Proteomics Analysis and Protein Expression during Sporozoite Excystation of *Cryptosporidium parvum* (Coccidia, Apicomplexa)*

William J. Snelling‡§, Qishan Lin¶, John E. Moore‖, B. Cherie Millar‖, Fabio Tosini**‡‡, Edoardo Pozio**‡‡, James S. G. Dooley‡, and Colm J. Lowery‡§§

Cryptosporidiosis, caused by coccidian parasites of the genus *Cryptosporidium*, is a major cause of human gastrointestinal infections and poses a significant health risk especially to immunocompromised patients. Despite intensive efforts for more than 20 years, there is currently no effective drug treatment against these protozoa. This study examined the zoonotic species *Cryptosporidium parvum* at two important stages of its life cycle: the non-excysted (transmissive) and excysted (infective) forms. To increase our understanding of the molecular basis of sporozoite excystation, LC-MS/MS coupling with a stable isotope N-terminal labeling strategy using iTRAQ™ reagents was used on soluble fractions of both non-excysted and excysted sporozoites, *i.e.* sporozoites both inside and outside oocysts were examined. Sporozoites are the infective stage that penetrates small intestinal enterocytes. Also to increase our knowledge of the *C. parvum* proteome, shotgun sequencing was performed on insoluble fractions from both non-excysted and excysted sporozoites. In total 303 *C. parvum* proteins were identified, 56 of which, hitherto described as being only hypothetical proteins, are expressed in both excysted and non-excysted sporozoites. Importantly we demonstrated that the expression of 26 proteins increases significantly during excystation. These excystation-induced proteins included ribosomal proteins, metabolic enzymes, and heat shock proteins. Interestingly three Apicomplexa-specific proteins and five *Cryptosporidium*-specific proteins augmented in excysted invasive sporozoites. These eight proteins represent promising targets for developing vaccines or chemotherapies that could block parasite entry into host cells. *Molecular & Cellular Proteomics 6:346–355, 2007.*

From the ‡Centre for Molecular Biosciences, School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine, County Londonderry BT52 1SA, Northern Ireland, ¶UAlbany Proteomics Facility, Centre for Functional Genomics, University of Albany, Rensselaer, New York 12144, ‖Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 7AD, Northern Ireland, and **Department of Infectious, Parasitic, and Immunomediated Diseases, Istituto Superiore di Sanità, Rome 00161, Italy

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The protozoan parasite *Cryptosporidium parvum*, which causes acute gastroenteritis in both humans and animals, is considered an important pathogen all over the world (1). *Cryptosporidium* is one of several genera in the phylum Apicomplexa, whose members share a common apical secretory apparatus mediating locomotion and tissue or cellular invasion (2). There are currently 16 recognized species in this genus (3), all of which have four naked sporozoites contained within a thick walled oocyst (Fig. 1*A*), the resistant and transmissive form of these parasites (4). Species of the genus *Cryptosporidium* have a complex life cycle. Upon ingestion, excystation of viable oocysts (4–6-μm diameter for intestinal species; gastric species are slightly larger) is triggered (5, 6). Following excystation, sporozoites (Fig. 1*B*; the infective stage) attach to the intestinal epithelium, are enveloped by the apical membrane, and reside in an intracellular, extracytoplasmic parasitophorous vacuole (5, 7). In this peculiar niche, the parasite undergoes an endogenous asexual and sexual reproductive development (Fig. 1*C*), culminating in the production of an encysted stage discharged in the feces of their host (6, 8). Coccidian oocyst stages are highly resistant to environmental stress and treatments, *e.g.* chemical disinfection; this is attributed to a durable oocyst wall, a complex protective barrier consisting of a double layer of a protein-lipid-carbohydrate matrix, allowing the parasite to stably persist outside hosts (2, 6). *C. parvum* causes self-limited watery diarrhea in immunocompetent subjects but is life-threatening in immunocompromised patients (1, 9, 10). Cryptosporidiosis is also responsible for significant neonatal morbidity in farmed livestock and causes weight loss and growth retardation, leading to large economic losses (11). *C. parvum* is transmitted by fecal contamination of food or water or by accidental ingestion of infectious oocysts during water-related recreational activities (12). In 1993, the largest *Cryptosporidium* spp. outbreak was registered in Milwaukee, WI where 403,000 people were infected through contaminated drinking water (13) with associated economic costs of $31.7 million in medical costs and $64.6 million in productivity losses (14). Over the last 2 decades increasing numbers of cryptosporidiosis outbreaks have been recorded in developed countries, and the importance of the zoonotic species *C. parvum* is being recognized by both government agencies and the global scientific community (15, 16). Unlike many organisms belonging to the phylum Apicomplexa, such as *Plasmodium* spp. and

