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The secretome of the filarial parasite, *Brugia malayi*: Proteomic profile of adult excretory–secretory products

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ABSTRACT

The secretome of a parasite in its definitive host can be considered to be its genome *in trans*, to the extent that secreted products encoded by the parasite fulfill their function in the host milieu. The 'extended phenotype' of the filarial parasite, *Brugia malayi*, is of particular interest because of the evidence that infection results in potent down-modulation of the host immune response. We collected *B. malayi* 'excretory–secretory' (BES) proteins from adult parasites and using a combination of shotgun LC–MS/MS and 2D gel electrophoresis, identified 80 *B. malayi* and two host proteins in BES, of which 31 (38%) were detectable in whole worm extract (BmA). Products which were enriched in BES relative to BmA included phosphatidylethanolamine-binding protein (PEB), leucyl aminopeptidase (LAP, homologue of ES-62 from the related filaria *Acanthocheilonema viteae*), *N*-acetylglucosaminyltransferase (GlcNAcT) and galectin-1, in addition to the previously described major surface glycoprotein, glutathione peroxidase (gp29, GPX-1) and the cytokine homologue macrophage migration inhibitory factor (MIF-1). One of the most abundant released proteins was triose phosphate isomerase (TPI), yet many other glycolytic enzymes (such as aldolase and GAPDH) were found only in the somatic extract. Among the more prominent novel products identified in BES were a set of 11 small transthyretin-like proteins, and three glutamine-rich-repeat mucin-like proteins. Notably, no evidence was found of any secreted protein corresponding to the genome of the *Wolbachia* endosymbiont present in *B. malayi*. Western blotting with anti-phosphorylcholine (PC) monoclonal antibody identified that GlcNAcT, and not the ES-62 homologue, is the major PC-bearing protein in BES, while probing with human filariasis sera showed preferential reactivity to galectin-1 and to processed forms of myotactin. Overall, this analysis demonstrates selective release of a suite of newly identified proteins not previously suspected to be involved at the host–parasite interface, and provides important new perspectives on the biology of the filarial parasite.

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1. Introduction

Helminth parasites continue to be a major global health problem with over 2 billion people infected across the world [1]. Their persistence is, in part, due to their success in escaping host immunity and surviving for years within the human body. Their long lifespan is considered to reflect the evolution of highly successful immune evasion strategies [2]. Manipulation of the host immune system is most likely to be mediated by molecules released from live parasites, and hence the definition of parasite secretomes will identify candidate molecules involved in immune modulation [3]. Further, if the function of parasite secretions can be blocked by host antibodies, than secreted antigens may be critical in the formulation of future vaccines against parasitic helminth infections [4,5]. For these reasons, we have analysed the spectrum of proteins secreted by adult parasites of the human filarial nematode, *Brugia malayi*, aided by the recent release of the draft genome sequence of this pathogen [6].

In both human populations and experimental model systems, filarial infections with *B. malayi* and the closely related *Wuchereria bancrofti* are associated with immunological down-regulation. In infection, there is a dominance of regulatory cytokines, such as IL-10 and TGF-β, which inhibit parasite-specific T cell proliferation and effector function [7,8], leading to a diverted Th2 phenotype characterised by high IL-4 and IgG4 isotype antibodies, but low levels of eosinophil-activating IL-5 and of reagenic IgE antibody [9].

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T cells from infected individuals are associated with the presence of a number of markers linked to regulatory T cells [10,11]. Defects in the antigen presenting cell (APC) population are also evident [12], as exemplified by reduced monocyte responsiveness to inflammatory stimuli [13]. Studies with mouse models confirm that, as in humans, both innate and adaptive arms of host immunity are targetted by filarial parasites [14–16].

The molecular basis of helminth-induced hyporesponsiveness has yet to be defined. One approach to investigate this is to characterise the molecules at the host–pathogen interface, given that these will have the opportunity to interact with, and potentially subvert, host immune cells. Early work used radiolabelling techniques to characterise the major surface and secretory products of adult *B. malayi* [17,18]. Subsequent *in vivo* studies have shown that *B. malayi* excretory–secretory (ES) products (hereafter referred to as BES) can inhibit parasite-specific proliferative responses [19], perhaps via the induction of a suppressive macrophage population [20].

More recently, as a result of large-scale parasite sequencing (genomic and EST) projects (http://www.sanger.ac.uk/Projects/Helminths/), the proteomic identification of helminth ES products has become possible. Among nematodes, numerous ES products from *Haemonchus contortus* [3], *Trichinella spiralis* [21], and *Teladorsagia circumcincta* [22] have been identified by reference to EST datasets. *Schistosoma mansoni* secretions from larvae [23,24], eggs [25] and adult guts [26], have also been determined by reference to transcriptomic data. However, proteomic studies on the secretions of filarial parasites revealed few matches to ESTs (Harcus, Curwen, Wilson and Maizels, unpublished). The release of the draft genome of *B. malayi* [6] has offered the opportunity for a more systematic characterisation of BES, and we now report on the use of two parallel proteomic approaches, gel-free (shotgun) LC–MS/MS and 2D gel electrophoresis to probe the adult *B. malayi* secretome.

2. Materials and methods

2.1. Parasites

B. malayi, obtained originally from TRS Laboratories, was maintained through *Aedes aegypti* mosquitoes and *Meriones unguiculatus* gerbils. Adult worms were recovered from the peritoneum approximately 4 months post-infection.

2.2. In vitro culture and collection of BES and BmA

Culture medium comprised serum-free RPMI-1640 supplemented with 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin (all Gibco) and 1% glucose (Sigma). Male and female parasites were washed extensively with culture medium, and then cultured at approximately 50 worms/50 ml medium at 37 °C in 5% CO_2. Spent media were collected five times over an 8-day culture period. Media were filtered through a 0.22-μm syringe filter (Sartorius) to remove microfilariae, and then stored at −20 °C. Media from 2–3 batches of worms were concentrated and then diafiltrated into 20 mM Tris–HCl pH 7.3 using an amicon ultrafiltration cell with regenerated cellulose 3000 MW cut-off (MWCO) filter, followed by further concentration using an centrifugal filter with a 5000 MWCO (all Millipore). Somatic extract of adult worms (BmA) was prepared by homogenisation of male and female worms on ice in 20 mM Tris pH 7.3, followed by centrifugation at 10,000 × *g* for 20 min at 4 °C. Protein concentrations were determined using Commassie Plus (Pierce) and samples were stored at −80 °C until use.

2.3. 1D gel electrophoresis

Independently prepared batches of BES or BmA (2 μg) were heated at 95 °C for 10 min in NuPAGE LDS sample buffer (Invitrogen) with 0.5 M 2-mercaptoethanol (Sigma), and then separated on NuPAGE 4−12% Bis–Tris gels using an X cell Surelock Minicell system with NuPAGE MES SDS running buffer (all Invitrogen) for 40 min at constant 200 V. Gels were silver stained using PlusOne (Amersham Biosciences, modified according to Ref. [27]) then scanned using a Linoscan (Heidelberg).

2.4. 2D gel electrophoresis

Parasite protein (10–20 μg) was diluted into a final volume of 125 μl with rehydration solution (7M urea, 2M thiourea (both BDH), 4% CHAPS (Sigma), 65 mM DTE (Fluka), 0.8% IPG buffer 3–10 (GE healthcare), trace bromophenol blue (Sigma)), and used to rehydrate 7 cm pH 3–10 IPG strips (Immobiline; GE healthcare) for 14 h at 20 °C. Isoelectric focusing was carried out (500 V for 30 min; 1000 V for 30 min; gradient to 8000 V for 5 h; total ∼20 kV h) using an IPGphor (Pharmacia Biotech). Strips were reduced and alkylated as previously described [28] before electrophoresis in the second dimension using NuPAGE 4–12% Bis–Tris ZOOM gels and NuPAGE MES SDS running buffer (both Invitrogen) for 2 h 10 min at constant 100 V. Gels were silver stained and scanned as described above.

2.5. MALDI-ToF/ToF and LC–MS/MS

Protein spots were excised, destained in Farmer's reagent (20% sodium thiosulphate, 1% potassium ferricyanide), washed twice in 50% acetonitrile with 25 mM ammonium bicarbonate, and once with acetonitrile before vacuum drying. Samples were digested overnight at 37 °C with 10 μl of 0.01 μg/μl sequencing grade modified trypsin (Promega) in 25 mM ammonium bicarbonate, and then added to a MALDI target plate with an equal volume of α-cyanohydroxycinnamic acid (5 mg/ml; Sigma) in 50% acetonitrile containing 0.1% TFA. For LC–MS/MS, samples were diluted to 100 μL with 0.5 M triethylammonium bicarbonate. Disulphide bonds were reduced with 5 mM tris-(2-carboxyethyl)phosphine and thiols modified with 10 mM methyl methanethiosulphonate, then proteins were digested overnight at 37 °C with 1/20 (w/w) trypsin. Samples were acidified (pH 2.5) with TFA, passed through a strata C18-E solid phase extraction cartridge (55 μm, 70A, Pheonomenex), and then dried and reconstituted in 10 μl 0.1% TFA. Peptides (3 μl aliquots) were injected onto a polystyrene-divinylbenzene polymeric monolithic column (200 μm i.d. × 5 cm; LC Packings, NL) linked to an Ultimate nano-HPLC system (Dionex), and separated using a linear gradient of 3–51% acetonitrile in 0.1% heptafluorobutyric acid over 20 min (3 μl/min flow rate). UV absorbance at 214 nm was monitored. Fractions were collected every 6 s onto a MALDI target plate, using a probot microfraction collector (Dionex), followed by post-column addition of 0.9 μl/min matrix and 6 mg/ml α-cyanohydroxycinnamic acid in 60% acetonitrile.

Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in reflectron mode. MS spectra were acquired over a mass range of *m/z* 800–4000 and monoisotopic masses were obtained from centroids of raw, unsmoothed data. CID-MS/MS was performed on the 20 strongest peaks with a signal to noise greater than 50 for both the gel spots and each LC–MS/MS fraction. For the latter, a fraction-to-fraction precursor exclusion of 200 ppm was used. For CID-MS/MS, a source 1 collision energy of 1 kV was used, with air as the collision gas. The precursor mass window was set to a relative resolution of 50, and the metastable suppressor was enabled. The default calibration was used for

MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky-Golay with three points across a peak and polynomial order 4); peak detection used a minimum S/N of 5, local noise window of 50 m/z, and minimum peak width of 2.9 bins. Filters of S/N 20 and 30 were used for generating peak lists from MS and MS/MS spectra, respectively.

2.6. Database searching and bioinformatics

Mass spectral data from protein spots were submitted to database searching using a locally running copy of the Mascot program (Matrix Science Ltd., version 2.1). Batch-acquired MS/MS data was submitted to a MS/MS ion search through the Applied Biosystems GPS Explorer software interface (version 3.6) to Mascot. Mass spectral data from LC–MS/MS were submitted to database searching using TS2Mascot (Matrix Science, version 1.0.0) and Mascot 2.1. Search parameters allowed a maximum of one missed cleavage, the modification of cysteine, the possible oxidation of methionine, peptide tolerance of 100 ppm and MS/MS tolerance of 0.1 Da. Spectra were searched against both a recent version of the NCBI non-redundant protein database and a *Brugia* coding sequence database composed of both genomic (http://www.tigr.org/tdb/e2k1/bma1/intro.shtml) and EST (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gireport.pl?gudb=b_malayi) sequences. The significance threshold was set at $p < 0.05$, and identification required that each protein contained at least one peptide with an expect value <0.05. By using a decoy database, the LC–MS/MS false discovery rate for single peptide matches was estimated to be 6.1% for BES and 4.7% for BmA. Single peptide matches are marked (*) in Tables 1 and 2 . For each protein match identified by MASCOT, the programme calculated the corresponding exponentially modified protein abundance index (emPAI) as the transformed ratio of the number of experimentally observed peptides to the total number of peptides that can theoretically be detected within the operating mass range and retention range of the instrument. To establish whether the proteins present in BES contained predicted signal sequences, sequences were analysed using SignalP [29] and SecretomeP [30]. ClusterW sequence alignment and phylogenetic tree construction (bootstrap, midpoint, 1000 replications) was performed using MacVector version 9.5.2.

2.7. Western blotting

BES was separated by 2D gel electrophoresis as described above, transferred to nitrocellulose membrane (Biorad) using an Xcell II blot module (90 min, 30 V; Invitrogen), and blocked overnight at 4 °C (Starting Block T20; Pierce). To detect phosphorylcholine (PC)-bearing proteins, membranes were probed with 1/1000 Bp-1 [31] overnight at 4 °C, washed extensively in TBS with 0.05% tween (TBST), and incubated with 1/2000 rabbit anti-mouse Ig HRP (1 h, room temperature; DakoCytomation). To assess the binding of human IgG to BES, sera from

Table 1
Proteins present in BES identified by LC MS/MS

	Name	Pub_locus	TIGR locus	Gene index	Score	SS?	emPAI	Spot
Cytosol stress response/chaperones								
1	14-3-3-like protein 2	Bm1_10970	13662.m00125		74	No	0.43	
2	Chaperonin-10 kDa	Bm1_56470	15397.m00021		34*	No	0.35	
3	Heat shock protein 70 kDa	Bm1_43675	14977.m04983		113*	SS	0.33	
4	Small heat shock protein P27	XXX	XXX	TC7940	33*	NCSS	0.18	
Anti-oxidants								
5	Glutathione peroxidase, major surface antigen gp29	Bm1_40465	14972.m07803		150	SS	1.31	
6	Oxidoreductase, aldo/keto reductase	Bm1_28070	14922.m00060		52	No	0.12	
7	Superoxide dismutase	XXX	XXX	TC8075	191	No	1.07	
8	Superoxide dismutase precursor (Extracellular)	XXX	XXX	AA509030	64	SS	0.30	
9	Thioredoxin peroxidase 2	XXX	XXX	TC7730	45	NCSS	0.72	
10	Translationally controlled tumor protein	Bm1_31480	14955.m00256		39*	NCSS	0.21	
Cytosol energy metabolism								
11	Enolase	Bm1_24115	14703.m00079		346	NCSS	0.83	13
12	Glycosyl hydrolases family 31 protein	Bm1_40580	14972.m07829		69	SS	0.11	
13	Inorganic pyrophosphatase	Bm1_16955	14271.m00285		51	SS	0.14	
14	MF-1 antigen (endochitinase)	Bm1_17035	14274.m00229	TC8062	115	SS	0.19	
15	6-Phosphofructokinase	Bm1_01930	12616.m00133		39	No	0.05	
16	Triose phosphate isomerase	Bm1_29130	14940.m00172		796	No	11.11	5, 6, 7,8
Structural/cytoskeletal								
17	Actin	Bm1_16810	14258.m00140		60	No	0.13	
18	Actin 1	Bm1_34925	14965.m00431		59	No	0.28	
19	Calmodulin	Bm1_50415	14992.m10856		34*	NCSS	0.35	
20	Calsequestrin family protein	Bm1_40185	14972.m07743		41	SS	0.24	
21	Lethal protein 805, isoform d	Bm1_12945	13929.m00009		166	SS	0.62	17
22	Major sperm protein	Bm1_55755	15304.m00111		125	NCSS	0.21	
23	Major sperm protein 2	Bm1_13600	14015.m00090		281	NCSS	4.40	
24	Major sperm protein 3	Bm1_16920	14269.m00019		44	No	0.31	
25	Muscle positioning protein 4	XXX	XXX	AI783143	88*	NCSS	0.20	
26	Muscle positioning protein 4	Bm1_05930	13207.m00046		45	NCSS	0.17	
27	Myotactin form B	Bm1_53510	15059.m00091		194	No	0.08	21, 22, 23
28	Secretory protein (LS110p)	XXX	XXX	TC9625	173	SS	2.22	
29	Tropomyosin family protein	Bm1_02060	12630.m00063		69	No	0.34	
Protein digestion and folding								
30	Calreticulin precursor	Bm1_23560	14677.m00169		71	SS	0.36	
31	Cyclophilin-2 cyp-2	Bm1_55850	15309.m00029		139	No	0.53	4
32	Cyclophilin-5 cyp-5	Bm1_24035	14702.m00390		38	SS	0.26	1
33	FKBP-12	Bm1_49010	14990.m07926		55	NCSS	1.41	
34	γ-Glutamyltranspeptidase family protein	Bm1_09950	13531.m00015	TC8816	431	SS	4.91	14, 15, 16

Table 1 (*Continued*)

