482
HtpG	C4	BH0560	71.3	27–71
GroEL	D1–D3	BH0649	60.0	150–316
Adhesin				
P66	76–78	BH0603	66.0	60–462
ABC transporter proteins				
OppA‡ II	D5–D8	BH0329	60.5	39–91
OppA‡ IV	70–72	FJ446702	20.5	41–191
Nucleoside-binding protein	2–5§	BH0383	37.2	68–85
Zn-binding protein	42, 44	BH0713	29.5	35, 86
Flagellar proteins				
Flagellin	14, 19, 20, 21	BH0147	35.5	91–618
Flagellar basal-body rod protein	40	BH0774	28.7	77
Housekeeping proteins				
Enolase	6–9, 13	BH0337	47.4	122–341
Cell-division protein FtsZ	10	BH0299	43.6	67
GAPDH‖	F1–F4	BH0057	36.2	150–571
GlpQ¶	24, 31–33	BH0241B	39.0	78–367
Ef-Tu	17, 23, 30	BH0476	43.4	41–375
Fructose-bisphosphate aldolase	27	BH0445	40.0	33–41
Triosephosphate isomerase	45	BH0055	27.7	211
Phosphoglycerate mutase	52, 53	BH0658	28.5	206–323
Variable membrane proteins				
Variable large protein 5	26, 28, 34–36, 38, 39	ABF21151	34.8	153–443
Variable large protein C54silD	12§, 13§	ABF82211	35.0	192–288
Variable small protein 3 precursor	E1–E11	AAA22967	18.6	167–318
Variable small protein 22 silD	E1–E11	ABF82176	23.0	48–77
Variable small protein 6	E1, E2, E4, E5	AAZ94628	23.0	31–86
Variable small protein 8 precursor	E1, E3–E5	AAA59225	23.0	61–98
Variable small protein 58	E3	ABE73350	23.0	80
Variable small protein 2 precursor	E3–E5	AAF73948	23.0	60–71
Variable tick protein	61–63	AAT99985	21.0	172–291
Immune evasion protein				
Factor H-binding protein	68, 69	EF411143	21.6	38–153
Proteins with unknown function				
Hypothetical	4	FJ446703	56.7	32
Hypothetical	41, 46, 47, 48	BH0238	30.6	33–369
P93	1, 73, 74	BH0744	96.3	94–98

*Predicted molecular mass given in kDa.
†MASCOT scores given are a low and high range for a respective protein. Scores above 24 are statistically significant with $P<0.05$.
‡Oligopeptide permease homologue.
§Protein plug numbers in a 2D gel with an isolelectric focusing range of pH 4–7.
‖Glyceraldehyde-3-phosphate dehydrogenase.
¶Glycerophosphodiester phosphodiesterase.

of 1 : 100 to probe immunoblots. To differentiate between IgM and IgG responses, goat-anti-mouse IgM-HRP or HRP-recombinant-Protein A (ZYMED) were used at a 1 : 4000 dilution.

rBH0238 produced in the pBAD/TOPO ThioFusion system (Invitrogen) was examined as follows. *E. coli* harvested prior to and 2 h after induction or *B. recurrentis* lysate and 2 μg of purified rBH0238 were separated by SDS-PAGE (Schwan *et al.*, 1989), and stained with the colloidal Coomassie SimplyBlue SafeStain (Invitrogen) or transferred onto a nitrocellulose membrane as stated above. Immunoblots were probed with a monoclonal anti-poly-histidine peroxidase conjugate (Sigma-Aldrich) at a 1 : 2000 dilution, immune serum from mice infected with *B. hermsii* at a 1 : 100 dilution, or acute and convalescent serum from Ethiopian patients infected with *B. recurrentis* at a 1 : 500 dilution. The secondary molecule used was HRP-recombinant-Protein A (ZYMED). In addition, convalescent-phase serum samples were used to determine seroconversion rBH0238.