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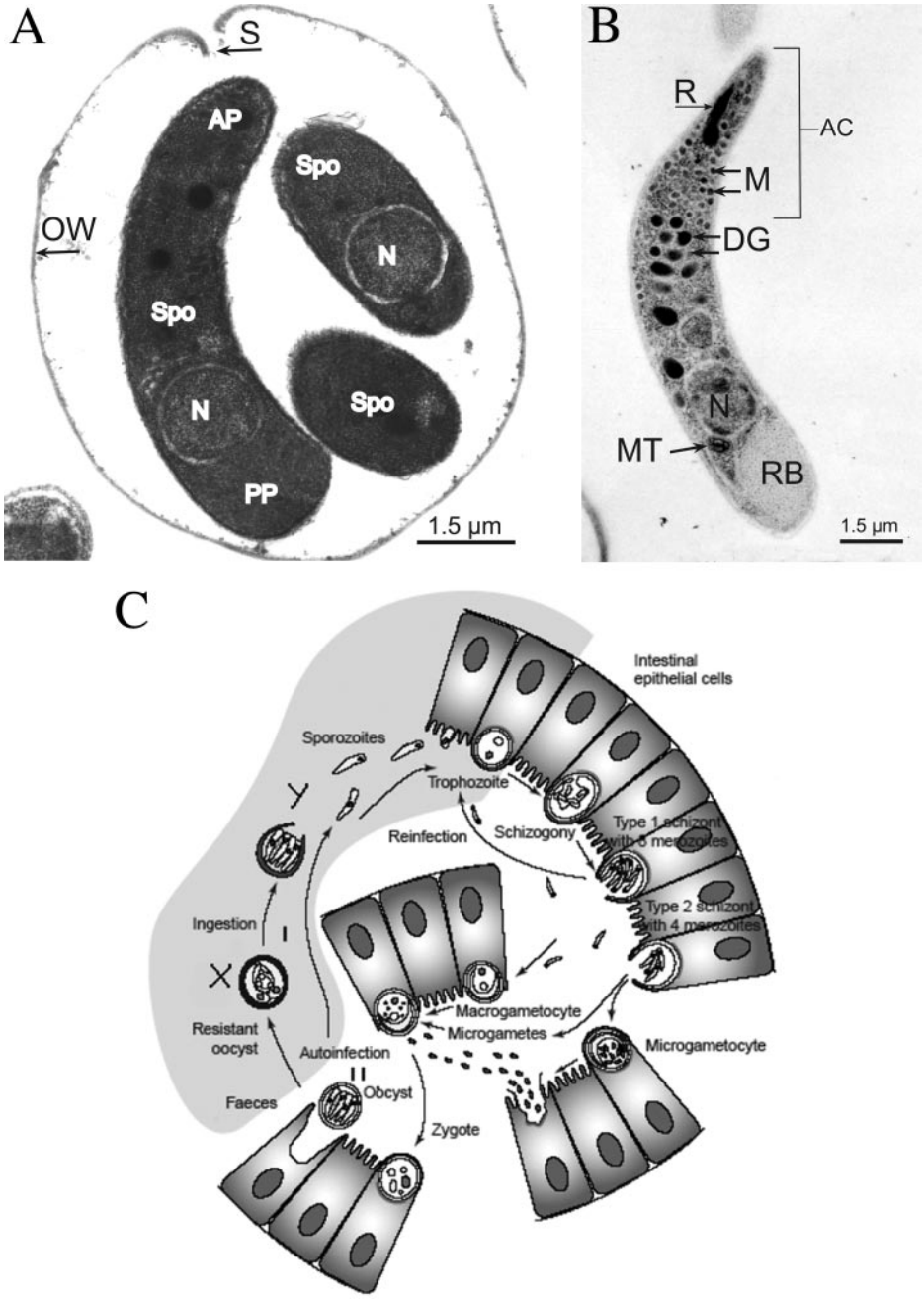