	Name	Pub_locus	TIGR locus	Gene index	Score	SS?	emPAI	Spot
35	Leucyl aminopeptidase	Bm1_56305	15373.m00009		568	SS	2.18	18
36	Protein disulphide isomerase	Bm1_39250	14972.m07552		36	SS	0.09	
37	Serine carboxypeptidase	Bm1_43130	14977.m04868		71*	SS	0.10	
38	Ubiquitin	Bm1_25395	14761.m00205		95	No	0.35	1
39	Ubiquitin-like protein SMT3	Bm1_45210	14979.m04551		59	NCSS	1.32	
Lectins and glycosyltransferases								
40	*N*-Acetylglucosaminyltransferase	Bm1_07275	13311.m00333	TC7929	370	NCSS	3.70	20
41	C-Type lectin domain containing protein	Bm1_40520	14972.m07815		45*	NCSS	0.08	
42	Galectin Bm-GAL-1	Bm1_24940	14731.m01012		556	No	6.13	9, 10
43	Galectin Bm-GAL-2	Bm1_46750	14981.m02389		101	No	0.18	
Protease inhibitors								
44	Cystatin CPI-2	XXX	XXX	TC7871	62	SS	0.22	
Lipid binding								
45	Nematode polyprotein allergen (gp15/400), NPA-1	Bm1_50995	14992.m10973		274	No	0.45	
46	Phosphatidylethanolamine-binding protein 1	Bm1_41005	14973.m02599		279	NCSS	8.36	
47	Phosphatidylethanolamine-binding protein 2	Bm1_31500	14956.m00481		44	NCSS	0.71	
Host cytokine homologues								
48	Macrophage migration inhibitory factor 1	Bm1_28435	14930.m00337		176	NCSS	2.38	2
Nuclear								
49	DNA repair protein Rad4 containing protein	Bm1_03115	12787.m00392		39	NCSS	0.06	
50	Snf5 homologue	Bm1_46120	14980.m02744		71	NCSS	0.19	
51	High mobility group protein	Bm1_25620	14768.m00190		46	No	0.33	
Transthyretin-like family proteins								
52	Transthyretin-like family protein	Bm1_26590	14830.m00078		194	SS	1.58	
53	Transthyretin-like family protein	XXX	XXX	TC7985	182	SS	1.11	
54	Transthyretin-like family protein	XXX	XXX	TC8258	161	SS	3.24	
55	Transthyretin-like family protein	XXX	XXX	TC8116	131	SS	1.35	3
56	Transthyretin-like family protein	XXX	XXX	TC7986	86	SS	2.29	
57	Transthyretin-like family protein	XXX	XXX	AA592049	59*	TRUN	0.32	
58	Transthyretin-like family protein	Bm1_20065	14486.m00069	AI105565	50	SS	0.33	
59	Transthyretin-like family protein	Bm1_15250	14164.m00122		53	SS	0.86	
60	Transthyretin-like family protein	Bm1_04380	12984.m00011		48*	SS	0.25	
61	Transthyretin-like family protein	Bm1_26585	14830.m00077		41	SS	0.87	
62	Transthyretin-like family protein	Bm1_06445	13250.m00031	TC8095	35	SS	0.30	
Proteins of undetermined function								
63	Conserved cysteine-glycine protein 1	Bm1_38150	14972.m07327		124	SS	0.52	
64	Conserved hypothetical protein	Bm1_19065	14396.m00009		35	No	0.06	
65	DJ-1 family protein	Bm1_07685	13325.m00230		49	No	0.68	
66	Fasciclin domain containing protein	Bm1_17270	14284.m00379		50	No	0.08	
67	G15-6A protein-related	Bm1_49930	14990.m08112		35	SS	0.22	
68	Hypothetical protein	Bm1_57465	15533.m00023	TC7900	107	No	0.09	
69	Hypothetical protein	Bm1_19875	14469.m00102		65	TRUN	0.42	
70	Hypothetical protein	Bm1_46475	14980.m02820		36	SS	0.20	
71	Hypothetical (mucin-like) protein	Bm1_11505	13734.m00156		207	No	0.24	
72	Hypothetical (mucin-like) protein	Bm1_01245	12551.m00090		188	No	0.31	
73	Hypothetical (mucin-like) protein	Bm1_09845	13513.m00023		145	No	0.31	
74	Immunogenic protein 3	Bm1_07780	13333.m00082	TC8120	133	SS	1.09	
75	Immunoglobulin I-set domain containing protein	Bm1_45475	14979.m04613		73	No	0.02	
76	Immunoglobulin I-set domain containing protein	Bm1_35295	14968.m01452		63	NCSS	0.26	
77	Major allergen	XXX	XXX	TC8813	272	SS	2.13	11, 12
78	PDZ-domain protein scribble	Bm1_33310	14961.m05155		35	No	0.04	
79	Recombinant antigen R1	Bm1_11105	13673.m00035		35	SS	0.23	
80	SXP-1 protein	Bm1_42870	14975.m04515		125	SS	1.10	
	Name	Pub_locus	Locus	Gene index	Score	SS?	emPAI	Spot
Host								
81	Fibronectin	n/a	P11276		352	n/a	0.10	
82	Serum albumin	n/a	P02769		256	n/a	0.25	19

Pub_locus is the stable gene ID, where the draft genome sequence includes the gene locus; XXX denotes gaps in the draft genome. TIGR locus is the original annotation. Gene index indicates an EST, and is given either when the genomic sequence appears truncated compared to an EST or is not present. Score refers to the mascot score. SS? shows the presence of a classical N-terminal signal sequence (“SS” from signalP), an internal non-classical sequence (“NCSS” from secretomeP), or the lack of a signal sequence (“no” negative for both signalP and secretomeP). TRUN indicates that the sequence is truncated at the N-terminus and so the presence of signal sequences could not be determined. Exponentially modified protein abundance index (emPAI) gives an approximate quantification of the protein. Spot refers to the spot number in Fig. 2C.

* Indicates single peptide hits.

previously characterised microfilaraemic or pathology individuals [32] were pooled (five representative individuals per group). Normal human sera were obtained from non-exposed UK residents. Membranes were probed with 1/500 sera dilutions in TBST overnight at 4 °C, washed in TBST, and then with 1/2000 HRP conjugated rabbit anti-human IgG (1 h room temperature; DakoCytomation P0214). Following further washing in TBST, all blots were developed using ChemiGlow West, according to the manufacturers instruc-

Table 2
Proteins present in BmA identified by LC–MS/MS

	Name	Pub_locus	TIGR locus	Gene index	Score	emPAI	Ratio
Cytosol stress response/chaperones							
1	14-3-3-like protein 2	Bm1_10970	13662.m00125		82	1.46	0.3
2	Chaperonine protein HSP60	Bm1_56580	15413.m00008		52*	0.16	xxx
3	Heat shock protein 70	Bm1_43675	14977.m04983	TC7686	613	1.56	0.2
4	Heat shock protein 70C	Bm1_17800	14318.m00070		98	0.16	xxx
5	HSP-like 86.9 kDa C30C11.4	Bm1_23190	14656.m00228		96	0.19	xxx
6	Heat shock protein 90	Bm1_51495	14992.m11078		269	0.65	xxx
7	Small heat shock protein	Bm1_19805	14459.m00246	TC8028	52	0.47	xxx
8	Small heat shock 19.4 kDa protein ZC395.10	Bm1_30230	14950.m01862		52*	0.27	xxx
9	Small heat shock protein OV25-1	Bm1_14535	14083.m00056		95	0.68	xxx
10	Small heat shock protein P27	XXX	XXX	TC7940	242	0.98	0.2
Anti-oxidants							
11	Glutathione peroxidase, major surface antigen gp29	Bm1_40465	14972.m07803		47*	0.19	6.9
12	Oxidoreductase, aldo/keto reductase	Bm1_28070	14922.m00060		94	0.27	0.4
13	Thioredoxin	Bm1_46700	14981.m02379	TC7847	80*	0.22	xxx
14	Thioredoxin peroxidase 2	XXX	XXX	TC7730	63	0.47	1.5
Cytosol energy metabolism							
15	Adenylate kinase isoenzyme 1	Bm1_13790	14037.m00196		148	0.61	xxx
16	Carbohydrate phosphorylase	Bm1_16060	14231.m00144		119	0.25	xxx
17	Enolase	Bm1_24115	14703.m00079	TC7838	541	0.65	1.3
18	Fructose-bisphosphate aldolase 1	Bm1_15350	14176.m00093		306	0.65	xxx
19	Glyceraldehyde 3-phosphate dehydrogenase	Bm1_41940	14975.m04318	TC7820	206	0.49	xxx
20	Inorganic pyrophosphatase	Bm1_16955	14271.m00285		51*	0.15	0.9
21	Phosphoenolpyruvate carboxykinase (GTP)	Bm1_25195	14749.m00214		86	0.23	xxx
22	Phosphoglycerate kinase	Bm1_01925	12616.m00132		117	0.43	xxx
23	Triosephosphate isomerase	Bm1_29130	14940.m00172		390	0.65	17.1
Structural/cytoskeletal							
24	Actin 1	Bm1_34925	14965.m00431		482	0.65	0.4
25	Actin	Bm1_16810	14258.m00140		432	0.65	0.2
26	Calponin protein 3	XXX	XXX	TC8546	295	0.65	xxx
27	Major sperm protein	Bm1_55755	15304.m00111	TC7970	96*	0.22	1.0
28	Major sperm protein 2	Bm1_13600	14015.m00090		112	0.90	4.9
29	Myosin essential light chain	XXX	XXX	TC7908	104	0.40	xxx
30	Myosin heavy chain	Bm1_40715	14972.m07860	TC7898	52	0.05	xxx
31	Myosin regulatory light chain 1	Bm1_40180	14972.m07742	TC8201	117	0.26	xxx
32	Profilin family protein	Bm1_21620	14590.m00346		57	0.43	xxx
33	Secretory protein (LS110p)	XXX	XXX	TC9625	52*	0.27	8.2
34	Tropomyosin family protein	Bm1_02060	12630.m00063		88	0.35	1.0
35	Troponin-like EF hand family protein	Bm1_48810	14990.m07885	TC7791	42	0.27	xxx
36	Tubulin alpha chain	Bm1_38680	14972.m07435	TC7853	67	0.20	xxx
37	Tubulin alpha chain	Bm1_12120	13818.m00233		43	0.11	xxx
38	Tubulin beta chain	Bm1_25780	14773.m00919	TC7923	86	0.09	xxx
Protein digestion and folding							
39	Cyclophilin-2 cyp-2	Bm1_55850	15309.m00029	TC7957	108	0.53	1.0
40	Cyclophilin-5, cyp-5	Bm1_24035	14702.m00390		83*	0.26	1.0
41	FKBP-12	Bm1_49010	14990.m07926	TC8539	50*	0.32	4.4
42	γ-Glutamyltranspeptidase family protein	Bm1_09950	13531.m00015	TC8816	83	0.36	13.6
43	Leucyl aminopeptidase	Bm1_56305	15373.m00009		56	0.12	18.2
44	Proteasome subunit alpha type 1	Bm1_17805	14318.m00071		59	0.20	xxx
45	Protein disulphide isomerase	Bm1_39250	14972.m07552	TC8291	49	0.09	1.0
46	Transglutaminase	Bm1_28935	14937.m00487		96	0.10	xxx
47	Ubiquitin	Bm1_09050	13432.m00246		61	0.27	xxx
48	Ubiquitin-like protein SMT3	Bm1_45210	14979.m04551		49*	0.53	2.5
Lectins and glycosyltransferases							
49	*N*-Acetylglucosaminyltransferase	Bm1_07275	13311.m00333	TC7929	42	0.33	11.2
Protease inhibitors							
50	Cystatin CPI-2	XXX	XXX	TC7871	77	0.23	1.0
Lipid binding							
51	Nematode polyprotein allergen (gp15/400), NPA-1	Bm1_50995	14992.m10973		332	0.65	0.7
52	Phosphatidylethanolamine-binding protein	Bm1_41005	14973.m02599	TC8147	107	0.41	20.4
Nuclear							
53	Nucleosome assembly protein 1	Bm1_27810	14919.m00204	TC8159	50	0.15	xxx
Proteins of undetermined function							
54	AT19640p-related	Bm1_34700	14963.m01811		43*	0.11	0.9
55	Conserved cysteine-glycine protein 1	Bm1_38150	14972.m07327		108	0.15	3.5
56	Disorganized muscle protein 1	Bm1_40320	14972.m07771		68	0.16	xxx
57	Dosage compensation protein dpy-30	XXX	XXX	TC9176	64	0.19	xxx
58	G15-6A protein-related	Bm1_49930	14990.m08112	TC8020	65	0.50	0.4
59	Hypothetical protein	Bm1_06545	13257.m00082		39	0.04	xxx