ELISA with rGlpQ. IgM reactivity to rGlpQ was determined by ELISA as previously described, with minor modifications (Porcella *et al.*, 2000). Sera from Ethiopian patients infected with *B. recurrentis* were available from their acute phase of infection. Immune and negative control sera were diluted 1 : 100 in diluent (1 × PBS, 5 % horse serum, 0.05 % Tween 20, 0.001 % dextran sulfate) and tested in triplicate. IgM binding to rGlpQ was determined using goat-anti-human IgM-peroxidase (Sigma-Aldrich) at 1 : 20 000 followed by the addition of a substrate consisting of 50 % 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate). After 30 min incubation, the A_{405} was measured with a Labsystems Multiskan Plus microtitre plate reader (Fisher Scientific). Samples were considered positive if their mean absorbance was more than three standard deviations above the mean of negative control serum samples.

RESULTS

Evaluation of mouse and patient serum samples infected with *B. hermsii*

All mice inoculated with *B. hermsii* DAH became spirochaetaemic within 3 days after injection (data not shown). IgM from these mice and patients infected with *B. hermsii* recognized antigens ranging from 20 to 100 kDa (Fig. 1A–C), while murine preimmune sera or sera from uninfected human patients had little reactivity to *B. hermsii* whole-cell lysate (Fig. 1E and F).

Protein spot selection for mass spectrometry

Image overlays from 2D electrophoresis gels and 2D immunoblots probed with immune serum from mice or humans identified 114 protein candidates for mass spectrometry analysis within the isoelectric focusing range of pH 5–8 (Fig. 1A–D). Vsps, Vlp 5, and the nucleoside-binding proteins were the only IgM-reactive proteins identified in pH 4–7 and pH 7–10 2D gels (data not shown), and of these proteins, Vlp C54silD and the nucleoside-binding protein were the only additional proteins not identified in the pH range 5–8 (Table 1). Also, there were no apparent differences between protein fractions that were tested by 2D electrophoresis and immunoblotting.

Table 2. Percentage amino acid identity of *Borrelia* orthologues compared to *B. hermsii*

Protein annotation	*B. burgdorferi*	*B. recurrentis*	*B. duttonii*	*B. turicatae*
Heat-shock proteins				
DnaK, BH0518	92	96	96	98
HtpG, BH0560	84	87	87	93
GroEL, BH0649	92	95	95	97
Adhesin				
P66, BH0603*	55	66	66	79
ABC transporter proteins				
OppA† II, BH0329	65	79	79	82
OppA† IV, FJ446702	47	72	72	78
Nucleoside-binding protein, BH0383	48	64	65	75
Zn-binding protein, BH0713	73	91	91	93
Flagellar proteins				
Flagellin, BH0147	91	90	90	93
Flagellar basal-body rod protein, BH0774	84	89	89	95
Housekeeping proteins				
Enolase, BH0337	90	95	95	96
Cell division protein FtsZ, BH0299	91	97	98	98
GAPDH‡, BH0057	92	95	96	97
GlpQ§, BH0241B	-	81	82	89
Ef-Tu, BH0476	94	97	97	97
Fructose-bisphosphate aldolase, BH0445	88	93	93	96
Triosephosphate isomerase, BH0055	77	86	86	91
Phosphoglycerate mutase, BH0658	83	90	91	96
Immune evasion protein				
Factor H-binding protein, EF411143*	–	–	–	61
Proteins with unknown function				
Hypothetical, FJ446703*	–	46	47	58
Hypothetical, BH0238*	53	72	73	83
P93, BH0744	49	67	61	74

*Proteins identified as unique within the *Borrelia* genus.
†Oligopeptide permease homologue.
‡Glyceraldehyde-3-phosphate dehydrogenase.
§Glycerophosphodiester phosphodiesterase.

B. hermsii database analysis and amino acid identity of proteins reactive with immune IgM

Proteins identified by mass spectrometry were grouped into eight categories where, except for Vsps, protein plugs containing an individual protein are shown (Table 1). Given that several Vsps were identified within a protein plug, mapping peptides identified by mass spectrometry to their respective Vsp confirmed that unique peptides were identified from each Vsp (data not shown).

While 114 protein samples were analysed by mass spectrometry, identifying the same protein in several spots was common. This could be due to post-translational modifications including lipidation of the identified variable membrane proteins and several of the ABC transporter proteins (Bono *et al.*, 1998; Burman *et al.*, 1990; Kornacki & Oliver, 1998; Shang *et al.*, 1998). Also, protein binding to residual DNA and RNA could cause heterogeneous protein migration.