FIG. 1. ***A–C*, the structure of *C. parvum* and its life-cycle stages.** *A*, a transmission electron microscopy image of a non-excysted *C. parvum* oocyst, which contains four sporozoites. Three of the sporozoites (of four) are visible, one in a longitudinal plane and the other two in transverse planes. *AP*, anterior pole; *OW*, oocyst wall; *N*, nucleus; *PP*, posterior pole; *S*, scissure; *Spo*, sporozoite. *B*, transverse section of a free excysted sporozoite showing the apical complex and the organelles. *AC*, apical complex; *DG*, dense granules; *M*; micronemes; *MT*, mitochondrion; *N*, nucleus; *R*, rhoptry; *RB*, refractile body. *C*, the stages of *Cryptosporidium* spp. life cycle (5). During this investigation oocysts were analyzed at both non-excysted (*x*) and (excysted) (*y*) stages of this cycle. Upon ingestion by the host, sporozoites are released and adhere directly to the intestinal epithelial cells of the host. Cell invasion by sporozoite is followed by intracellular development to trophozoite. Trophozoite undergo schizogony to form schizonts. Asexual replication occurs by reinfection of merozoites released by type I schizonts. Development of type II from type I schizonts is the initial step of the asexual reproductive cycle. Type II merozoites are released and reinfect neighboring cells where they develop into microgametocytes (male) or macrogametocytes (female). The macrogametocyte is fertilized by released microgametes and matures into a zygote, which undergoes further development into an oocyst. Two types of oocysts are released: thick walled oocysts, which are excreted in the feces (*I*), or thin walled oocysts for endogenous reinfection (autoinfection) (*II*).

Toxoplasma gondii, there is no clinically proven effective drug treatment against *Cryptosporidium* spp. (17). Proteomics profiling is a useful approach for obtaining a global overview of the proteins present in a system under differing conditions and can aid in understanding the molecular determinants involved with pathogenesis and vaccine development (18). Compared with other apicomplexans, *e.g. Toxoplasma gondii*, both the limited supply of purified parasite material and the lack of transfection systems have restricted analyses of proteins in parasites of the genus *Cryptosporidium* (15). The genomics analysis of *C. parvum* and the closely related *Cryptosporidium hominis* (19) has revealed extremely streamlined metabolic pathways and a reliance on hosts for nutrients (20). *C. parvum* has an animal-type *O*-linked glycosylation pathway and >30 predicted surface proteins with mucin-like segments (21).

The mechanisms involved in *Cryptosporidium* spp. excystation are incompletely understood; although roles for both host- and parasite-derived components are recognized, little is known about their precise involvements (5). *In vitro* excystation protocols for *C. parvum* mimic host-derived signals, including temperature (37 °C), pH fluctuations, and bile salts (5). We adopted an accepted *in vitro* excystation method (22) and analyzed *C. parvum* proteins at two key stages, both the

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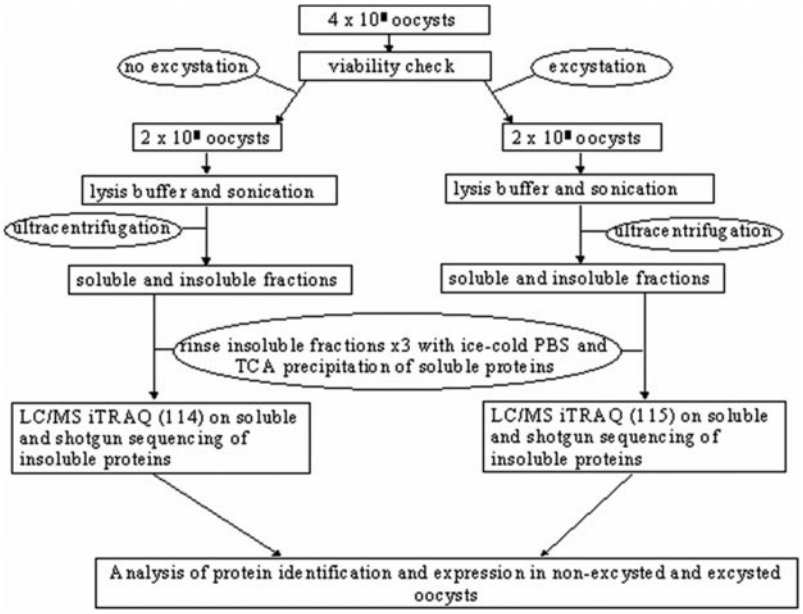