Table 2 (*Continued*)

	Name	Pub_locus	TIGR locus	Gene index	Score	emPAI	Ratio
60	Immunoglobulin I-set domain containing protein	Bm1_35295	14968.m01452	TC7669	145	0.51	0.5
61	Thrombospondin type 1 domain containing protein	Bm1_13945	14041.m00077		48	0.05	xxx
62	TPR domain containing protein	Bm1_46100	14980.m02740		49	0.18	xxx
63	Tumor domain containing protein	Bm1_52240	14992.m11233		36	0.04	1.0

Pub_locus is the stable gene ID, where the draft genome sequence includes the gene locus; XXX denotes gaps in the draft genome. TIGR locus is the original annotation. Gene index indicates an EST, and is given either when the genomic sequence appears truncated compared to an EST or is not present. Ratio refers to the ratio of emPAI values for proteins detected in both BES and BmA (BES: BmA). Identities are from *Brugia malayi* coding sequence database; no host proteins were detected in BmA when peptides were searched against the NCBI database.

* Indicates single peptide hits.

tions (Alpha Innotech) and imaged using a FluorChem SP (Alpha Innotech).

2.8. RT-PCR of N-acetylglucosaminyltransferase

Total RNA was extracted from adult mixed sex worms using TRIzol (Invitrogen), and reverse transcribed with MMLV reverse transcriptase (Stratagene) using standard protocols. GlcNAcT was amplified using a forward primer specific for the 5′-end of the gene coding sequence Bm1_07275 (ATGCGTTTACTGCCTCTCATT) and a reverse primer specific for the 3′-end of EST TC7929 (TAGCACTCAAACATTGATATATT). An N-terminal extension to the protein was predicted from an open-reading frame in the designated non-coding sequence of Bm1_07275, and amplified using the above reverse primer and the forward primer ATGAAACCTGAAATGTTCTCG. PCR conditions were as follows: 40 cycles of 95 °C 30 s, 60 °C 30 s, and 72 °C 2 min. Reaction products were separated on 1% agarose gels and visualised using ethidium bromide.

3. Results and discussion

3.1. Comparison of BES with whole worm extract (BmA)

The secretions of *in vitro* cultured adult *B. malayi* were collected under serum-free conditions using previously optimised protocols [18]. Preliminary 1D SDS-PAGE analysis (Fig. 1) of several different batches of concentrated BES revealed a general consistency, both in protein concentration and composition, although minor differences were evident. Protein yields from different batches averaged 51 ± 4 μg/L culture media, which is equivalent to ~30 ng per worm per day. To address the question of whether adult *B. malayi* worms selectively secrete a specific subset of proteins, we compared BES to a soluble worm homogenate (BmA). 1D gel electrophoresis revealed that BES and BmA had distinct banding patterns (Fig. 1). 2D gel electrophoresis confirmed the differing protein compositions of the two parasite preparations (Fig. 2A and B), although visual inspection reveals a number of common proteins. Importantly, 2D gel electrophoresis shows that BES (comprising approximately 20 strongly staining spots and 70+ additional molecular species) is markedly less complex than BmA (in excess of 200 spots). This is consistent with the adult parasites differentially secreting a defined set of proteins, and argues against the ES simply reflecting non-specific leakage of proteins from the worms during the *in vitro* culture period.

To identify the proteins present in BES, selected spots were picked from 2D gels and subjected to MALDI-ToF/ToF analysis (Fig. 2C shows spots; Fig. 2D gives identities). Because 2D gel electrophoresis does not favour the identification of less abundant protein species or those with extremes of p*I*, we performed shotgun LC–MS/MS as a complementary approach, which allows the simultaneous identification of multiple proteins in a complex mixture. LC–MS/MS was also carried out on BmA to confirm differences in protein composition compared to BES, as suggested by 1D and 2D gel electrophoresis (Figs. 1, 2A and B). By using LC–MS/MS, we were able to identify 80 *B. malayi* and 2 host proteins in BES (Table 1), and a further 63 *B. malayi* proteins in BmA (Table 2). Of the 82 positively identified BES proteins, 38% (31/82) were also detected in BmA. Thirty spots were subjected to individual LC–MS/MS analysis and produced adequate spectra for database searching (Fig. 2C); of these 23 were positively identified (Fig. 2D). All identified proteins spots were also present in the LC–MS/MS analysis (Table 1). One spot (19) corresponded to host serum albumin.

We also compared the relative abundance of proteins in BES and BmA by their emPAI values (exponentially modified Protein Abundance Index [33]). While this measure may not define the absolute abundance of different proteins, it is a useful means of estimating abundance of the same protein in different mixtures. Such analysis has been previously used to estimate changes in bacterial protein expression during different growth conditions [34]. Hence, we were able to infer that the concentration of a number of proteins differed substantially between BES and BmA, as graphically represented in Fig. 3. Specifically, phosphatidylethanolamine-binding protein (PEB Bm1_41005, 20-fold enrichment in BES), leucyl aminopeptidase (LAP, 18-fold), γ-glutamyltranspeptidase (14-fold)

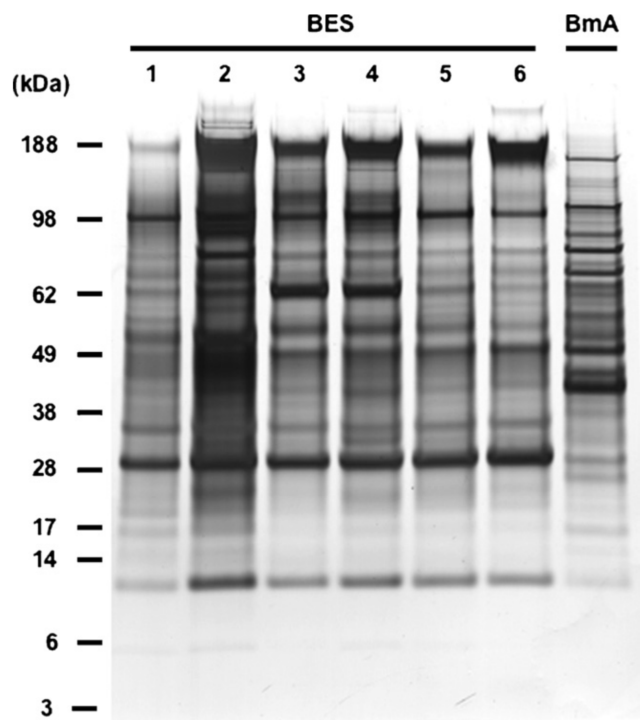
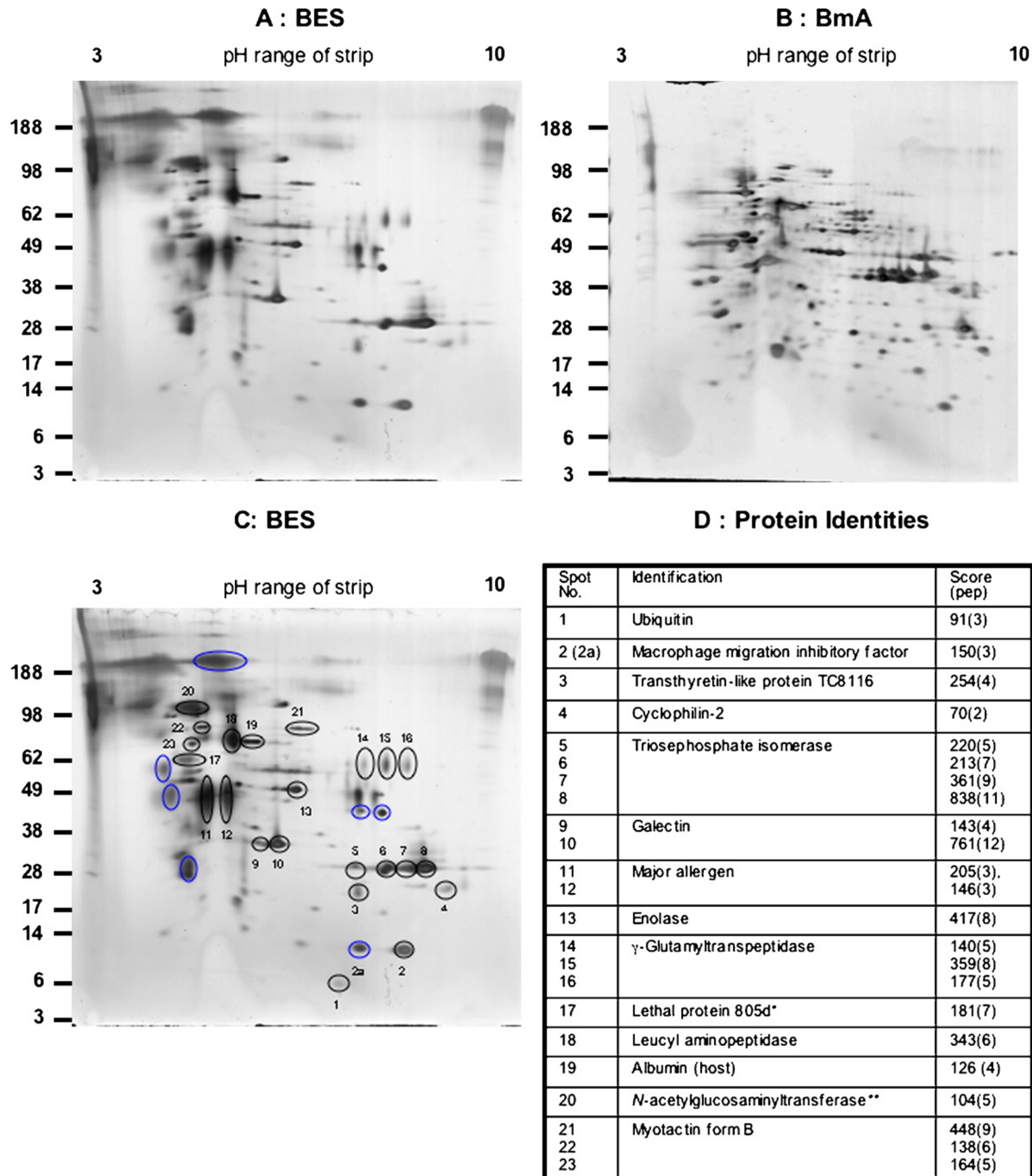