Amino acid alignments of non-variable membrane proteins indicated the conserved nature of the identified proteins when compared to *B. hermsii* (Table 2). Similar to GlpQ and the factor H/plasminogen-binding protein (FhbA) (Hovis *et al.*, 2006; Rossmann *et al.*, 2007; Schwan *et al.*, 1996), no orthologue of hypothetical protein FJ446703 was identified in *Borrelia burgdorferi* (Table 2). Additionally, BLAST analysis identified P66, FhbA, and hypothetical proteins FJ446703 and BH0238 as unique proteins within the genus *Borrelia*.

Comparisons of IgM-reactive protein candidates between human patients and mice infected with *B. hermsii*

Immunoblot comparisons confirmed similar IgM profiles in human patients and mice infected with *B. hermsii*, where 74 % of the protein spots were recognized by both human and mouse serum samples (Fig. 2). In addition, lack of IgM reactivity to Vlp 5 from human serum samples was likely due to the expression of other *vlps* during their infection.

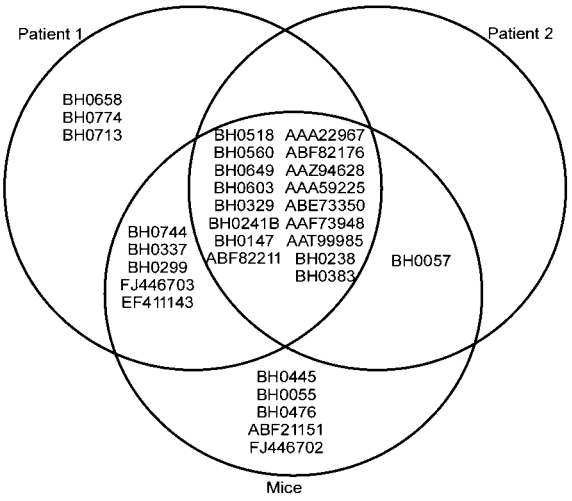

Fig. 2. Venn diagram demonstrating the reactivity of human and murine IgM against *B. hermsii* proteins. Most proteins were recognized by IgM from both human patients and mice.

IgM reactivity to rGlpQ during early *B. hermsii* and *B. recurrentis* infection

Murine and human IgM bound to rGlpQ during early spirochaete infection (Figs 3 and 4). Murine IgM bound to rGlpQ at the expected molecular mass by the fourth day after spirochaete infection (Fig. 3A), and prior to detection of IgG antibodies (Fig. 3B), while preimmune serum was negative to the antigens tested (Fig. 3C).