FIG. 2. **Scheme of the overall workflow used for the proteomics analysis of *C. parvum*.**

transmissive (oocysts) and infective stages (sporozoites), of its life cycle (5, 6). Thus, as well as evaluating the proteome of *C. parvum*, a major aim of our study was to identify proteins that demonstrated significant increases of expression during oocyst excystation, a vital step of the pathogenesis of these coccidian parasites (6). This was achieved by using LC/MS and iTRAQ™ reagent isotope labeling, providing a powerful tool for the identification and quantification (23, 24) of soluble *C. parvum* proteins. Using LC/MS with iTRAQ the protein expression in non-excysted and excysted soluble *C. parvum* oocyst fractions was compared. Then using shotgun peptide sequencing (25) the insoluble protein content of non-excysted and excysted oocysts was also analyzed.

EXPERIMENTAL PROCEDURES

Oocyst Isolation and Sporulation—To recover high numbers of *C. parvum* oocysts, with minimal fecal and bacterial contaminants (26), 4×10^8 *C. parvum* oocysts (strain code ISSC162) were recovered from experimentally infected calves using feces purification with sucrose and Percoll density gradients (27). Purified oocysts were stored at 4 °C for no longer than 3 weeks. Using 4′,6′-diamidino-2-phenylindole-propidium iodide assays (28), oocyst viability was determined to be at least 92%. To obtain free sporozoites, half of the oocysts (2×10^8) were excysted as described previously (Fig. 2) (22). Briefly oocysts were resuspended in 10 mM HCl (Sigma) and incubated at 37 °C for 10 min. The suspension was centrifuged at 3,000 × *g* for 5 min. The pellet was then resuspended in 2 mM sodium taurocholate in PBS (Sigma) and incubated at 15 °C for 10 min followed by incubation at 37 °C for 8 min, and a high level of excystation, *i.e.* at least 90%, was confirmed (29).

C. parvum Lysis, Protein Precipitation, and Fraction Preparation—All excysted and non-excysted oocysts were solubilized (30 min at 4 °C) in lysis buffer (50 mM Tris (Bio-Rad) 5 mM EDTA (Sigma), 5 mM iodoacetamide (Sigma), 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), 1% (w/v) octyl glucoside (Roche Applied Science)) and stored at −80 °C (30). Upon thawing, samples were sonicated with 20 10-s pulses (50 watts) on ice with 1-min intervals and were then ultracentrifuged at 4 °C (20,000 × *g* for 30 min) to separate the soluble and insoluble fractions (30). Insoluble fractions were then rinsed three times with ice-cold PBS and stored at −80 °C. Proteins in soluble lysate fractions were then precipitated by mixing oocyst lysates with 8 volumes of ice-cold acetone, 1 volume of TCA, and 0.7% (w/v) 2-mercaptoethanol at −20 °C for 1 h and then centrifuged at 18,000 × *g* for 15 min at 4 °C (31, 32). Pellets of precipitated soluble proteins were then washed with 1 ml of ice-cold acetone, centrifuged at 18,000 × *g* for 15 min at 4 °C, covered in a layer of ice-cold acetone, and stored at −80 °C.

Trypsin Digestion, Shotgun Peptide Sequencing, and iTRAQ Isobaric Labeling—The pellets (soluble and insoluble fractions) were resuspended in 400 μl of sample preparation buffer (100 mM Tris-HCl, 8 M urea, 0.4% SDS, 5 mM tributylphosphine, pH 8.3) and placed on ice (33). Sample mixtures were sonicated with five 20-s pulses (50 watts) with 1-min intervals on ice followed by iodoacetamide alkylation at room temperature for 1 h (34). Samples were then ultracentrifuged at 100,000 × *g* for 20 min at room temperature. Protein concentrations were measured using a MicroBCA protein assay kit (Pierce) according to the manufacturer's instruction (35) before reactions were quenched by the addition of DTT (33). The protein mixtures were diluted 8-fold in 50 mM Tris-HCl. Modified trypsin (Sigma) was added to a final substrate-to-enzyme ratio of 30:1, and the trypsin digests were incubated overnight at 37 °C. The peptides from each digest solution were acidified to pH 3.0 with formic acid (FA)[1] and loaded onto a Discovery DSC-18 cartridge (Sigma). Then peptides were desalted (5 ml of 0.1% FA) and eluted with 5 ml of a solution composed of 50% ACN with 0.1% FA.