Fig. 1. One-dimensional SDS-PAGE of BES and BmA. Six independent batches of BES were compared to BmA by 1D SDS-PAGE; each lane was loaded with 2 μg of protein. Molecular weight markers are indicated on the left.



Spot No.	Identification	Score (pep)
1	Ubiquitin	91(3)
2 (2a)	Macrophage migration inhibitory factor	150(3)
3	Transthyretin-like protein TC8116	254(4)
4	Cyclophilin-2	70(2)
5 6 7 8	Triosephosphate isomerase	220(5) 213(7) 361(9) 838(11)
9 10	Galectin	143(4) 761(12)
11 12	Major allergen	205(3). 146(3)
13	Enolase	417(8)
14 15 16	γ-Glutamyltranspeptidase	140(5) 359(8) 177(5)
17	Lethal protein 805d*	181(7)
18	Leucyl aminopeptidase	343(6)
19	Albumin (host)	126 (4)
20	*N*-acetylglucosaminyltransferase**	104(5)
21 22 23	Myotactin form B	448(9) 138(6) 164(5)

Fig. 2. Two-dimensional SDS-PAGE of BES and BmA. (A) BES (20 μg) was separated by isoelectric focusing on immobilized pH 3–10 gradients and by second dimension SDS-PAGE electrophoresis. Molecular weight markers are indicated on the left. Gel is representative of six different batches of BES. (B) BmA (20 μg) separated as in (A). (C) 2D SDS-PAGE as shown in (A), with identified proteins circled in black and numbered in accordance with Table 1, whereas non-identified spots are circled in blue. One spot (2a) could not be unequivocally identified although it was a weak match to MIF-1 (*cf.* spot 2). (D) Table of 23 spots identified from gel C. Score refers to the mascot score and pep refers to the number of matching peptides identified. *Unique peptides from spot matched two different genomic sequences (13507.m000111 and 13929.m00009) that overlap significantly (data not shown) and so are likely to be the same protein. Score is derived from putative full-length consensus. **Score derived from putative full-length GlcNAcT as detailed in Supplementary Fig. 1.

and *N*-acetylglucosaminyltransferase (11-fold) appear to be preferentially secreted proteins, given that they are substantially over-represented in BES when compared to BmA. Furthermore, several proteins that are highly represented in BES, as judged by both 2D gel electrophoresis and emPAI (Fig. 2C and Table 1), such as the galectin Bm1_24940 (spots 9 and 10) and macrophage migration inhibitory factor-1 (MIF-1) (spot 2) were at undetectable levels in BmA using LC–MS/MS.

As might be expected, the majority of full-length parasite proteins identified in BES (66%; 52/78) have some form of predicted signal sequence, either classical N-terminal (33/78) or non-classical internal (19/78). However, the remaining 33% of proteins lacked detectable signal sequences, including triose phosphate isomerase (TPI), which is the most abundant protein in BES, as judged by 2D gel spot intensity (Fig. 2C, spots 5–8), consistent with its high emPAI value (Table 1). It has been suggested that this is the result of signal peptide prediction programs being optimised using mammalian, rather than worm, proteins [25]. Alternatively, such proteins may be released into BES as a result of holocrine secretion, whereby the entire contents of a cell are released into the surroundings, as

has been previously described in *S. mansoni* [24]. Although holocrine secretion may be occurring to a certain extent, it cannot fully explain the abundance of TPI in BES (17-fold enrichment compared to BmA), given that the only other glycolytic enzyme detected in BES, enolase, is not particularly preferentially secreted (1.28 emPAI ratio BES:BmA). Additionally, abundant glycolytic enzymes, such as GAPDH and aldolase, present in BmA at similar levels to TPI, are undetectable in BES. TPI has been previously described in the secretions of schistosome larvae [23,24] and eggs [25], as well as adult *H. contortus* [3], indicating that it may play an important, as yet undiscovered, role at the helminth–host interface. These results also show it is inappropriate to use the lack of a detectable signal sequence as a reason to exclude a parasite protein from potentially fulfilling a role in the extracellular environment.

3.2. Abundant proteins in BES

The majority of BES comprises a relatively small number of abundant proteins (Fig. 2A). Prominent amongst these is the galectin Bm1_24940 (GAL-1), identified by both LC–MS/MS and 2D gel electrophoresis (Table 1 and Fig. 2C, spots 9 and 10). A second less abundant galectin (GAL-2; Bm1_46750) was also detected using LC–MS/MS. Galectins are an extensive family of sugar-binding proteins with an affinity for *N*-acetyllactosamines, through a conserved carbohydrate-recognition domain (CRD) [35]. Galectins have previously been identified in other helminths, with *B. malayi* GAL-1 being most similar to an *O. volvulus* galectin (AAA20541 [36]), and GAL-2 to a *H. contortus* homologue (O44126 [37]) (Fig. 4A). Both *B. malayi* galectins are tandem-repeat types, with duplicated CRDs; in each case, the C-terminal CRD contains a full sugar-binding consensus sequence [38], while N-terminal domains are divergent (Fig. 4B). No function has yet been ascribed to any helminth galectin, but it is interesting to note that a number of mammalian galectins have the ability to inhibit both Th1 and Th2-mediated inflammation [39,40]. Moreover, the suppressive phenotype of regulatory T cells is dependent on their preferential expression of galectins 1 and 10 [41,42].

The binding of mammalian galectins to their target glycoproteins is determined by the availability of their polylactosamine ligands. In turn, this is regulated by the activity of Golgi-resident glycosyltransferases, such as core 2 β-1,6-*N*-acetylglucosaminyltransferase (core 2 GnT) and β-1,6-*N*-acetylglucosaminyltransferase V (GnT V), which promote the addition of polylactosamine to *O*- and *N*-linked glycans, respectively [43]. These enzymes allow galectins to bind and cross-link

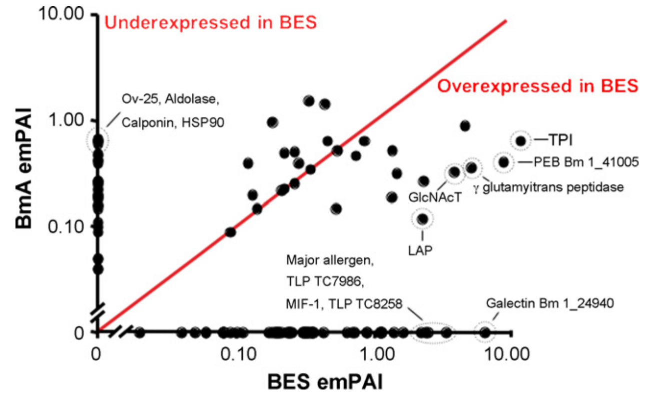


Fig. 3. Relative abundance of proteins in BES and BmA. Bivariant plot of emPAI values of proteins from BES and BmA. Proteins over-represented in either BES or BmA are highlighted.