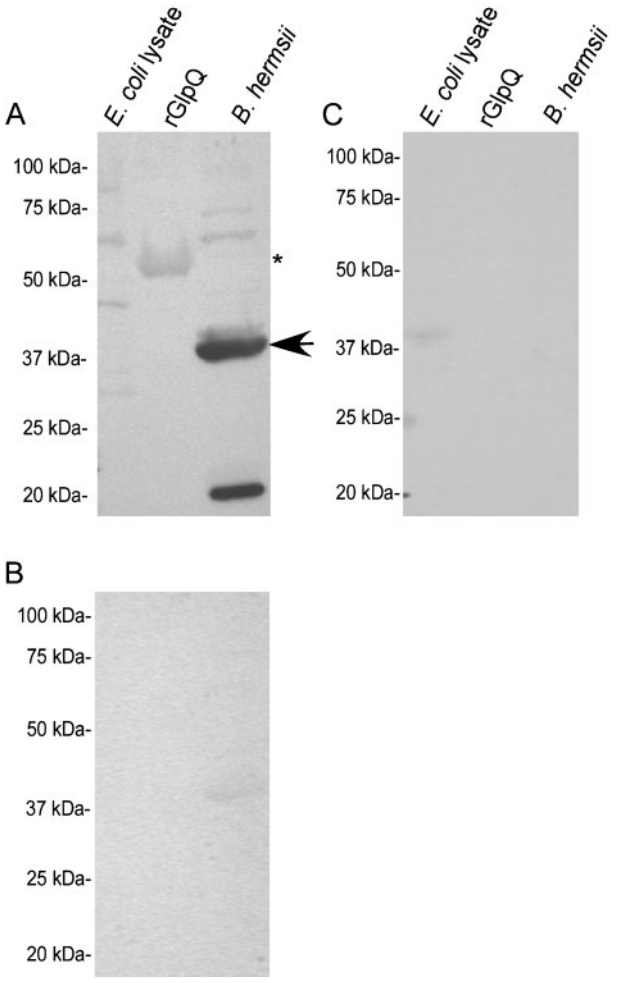

Fig. 3. Detection of IgM to rGlpQ during early *B. hermsii* infection. Murine IgM reactivity was detected to rGlpQ 4 days after infection (A), while an IgG response (B) and preimmune serum were negative to *E. coli* lysate, rGlpQ and *B. hermsii* (C). The arrow indicates the observed molecular mass of native GlpQ, while the asterisk indicates the molecular mass of rGlpQ. Molecular mass standards are shown on the left of each immunoblot.

IgM binding to rGlpQ was detected in acute-phase serum samples from eight of 15 Ethiopian patients infected with *B. recurrentis* (Fig. 4). In addition, these serum samples were negative when previously tested for IgG responses against rGlpQ (Porcella *et al.*, 2000).

Expression and immunogenicity of rBH0238

Given the conserved nature of BH0238, its apparent immunogenicity in *B. hermsii,* and preliminary data suggesting that its orthologue is immunogenic in *B. recurrentis* (data not shown), this gene was expressed as a potential diagnostic antigen. Inducing transformed *E. coli* with a final concentration of 0.2 % arabinose produced a protein band at the expected molecular mass of 48 kDa (Fig. 5A). Immunoblotting with an anti-polyhistidine mAb confirmed reactivity with a protein at 48 kDa and 35 kDa, suggesting degradation of rBH0238 (Fig. 5B). In addition, there was no mAb reactivity in uninduced *E. coli* (Fig. 5B).

Immunoblotting confirmed the immunogenicity of rBH0238 in mice (Fig. 6A). Convalescent serum from Ethiopian patients infected with *B. recurrentis* reacted with rBH0238 while their acute serum did not (Fig. 6B). Reactivity to a protein at approximately 37 kDa in acute serum samples 1A and 4A suggests reactivity to the immunogenic protein flagellin (Fig. 6B) (Barbour *et al.*, 1986, 2008; Coleman & Benach, 1989; Collins & Peltz, 1991; Schneider *et al.*, 1992).

DISCUSSION

The results reported here are from the first comprehensive identification of *B. hermsii* protein candidates that are bound by immune IgM. Furthermore, identifying the previously reported immunogenic proteins GlpQ, FhbA, flagellin, Vtp, Vsps and Vlps (Barbour *et al.*, 1986; Colombo & Alugupalli, 2008; Hovis *et al.*, 2006; Porcella *et al.*, 2000; Schwan *et al.*, 1996; Shang *et al.*, 1998) suggested that 2D electrophoresis and immunoblotting was an adequate method for identifying protein candidates that are reactive with IgM. In addition, while P66, DnaK, P93 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) are reactive with serum generated against *B. burgdorferi* (Anda *et al.*, 1994, 1996; Bunikis *et al.*, 1996, 1998; Coleman & Benach, 1992), we report that these proteins are also immunogenic during *B. hermsii* infection. Interestingly, in *B. burgdorferi*, DnaK, P93 and GAPDH are detected by the same IgM monoclonal antibody (Anda *et al.*, 1994, 1996); however, it is unknown if these proteins were identified in this study due to a common epitope.

Of the immunoreactive protein candidates identified, heat-shock, ABC transporter and housekeeping proteins have been reported as immunostimulators in other organisms (Bercic *et al.*, 2008; Brown *et al.*, 2001; Bunk *et al.*, 2008; Da'dara *et al.*, 2008; Delvecchio *et al.*, 2006; Garmory & Titball, 2004; Granato *et al.*, 2004; Harland *et al.*, 2007; Jomaa *et al.*, 2005; Lewthwaite *et al.*, 2002, 2001, 2007;