Peptides derived from insoluble fractions were analyzed qualitatively using shotgun peptide sequencing as described previously (36). For peptides derived from soluble fractions, equal amounts (100 μg) of sample were labeled with two iTRAQ reagents (Applied Biosystems, Foster City, CA) following the manufacturer's instruction: iTRAQ-114 (non-excysted) and iTRAQ-115 (excysted). Briefly after desalting on a C_{18} cartridge the peptide mixture was lyophilized and resuspended in 30 μl of 0.5 M triethylammonium bicarbonate

[1] The abbreviations used are: FA, formic acid; ARM, armadillo repeat; HSP, heat shock protein; LCCL, *Limulus* factor C, cochlear protein Coch-5b2, and late gestation lung protein; QqTOF, quadrupole time of flight.

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($N(Et)_3HCO_3$), pH 8.5. The appropriate iTRAQ reagent (dissolved in 70 μl of ethanol) was added, allowed to react for 1 h at room temperature, and then quenched with 300 μl of double distilled H_2O.

Off-line Strong Cation Exchange Chromatography—iTRAQ-labeled peptides were then concentrated, mixed, and acidified to a total volume of 2.0 ml. They were then injected into an Agilent 1100 HPLC system with a Zorbax 300-SCX column (15 cm × 4.6 mm, 5 μm) (Agilent, Waldbronn, Germany). Solvent A was 5 mM $KHPO_4$ and 25% ACN (pH 3.0), and solvent B was 350 mM KCl in solvent A. Peptides were eluted from the column with a 40-min mobile phase gradient of solvent B. A total of 30 fractions were collected, and samples were dried by a SpeedVac prior to LC-MS/MS analysis.

On-line Nano-LC ESI QqTOF MS Analysis—A nanobore LC system (Dionex, Sunnyvale, CA) that was interfaced to a QSTAR XL QqTOF mass spectrometer with a NanoSpray ion source (Applied Biosystems) was used for mass spectrometry. The Magic C_{18} column (100-Å pore, 75-μm inner diameter × 150 mm; Picofrit Woburn, MA) was packed in house. Solvent A was 3% ACN, 0.1% FA, and 0.01% TFA, and solvent B was 98% ACN, 0.1% FA, and 0.01% TFA. Peptide mixtures (reconstituted in 200 μl of 5% FA) were injected and eluted from the column with a 110-min mobile phase solvent B gradient (5–5% B in 5 min, 5–18% B in 10 min, 18–30% B in 65 min, 30–60% B in 10 min, 60–90% B in 10 min, and 90–90% in 5 min) at a flow rate of 250 nl/min. The mass spectrometer was operated in an information-dependent acquisition mode whereby following the interrogation of MS data (*m/z* 350–1500) using a 1-s survey scan ions were selected for MS/MS analysis based on their intensity (>15 cpm) and charge state (+2, +3, and +4). A total of three product ion scans (2, 3, and 3 s each) were set from each survey scan. Rolling collision energies were chosen automatically based on the *m/z* and charge state of the selected precursor ions. The integrated data appliance Extensions II script was set to one repetition before dynamic exclusion.

Protein Identification and Quantification—Identification and quantitation was performed using Pro ID (37) and ProQUANT software 1.1 (38) (Applied Biosystems) using the non-redundant database *C. parvum* subdatabase (9811 entries) with an MS and MS/MS mass tolerance of 0.15 Da (39). Variable modification on methionine (oxidation, +16 Da) and fixed modification on cysteine (carbamidomethylation, +57 Da) residues were considered during the searching (39). Protein identification with confidence scores of >95% were considered significant, and the false positive rate was determined from decoy database (39) searching (data not shown). The identifications of all detected *C. parvum* proteins were then verified using BLASTP at www.ncbi.nlm.nih.gov/BLAST/ or where appropriate at CryptoDB 3.3 (cryptodb.org/cryptodb/).