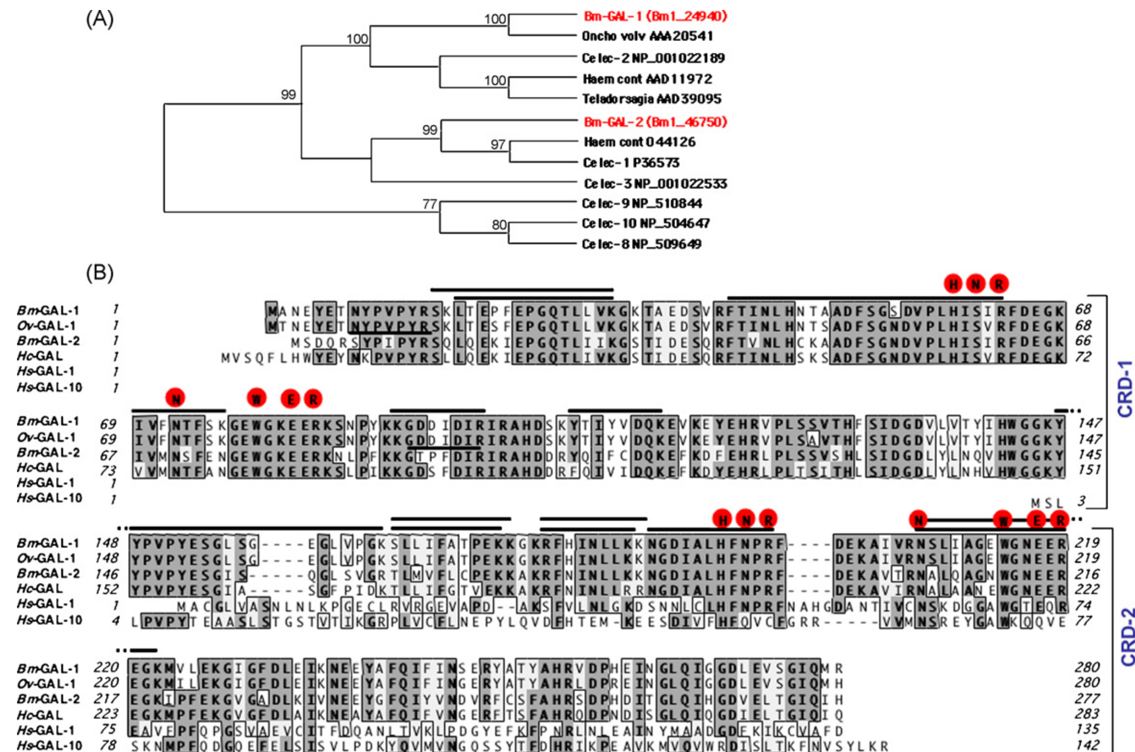


Fig. 4. Comparison of secreted *B. malayi* galectins with parasitic and human galectins. (A) Phylogenetic tree of parasitic nematode galectins. Closely related galectins from *C. elegans* are included for comparison. Values indicate % occurrence of relevant nodes. (B) Sequence alignment of BES galectins with their closest identified homolog. Human (Hs) galectin 1 and 10 are included for comparison. Dark grey boxes indicate identity and light grey boxes indicate similarity. Red circles indicate conserved residues known to interact with carbohydrate ligands [38]. Thick black lines indicate tryptic peptide fragments for the *B. malayi* galectins. Each CRD (carbohydrate recognition domain) is indicated.

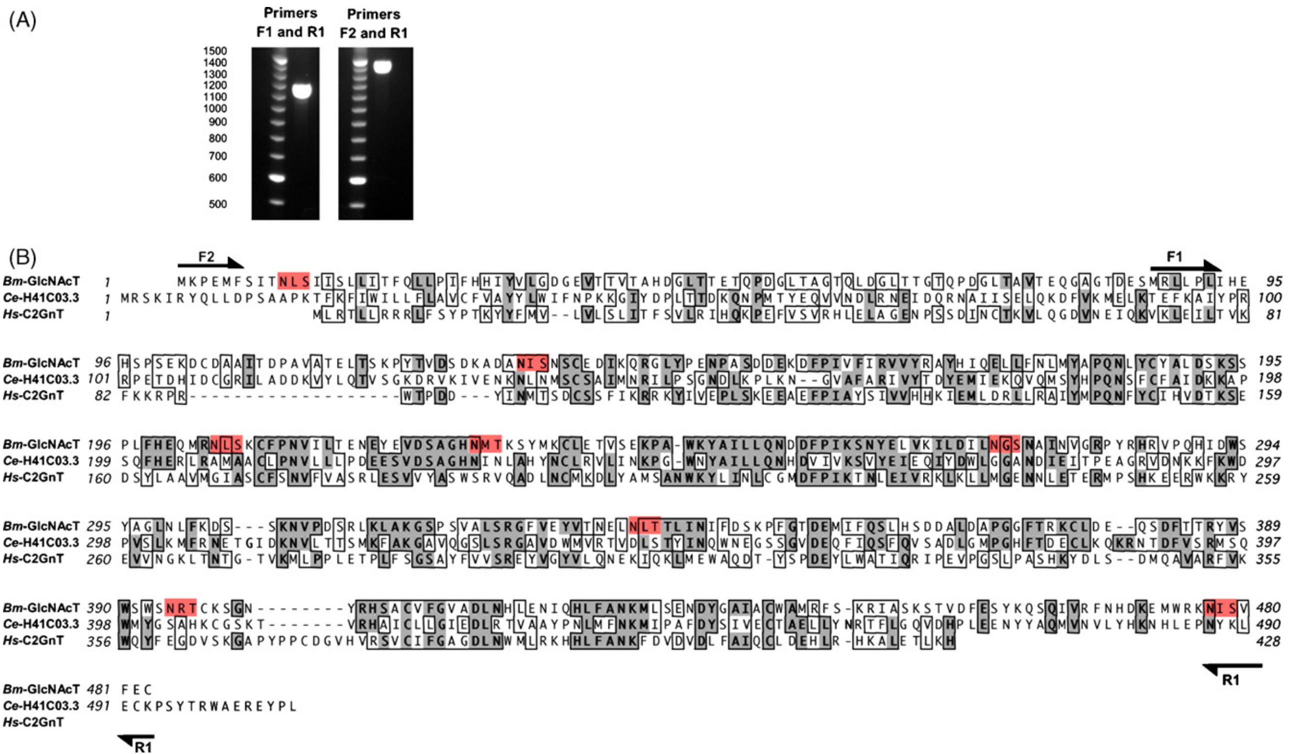


Fig. 5. Detection of putative full-length *N*-acetylglucosaminyltransferase. (A) Expression of the predicted GlcNAcT mRNA was confirmed by RT-PCR of adult *B. malayi* cDNA. Left panel: amplification using primers specific for the draft genome predicted N-terminus of Bm1_07275 (F1) and the predicted C-terminal coding sequence of EST consensus TC7929 (R1). Right panel: amplification of putative full-length GlcNAcT cDNA with primer F2, for upstream N-terminus based on homology between genomic sequence and *C. elegans* GlcNAcT, and R1 as in left panel. PCR product size is indicated by DNA ladder on the left. (B) Sequence alignment of *B. malayi* GlcNAcT with *C. elegans* homologue (H41C03.3) and human core 2 GnT1. Dark grey boxes indicate identity and light grey boxes indicate similarity. Red boxes indicate potential *N*-glycosylation sites of the *B. malayi* protein.

cell surface proteins, creating galectin–glycoprotein lattices, which can contribute to immune down-regulation by increasing the signalling threshold required for T cell activation [44,45]. In this regard, another abundant protein in BES was identified as *N*-acetylglucosaminyltransferase (GlcNAcT; Fig. 2C, spot 20), with different peptides assigned to either draft genome sequence (Bm1_07275) or to an overlapping EST consensus (TC7929) (Supplementary Fig. 1). We first verified by RT-PCR that these sequences corresponded to a single, longer gene (Fig. 5A). Inspection of upstream genome sequence indicated a further, N-terminal, exon that was again confirmed by RT-PCR (Fig. 5A), allowing us to infer the complete sequence of *B. malayi* GlcNAcT (Supplementary Fig. 1). The discrepancy between the calculated (54 kDa) and observed size (~98 kDa) of this protein may be explained by substantial post-translational modifications—the protein contains eight *N*-glycosylation consensus motifs (N-X-S/T), and none of these sequences were identified as tryptic peptides by MALDI-ToF/ToF, consistent with their modification (Fig. 5B and Supplementary Fig. 1). The *B. malayi* GlcNAcT protein has significant homology to human core 2 GnT, as well as to a hypothetical protein from *C. ele-*

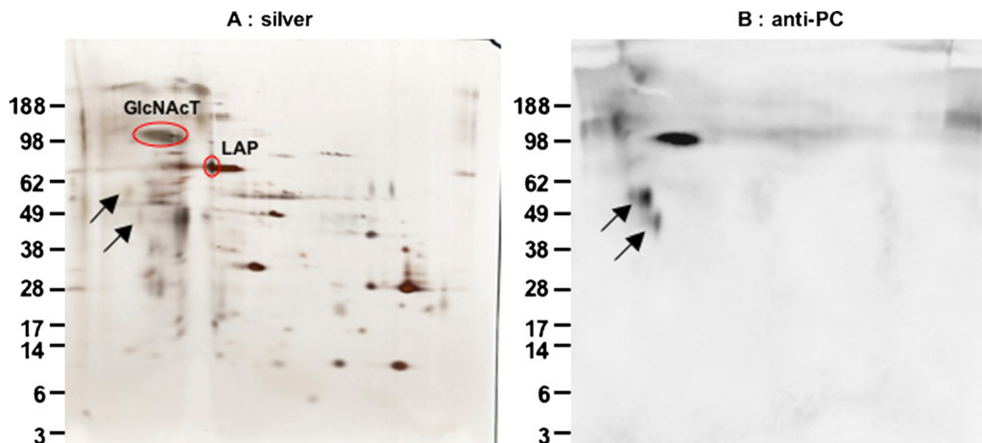


Fig. 6. Attachment of PC-residues to *N*-acetylglucosaminyltransferase. (A) 2D SDS-PAGE gel of BES (10 μg). Circles indicate GlcNAcT and LAP. (B) Duplicate of (A), Western blotted and probed with anti-PC Bp-1 monoclonal antibody. The PC-bearing spot was identified as GlcNAcT (Fig. 2C). Representative of two independent batches of BES. We were unable to identify the minor PC positive proteins (arrows).

gans (Fig. 5B). As yet, it is not known whether *B. malayi* GlcNAcT is capable of inducing protein glycosylation (and galectin binding) in the extracellular milieu, given that glycosyltransferases are usually functional in the Golgi. It is also possible that GlcNAcT may have an alternative extracellular function, as had been demonstrated for a secreted form of human GnT V, which can promote angiogenesis [46].