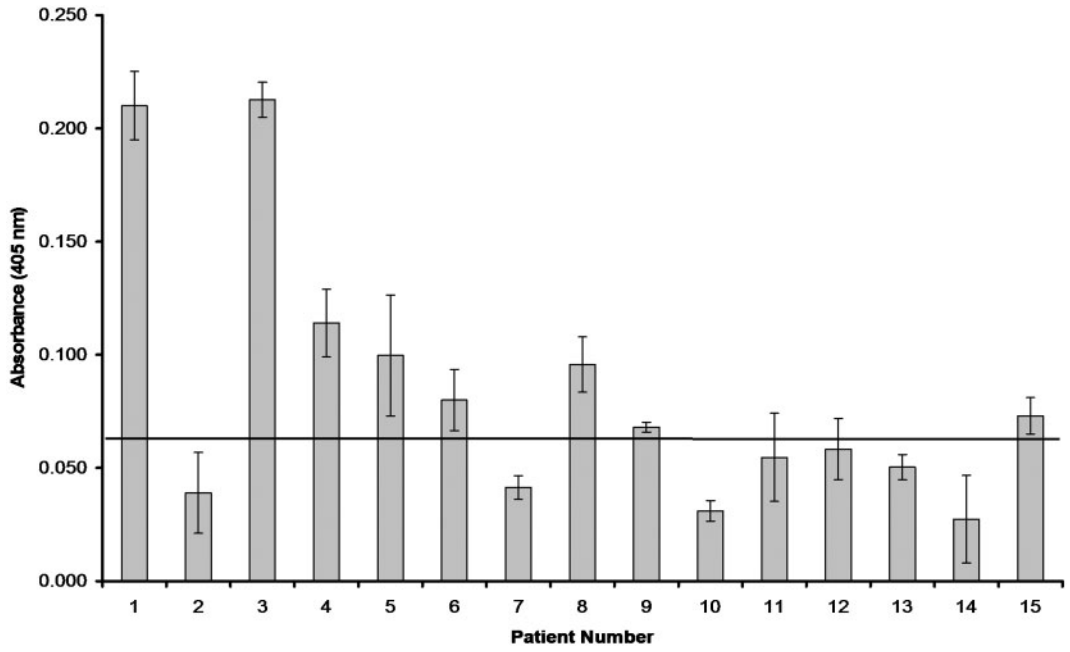

Fig. 4. An ELISA measuring IgM binding to rGlpQ using acute-phase serum samples from 15 Ethiopian patients infected with *B. recurrentis.* The standard deviation of each mean is indicated with error bars, and the horizontal line represents the threshold for determining a positive sample with a $P \leqslant 0.003$.

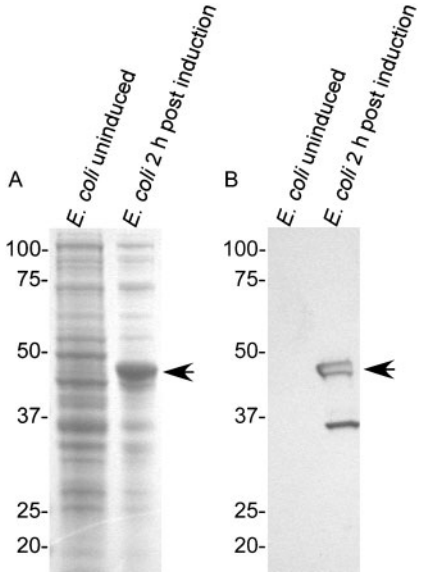

Fig. 5. Production of rBH0238. Coomassie-stained gel (A) and an immunoblot (B) probed with an anti-polyhistidine mAb of uninduced and induced *E. coli*. The arrowheads indicate protein bands of the expected size for rBH0238.

Ratnakar *et al.*, 1996; Sellman *et al.*, 2005; Shah & Swiatlo, 2006; Tanabe *et al.*, 2006; Tanghe *et al.*, 1999). For example, the adjuvant properties of DnaK aid in protecting against *Schistosoma japonicum* infection when delivered as a DNA-based vaccine (Da'dara *et al.*, 2008). The protective capacity of ABC transporter proteins is observed in mice, animals immunized with OppA being more resistant to *Yersinia pestis* infection compared to control mice (Tanabe *et al.*, 2006). While the surface localization of various heat-shock, ABC transporter and housekeeping proteins has been determined in other bacteria (Balasubramanian *et al.*, 2008; Delepelaire, 2004; Frisk *et al.*, 1998; Frisk & Lagergard, 1998; Huesca *et al.*, 1996; Lewthwaite *et al.*, 1998, 2002, 2001, 2007; Ratnakar *et al.*, 1996; Yamaguchi *et al.*, 1996), our attempts to confirm surface localization of these proteins were inconclusive.