Using ProQUANT software, quantification was based upon the signature peak areas (*m/z* 114 and 115) and corrected according to the manufacturer's instructions to account for isotopic overlap. Briefly non-excysted oocysts (control) were labeled with isobaric tag 114, and relative quantification ratios of the identified proteins were calculated, averaged, and corrected for systematic error in labeling from the iTRAQ peptides. For proteins in which the iTRAQ ratio of every peptide (labeled with tag 115) was 0, the average iTRAQ ratio was set to 0, an indication of a protein that may be highly down-regulated relative to the control. Similarly for those proteins where every (tag 115) ratio was 9999, the average was set to 9999 (indicating a protein that may be highly up-regulated). Statistically significant changes were weighted by the error factor (a measure of the variation between the different iTRAQ ratios for the reagent pair) and *p* value (95% confidence interval, which defined a range into which the true average iTRAQ ratio was 95% likely to fall). For instance, for a ratio of 1.0 (indicating no change in expression levels) with an error factor of 2 (and the corresponding 95% confidence interval of 0.5–2.0), the protein expression ratio and error were reported as 1.0 ($p < 0.05$ with an error factor of 2). Ratios in which the total peak area was <40 counts were omitted. If there were fewer than two peptides contributing toward an iTRAQ ratio average, the error factor and *p* values were not valid and were not calculated. Proteins that were augmented significantly during excystation had a *p* value <0.05 and an error factor <2.

Bioinformatics Analysis of 26 Proteins That Were Significantly Augmented during Excystation—Significantly overexpressed proteins were examined using BLASTP both on the National Center for Biotechnology Information (NCBI) database and on ApiDB (www.apidb.org/blast/) to find similarity with other apicomplexan proteins or with proteins from unrelated species. Only proteins identified by the BLASTP search with an expected value lower than 10^{-10} were considered homologs. Searches were also performed for molecular traits and domains of the putative proteins using SMART (smart.embl-heidelberg.de/). The predicted coding sequence for the hypothetical protein cgd6_4460 (N46) was extended (results are currently unfinished in CryptoDB), by downloading a larger portion of the AAEE01000002 contig. Gene sequences were predicted using Genscan (http://genes.mit.edu/GENSCAN.html).

RESULTS

Protein Expression in the Soluble Fractions of Excysted and Non-excysted Oocysts—Using LC/MS with ICAT, 142 proteins were detected in both soluble fractions of excysted and non-excysted oocysts (Supplemental Table 1). Ribosomal proteins constituted a significant proportion (35 proteins; 24.7%) of proteins that were detected in both of the soluble fractions. Twenty-five proteins (17.6%) were detected that had hitherto been described as being only hypothetical proteins, *e.g.* N77 (Fig. 3 and Table I). Six heat shock proteins and 17 secreted proteins were also expressed in both excysted and non-excysted oocysts. Many of the detected proteins are involved in infection/pathogenesis, energy pathways (*e.g.* glycolysis), cellular division and replication, and DNA modification. The protein expression of 26 proteins was significantly greater ($p < 0.05$ and error factor <2) in excysted oocysts (Table I).

Bioinformatics Analysis of the 26 Proteins That Were Augmented during Excystation—There were 18 ubiquitous proteins, which are highly conserved in many species from different phyla. Seven proteins that had hitherto been described as only being hypothetical proteins were augmented significantly during excystation. Four are associated with ribosomes, and four are heat shock proteins. A number of housekeeping (*e.g.* lactate dehydrogenase (N102)) or structural genes (*e.g.* histone H4 (N118)) showed significant increases of expression. The protein Chro.50226 (N35) was a ubiquitous ribosomal protein, L3. Eight proteins are restricted to the *Cryptosporidium* genus or to Apicomplexa and consequently could play a specific role in the invasion process (Table I). Five of these proteins appear to be restricted to the *Cryptosporidium* genus (Table I). The hypothetical protein with signal peptide EAK88888 (N77) is highly conserved both in *C. parvum* and *C. hominis* (Table I). EAK88888 demonstrates a low similarity with proteins of the genus *Plasmodium*; however, the *E* value (1×10^{-8}) is higher than the fixed cutoff point. Therefore, EAK88888 is probably more closely related to sim-