The *B. malayi* homologue of *Acanthocheilonema viteae* ES-62, LAP, was readily detectable in BES (Fig. 2C, spot 18). The anti-inflammatory properties of ES-62 are well known [47], and are dependent on its modification with phosphorycholine (PC) side chains [48]. Furthermore, the attachment of PC to *B. malayi* proteins allows them to modulate immune cells, demonstrated by their inhibition of mitogen-dependent T cell proliferation [49]. To probe LAP for PC side chains, we used the anti-PC monoclonal antibody Bp-1 [31] on 2D Western blots of BES (Fig. 6A and B). Surprisingly, we could find no evidence for the attachment of PC to LAP. Instead, we identified the most prominent PC-bearing species in BES as the GlcNAcT described above. This is consistent with previous detection of a ∼90 kDa PC-conjugated protein in BES [50]. We were unable to identify the less intense PC-bearing proteins of 45–55 kDa by MALDI-ToF/ToF. It has previously been noted that *B. pahangi* secretes 3-fold less LAP than *A. viteae* does of ES-62 [51], and our data indicate that in *B. malayi*, the majority of PC is coupled to a different carrier protein than LAP. Further functional comparisons between PC-conjugated proteins from the two species will doubtless yield extremely interesting results, and experiments designed to assess the ability of BES to modulate inflammatory diseases may also be warranted.

Also prominent in BES are two proteins, γ-glutamyltranspeptidase (Fig. 2C, three isoforms, spots 14–16) and a protein designated as “major allergen” (two isoforms, spots 11 and 12), that are known to be targets for host IgE, either in experimental animal models or human infection [52,53]. The function of “major allergen” is unknown, although it has weak homology to two proteins from *C. elegans* (ZC412.3 and T10G3.3).

3.3. Potential immune evasion proteins

B. malayi release a number of proteins whose function appears to directly interfere with host effector mechanisms. Prominent among these are several anti-oxidant proteins, which can detoxify the potentially damaging reactive oxygen and nitrogen intermediates produced by immune cells. Both the cytoplasmic and extracellular forms of superoxide dismutase were detected (Table 1; [54]). The major cuticular protein of adult *B. malayi*, gp29 (glutathione peroxidase; [55,56]), which may protect parasite lipids from reactive oxygen attack [57], was also released. Additionally, thioredoxin peroxidase 2 [58] was identified using LC–MS/MS. As well as the above enzymes, two scavenger proteins are present in BES which can act as an anti-oxidants in a non-enzymatic manner: translationally controlled tumour protein [59], and host albumin [60]. It is unclear how host albumin comes to be associated with adult *B. malayi*, although previous studies have detected it on the surface of the microfilarial stage of other filarial parasites [61,62].

Another mechanism by which filarial parasites may dampen the immune response is through the release of protease inhibitors [63]. *B. malayi* encodes three cysteine-protease inhibitors, cystatins (CPI-1, -2 and -3 [64]), and CPI-2 was identified in BES through LC–MS/MS (Table 1). CPI-2 homologues are prominent in the secretions of a range of filarial nematodes (reviewed in Ref. [63]). Bm-CPI-2 can inhibit host proteases involved in antigen processing and presentation, including the key enzyme asparaginyl endopeptidase [65]. Two PEB proteins identified in BES by LC–MS/MS (Table 1; Bm1_41005 and Bm1_31500) may represent additional protease inhibitors. Mouse PEB, to which the *B. malayi* proteins share significant sequence similarity (Supplementary Fig. 2), has been shown to be a novel type of serine protease inhibitor [66]. However, it is not yet known whether the *B. malayi* proteins, or homologues from other helminths (such as *O. volvulus* Ov-16 [67] and *Toxocara canis* TES-26 [68]) share this inhibitory function.

B. malayi secrete three members of the immunophilin family (Table 1), specifically two cyclophilins (CYP-2 (see also Fig. 2C, spot 4) and CYP-5) and FKBP (FK506-Binding Protein)-12. Immunophilins are characterised by peptidyl-propyl *cis–trans* isomerase activity, and have roles in protein folding and as molecular chaperones [69]. It is unclear what, if any, role these proteins have in parasite immune evasion, although secreted cyclophilins have been identified in the ES of *H. contortus* [3],and schistosome larvae and eggs [67]. Of note, a cyclophilin (CYP-18) from the protozoan parasite *Toxoplasma gondii* is directly involved in host–parasite crosstalk, as it can stimulate protective Th1 responses through its binding to the chemokine receptor CCR5 [70].

A wide variety of pathogens, from viruses to helminths, are able to subvert the host immune response through the production of host cytokine mimics [71]. *B. malayi* produces two homologues of macrophage migration inhibitory factor (Bm-MIF-1 and -2) [72,73]. In our analysis, MIF-1 is readily detectable in BES through both LC–MS/MS and 2D gel electrophoresis (Table 1 A and Fig. 2C, spot 2). It is known that MIF-2 is expressed at a lower level [73], and so its release may be below our lower limit of detection. Similarly, we have not detected the expression of homologues of host TGF-β (Bm-TGH1 and TGH-2), although secretion of the latter has been reported [74]. TGF-β family members are potent at extremely low concentrations, and again may be present below our lower detection threshold. There was also no evidence of secretion of two recently assigned cytokine homologues, similar to IL-16 and IL-17, which have recently been identified at the genomic level [6].

3.4. Newly identified secretory proteins and unidentified proteins

The benefit of applying a proteomic-based approach to the study of BES is it can identify previously undiscovered secretory proteins. In this respect, we have found that adult *B. malayi* secrete 11 members of the transthyretin-like protein (TLP) family (Table 1). The *B. malayi* draft genome reveals 15 TLP genes [6], of which 6 are detected in BES. The remaining five proteins (TC8116 – also identified as spot 3, Fig. 2C – as well as TC7985, TC8258, TC7986, AA592049) are tentative consensuses or ESTs from the *B. malayi* gene index project, and have no match in the current draft version of the genome. TLP are distantly related to both the classical transthyretin (TTR) and transthyretin-related protein (TRP) families (Fig. 7) [75]. TTR is responsible for the transport of thyroid hormones, as well as vitamin A and its derivative retinoic acid [76]. TLP are also secreted by the cattle helminths *Ostertagia ostertagi* (AJ31875; [77]) and *H. contortus* (BM139192, BM138829, BF423163 [3]), although their function is currently unknown.

In addition to newly discovered secreted proteins with close matches from other parasites, 7 ES products matched predicted proteins in the draft genome with no known homologue. Three closely related hypothetical proteins (Bm1_11505; Bm1_01245; Bm1_09845) were identified, which are unusual in being composed of multiple homologous repeats of ∼70 amino acids, including an acidic tract (QEEEEEEE), as well as being rich in serines and threonines (Supplementary Fig. 3). Together these characteristics support the assignation of mucin-like proteins in Table 1. Four other hypothetical proteins were identified, of which two had weak homology to entries from either *C. elegans* (Bm1_46475 to KO1A11.1) or *C. briggsae* (Bm1_19065 to CBG09470).

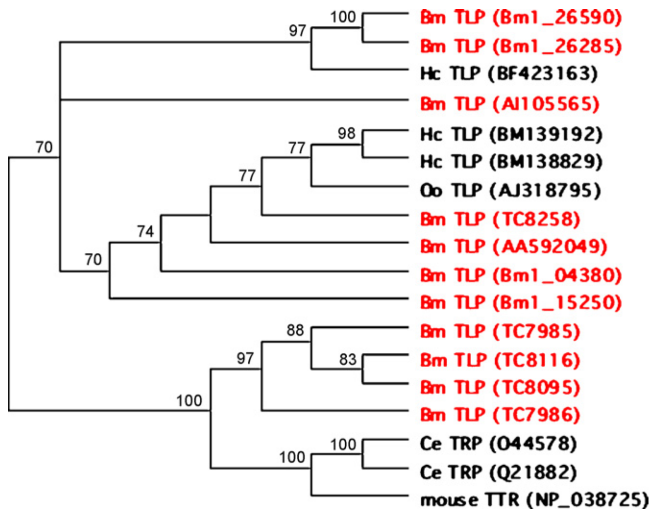


Fig. 7. Transthyretin-like proteins. Phylogenetic tree of TLP, TRP and TTR from *B. malayi* (Bm), *H. contortus* (Hc), *O. ostertagi* (Oo), *C. elegans* (Ce) and mouse. Values indicate % occurrence of relevant nodes.