An additional objective of our study was to determine early IgM reactivity to rGlpQ (Porcella *et al.*, 2000). In mice, IgM reactivity to rGlpQ was detected within 4 days of spirochaete infection. Furthermore, IgM responses to rGlpQ were detected from eight additional patients infected with *B. recurrentis*. While promising, these results also suggest the importance of identifying additional immunogens for early detection of relapsing fever spirochaetes, especially for infections where the organisms are below the level of detection.

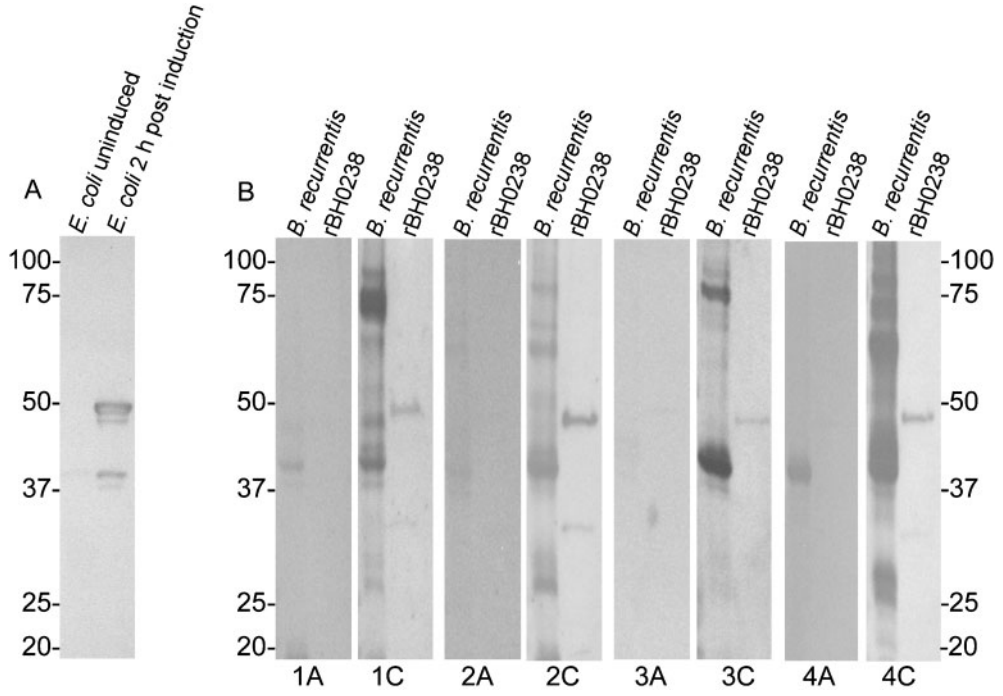

Fig. 6. Serum reactivity against rBH0238 using serum samples from mice infected with *B. hermsii* (A) or from patients infected with *B. recurrentis* (B). Immunoblots were probed with acute-phase serum samples (labelled A under *B. recurrentis* immunoblots) or with convalescent-phase serum samples (labelled C). Molecular mass standards (kDa) are shown next to each immunoblot.

Three diagnostic antigens of interest include FhbA (Hovis *et al.*, 2006), and hypothetical proteins FJ446703 and BH0238. The factor H-binding protein and hypothetical protein FJ446703 could be diagnostic antigens in North America because they discriminate between relapsing fever spirochaetes and Lyme-disease-causing spirochaetes. While rBH0238 was recognized with serum from patients infected with *B. recurrentis*, studies indicate that patients with Lyme disease also seroconvert to this antigen (data not shown), limiting rBH0238 as an antigen that could discriminate between the two species of *Borrelia*. However, given this protein's immunogenicity and BLAST analysis indicating that it is a *Borrelia*-specific protein, combining rBH0238 with additional immunogens may aid in diagnosing infection of relapsing fever spirochaetes.

Two factors complicating the treatment of relapsing fever patients include high spirochaete counts associated with a poorer prognosis (Melkert, 1991) and the reported misdiagnosis of relapsing fever *Borrelia* (Nordstrand *et al.*, 2007). Collectively, the data reported here identify proteins whose immunogenic properties can be further investigated and may be useful for early serodiagnosis. In addition, these findings may aid in managing relapsing fever *Borrelia* in endemic regions and have the potential to alleviate healthcare costs in developing countries.

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