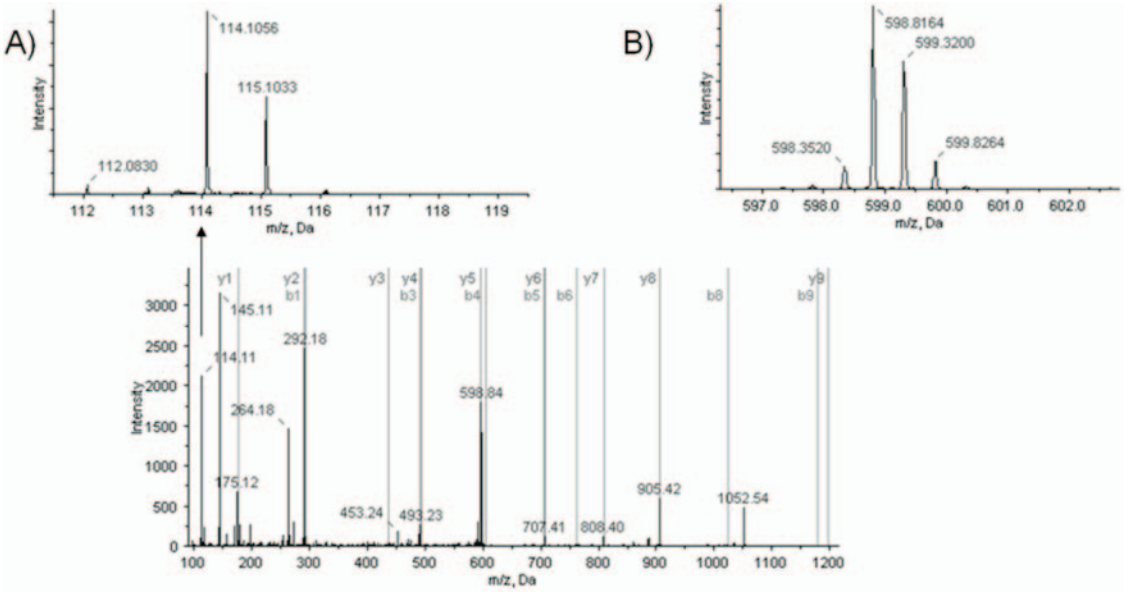

FIG. 3. **Example of MS/MS spectrum of peptide FPTLTGFNR (hypothetical protein with signal peptide EAK88888; N77) from a protein digestion mixture prepared by labeling two separate digests with tag 114 and 115, respectively, and combining the reaction mixtures in a 1:1 ratio.** *A*, isotopic distribution of a doubly charged precursor $[M + 2H]^{2+}$, *m/z* 598.8164. *B*, low mass region showing the signature ions used for quantification. The peptide was labeled by isobaric tags at the N terminus.

ilar proteins present in the genus *Plasmodium* than it appears from *C. parvum* and *C. hominis*. However, these proteins contain repetitive motifs that make the identification of a consensus sequence difficult. Three proteins (hypothetical protein cgd6_4460 (N46), hypothetical protein cgd7_4280 (N51), and Cpa135 protein (a135; N64)) demonstrate similarity with proteins from other Apicomplexa genera (*Plasmodium* and/or *Toxoplasma*) but not with proteins from other phyla (Table I).

Identification of Proteins from the Insoluble Fractions of Oocysts—Using shotgun sequencing 122 proteins were detected from the non-excysted insoluble oocyst fraction (Supplemental Table 2). Thirty-four (27.9%) of these detected proteins were ribosomal, and 26 of the proteins (21.3%) had hitherto been described as being only hypothetical proteins. Five heat shock proteins were also detected in non-excysted oocysts. As expected a number of surface antigens and oocyst wall-associated proteins were also detected in this fraction. In regard to the soluble oocyst fractions, many of the detected proteins in the insoluble non-excysted oocyst fraction are involved in glycolysis, cellular division and replication, and DNA modification. Also 14 proteins (with unique accession numbers) were only detected from the excysted insoluble oocyst fractions (Supplemental Table 3).

Surface, Cytoskeletal, and Extracellular Proteins—During our investigation, in both soluble and insoluble fractions, we detected a number of *C. parvum* cytoskeletal and surface proteins. Detected cytoskeletal proteins included actin (N36) and α-tubulin (N277; Table II). A number of proteins that are associated with extracellular protein secretion were detected in both soluble and insoluble fractions. These proteins included a predicted secreted protein signal peptide (N65), aminopeptidase N (N109), and the secreted GP900 (N88; Table II) (40). Moreover among the sporozoite secreted proteins that were identified, all the *C. parvum* predicted LCCL-containing proteins, namely CCp2 (N69) and CCp3 (N70; Table II), as well as the LCCL-containing protein Cpa135 (N64; Table I) were detected. Several oocyst wall and surface proteins were also detected, including glycoprotein Cp17 (N223), an oocyst wall protein precursor (N224), COWP1 (N225), and 15-kDa glycoprotein (N286; Table II).