As is normal with LC–MS/MS analysis, many peptide masses could not be unequivocally assigned to an individual gene product. However, we were surprised that 7 out of 30 spots from 2D analysis also could not be matched, despite generating good spectra and exhaustive searching of genomic and EST datasets (Fig. 2C, blue circles). Because the draft genome is estimated to be 90% complete, it may be that these proteins corresponded to genes that have yet to be identified at the genomic level. However, as sufficient sample quantities become available, *de novo* peptide sequence deduction will be carried out, from which experimental searching of genomic DNA with degenerate primers may allow us to isolate the corresponding gene sequences.

B. malayi contains a rickettsial endosymbiont, *Wolbachia* (*wBm*), which is essential for the survival and reproduction of the parasite [78]. It has also been postulated that *Wolbachia*-derived products may impact upon the host immune system. We therefore, specifically searched for matches to the 1.1 Mb genome of wBm [79], but found none. Hence, in our system at least, *Wolbachia* proteins are not strongly represented in the secretions of viable adult parasites. It is, of course, very likely that dying parasites would release *Wolbachia* proteins, and the relative influence of parasite and endosymbiont products on host immunity remains to be determined.

3.5. Human sera recognition

To define which, if any, of the identified BES proteins were targets for immune recognition in human filarial infections, Western blots were carried out using two pools of characterised sera from Indonesian filariasis patients. We have previously reported on the reactivity of individual members of these pools to extracts of whole worm [32], which will measure immune responses to the totality of somatic antigens, many of which are likely to be presented only after parasite demise. Immunoreactivity to BES, however, is likely to

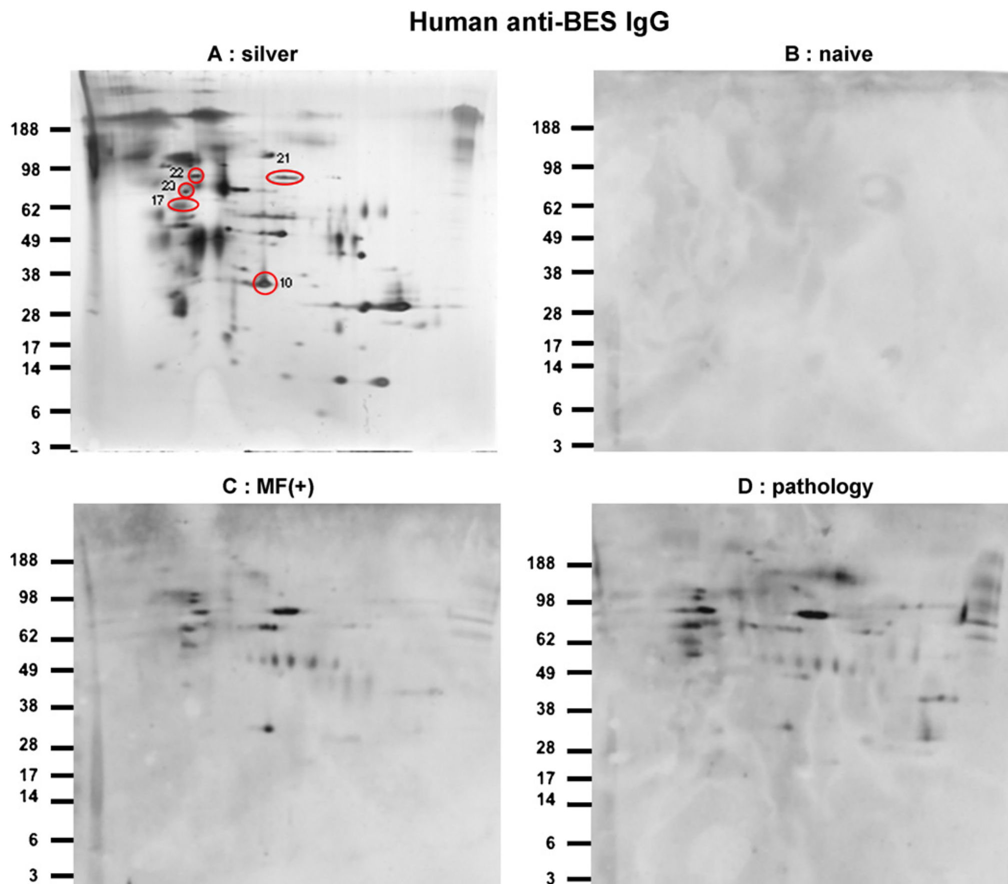


Fig. 8. Recognition patterns of anti-BES IgG in *B. malayi* infected patients. (A) One of 4 replicate 2D gels of BES, silver stained. (B) Replicate, Western blotted and probed with sera from non-exposed "naïve" individuals. (C) Replicate, Western blotted and probed with sera from microfilaraemic patients. (D) Replicate, Western blotted and probed with sera from elephantiasis pathology patients. Immunogenic proteins are circled in (A) and are numbered as in Fig. 2C with identities in Table 1.

reflect the immune response directed against live parasites. A particularly important question is whether clinical outcome can be correlated to the recognition of a particular antigen/group of antigens.

To begin to answer this question, we probed 2D blots of BES with pooled sera from filariasis cases and control individuals. The silver-stained replicate of BES proteins in this analysis is shown in Fig. 8A, and no significant reactivity was observed with sera from uninfected UK residents (naïve; Fig. 8B). However, asymptomatic microfilaraemic (MF+; Fig. 8C) or elephantiasis (pathology; Fig. 8D) serum pools both reacted with a number of common proteins. Overall, it is clear that sera from elephantiasis patients bind a broader spectrum of proteins than sera from microfilaraemic individuals. In general, protein abundance and immunogenicity correlated poorly. For instance, the most abundant protein in BES, TPI, was not a target for human sera (Fig. 8C and D). Instead, the most immunogenic protein in both groups was identified as myotactin form B, which is present as spots 21–23 (Figs. 2 and 8).

It is notable that myotactin B is represented by three distinct spots of 70–90 kDa on 2D gels. However, the predicted *B. malayi* myotactin B gene (Bm1_53510) encodes a much larger protein of 285 kDa. Analysis of the tryptic peptides generated by MALDI-ToF/ToF shows that the spots represent different regions of the full-length myotactin B protein; spot 21 peptides corresponding to amino acids 303–677, spot 22 to 983–1539, and spot 23 to 1020–1347 (Supplementary Fig. 4). This profile is likely to reflect proteolytic processing of the full-length protein, either directly from the parasite or following its secretion, although alternative splicing cannot be excluded. Another immunogenic protein (spot 17) was identified as lethal protein 805d. Both this protein and myotactin B are related to *C. elegans* myotactin (also known as lethal protein 805), a protein that links the contractile apparatus of the worm to its hypodermis [80]. The significance of this protein's secretion by *B. malayi* is unclear, although its immunogenicity in infected patients is consistent with a role as a decoy for the host immune response. Galectin Bm1_24940 was also an antibody target in both groups of filariasis patients, although perhaps surprisingly, it was recognised to a greater extent by (the generally less immunoreactive) MF+ sera (Fig. 8C). This work paves the way for future studies to analyse at the individual level the response of different human antibody isotypes, particularly IgG4 and IgE, to specific identified BES antigens.

4. Conclusions

The secretome of the long-lived mammalian stage of a successful parasite will encompass a spectrum of proteins required for every facet of the parasitic life-style, from metabolism, reproduction, and modification of the physiological environment, as well as immune evasion. For some of the adult BES proteins we have identified (such as the cystatin, CPI-2), a specific role in immune modulation has already been established [63,65]. Our analysis greatly extends the number of candidate immune evasion products, including a number of unexpected players, such as the galectins, *N*-acetylglucosaminyltransferase, the transthyretin-like proteins, and the phosphatidylethanolamine-binding proteins, each of which fully warrant further investigation.

Many of the additional secreted proteins identified here, however, have no obvious role in the host–parasite immune interaction. It is likely that some act in other arenas, for example in aiding parasite co-location and reproduction, but if so then they may serve equally well as the target of an antibody-inducing vaccine aimed at preventing transmission. Inevitably, some of the products designated as 'secretory', may in fact simply be cellular proteins which are sufficiently abundant and soluble to be released by live parasites over time. Nevertheless, if such release occurs *in vivo*, then the immunological consequences may be significant, not only as host innate immune receptors may have evolved to recognise these as pathogen-associated molecular patterns, but also (as in the case of filarial tropomyosin) because they may also be effective targets of vaccine-induced immunity [81,82]. It will be interesting to test TPI as a potential vaccine target in the same manner.

The analysis reported here has been limited in that quantities of BES proteins were, in some instances, less than desired; and also because both genomic and EST datasets offer incomplete coverage. Currently the draft *B. malayi* genome covers around 90% of coding regions (13 of 80, or 16%, of identities we report match ESTs but not genomic data). As the sensitivity of mass spectrometry increases, and the scope of filarial database information widens, additional BES proteins will be identified. The identification of individual ES proteins will now facilitate discovery of complementary host receptors, and host cell types, with which parasite products may interact. In terms of the wider molecular biology of parasite secretions, future work should also expand on our knowledge of post-translational modifications, in particular glycosylations and other side chains such as PC [48] and DMAE [83]. Attention should be paid to the source of ES, perhaps using identified proteins as markers (for example, where monoclonal antibodies are available), looking particularly to distinguish dedicated secretory organs such as the amphid glands, from the uterine contents of gravid females which may be voided together with newly released MF. Finally, and perhaps most importantly, the study of filarial ES proteins should encompass the immature stages, the blood-dwelling MF and the infective L3 stage, to complete our understanding of the *B. malayi* secretome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2008.02.007.

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