Metabolic Enzymes—A number of proteins involved in metabolic reactions were detected in both soluble and insoluble fractions. These enzymes included enolase (2-phosphoglycerate dehydratase) (N98), glycerol-3-phosphate dehydrogenase (N106), and fructose-1,6-bisphosphate aldolase (N236; Table II). A number of ATP-related enzymes were also detected, including secreted nucleoside-diphosphate kinase (N73; Table II). A Pdr17p-like protein (N110), which may regulate lipid synthesis, and a pleckstrin homology domain-containing protein (N124) were also detected (Table II).

Nucleic Acid and Protein Synthesis, Modification, and Replication—In both soluble and insoluble oocyst fractions a number of enzymes that are involved in nucleic acid synthesis, repair, and modification were detected. Examples of these included a small GTP-binding protein rab1a (N91) and guanine nucleotide-binding protein (N115; Table II). A number of enzymes were detected that would have key functions during the *Cryptosporidium* sp. replication, protein synthesis, and modification. Detected proteins with functions during

TABLE I

Summary of the bioinformatics analysis of the 26 proteins that showed significant expression increases during excystation

LC, largely conserved among different phyla; R, proteins involved in protein synthesis and/or associated with ribosomes; H, housekeeping genes or structural genes; S, heat shock proteins; HDAC, histone deacetylase; EST, expressed sequence tag; MPN, domain observed at N terminus of Mpr1p and Pad1p proteins.

N	Accession number	Protein name	Homologs	Domains
Ribosomal				
5	gi|66358116	40 S ribosomal protein S7	LC R	None detected
17	gi|32398896	60 S ribosomal protein-like, probable	LC R	None detected
31	gi|46229456	60 S ribosomal protein L35A, transcript identified by EST	LC R	None detected
35	gi|67594773	Hypothetical protein Chro.50226 (ribosomal protein L3), note: it is not present in the *C. parvum* annotated proteins	LC R	None detected
Undetermined function				
44	gi|66357812	Hypothetical protein cgd5_1370	*C. hominis*	Not detected
46	gi|66475922	Hypothetical protein cgd6_4460	Apicomplexa	HDAC-interacting domain and ARM domain
Heat shock				
58	gi|46229711	HSP70, transcripts identified by EST	LC S	None detected
59	gi|8515208	HSP70	LC S	None detected
61	gi|3273568	HSP90	LC S	None detected
62	gi|66359492	HSP90	U S	None detected
Secreted				
42	gi|66359420	Hypothetical protein cgd3_3370	*C. hominis*	Signal peptide
51	gi|66363232	Hypothetical protein cgd7_4280	Apicomplexa	Signal peptide
52	gi|32398968	Hypothetical garp protein, possible	*C. hominis*	Signal peptide
64	gi|20513131	Cpa135 protein	Apicomplexa	Signal peptide, ricin B, and LCCL
68	gi|46229371	Protein with signal peptide and 2 *Cryptosporidium*-specific paralogs, putative secreted protein EAK90189	*C. hominis*	Signal peptide
77	gi|46227968	Hypothetical protein with signal peptide EAK88888	*C. hominis*	Signal peptide
Metabolic enzymes				
102	gi|10444017	Lactate dehydrogenase	LC H	None detected
105	gi|46229140	Glyceraldehyde-3-phosphate dehydrogenase	LC H	None detected
107	gi|66359800	Acetaldehyde reductase plus alcohol dehydrogenase (AdhE) of possible bacterial origin	LC H	None detected
108	gi|46229859	Phosphoglycerate kinase 1	LC H	None detected
Nucleic acid and protein synthesis, modification, and replication				
84	gi|51951320	Thioredoxin peroxidase-like protein	LC H	None detected
118	gi|46228775	Histone H4	LC H	None detected
119	gi|32398654	protein-disulfide isomerase, probable	LC H	None detected
120	gi|32398904	Mov34/MPN/PAD-1 family proteasome regulatory subunit, probable	LC H	None detected
127	gi|46229603	EF-1γ (glutathione *S*-transferase family)	LC R	None detected
135	gi|32398975	EF-1α	LC R	None detected

replication included a GTP-binding nuclear protein ran/tc4 (N136), ribonucleotide reductase (N131), transcription regulator (N133), and a SEC14 domain-containing protein (N264; Table II). Also detected were a number of ribosomal proteins, translation elongation factor 2 (EF-2; N137), and ubiquitin-activating enzyme E1 (N123), which have key roles during protein synthesis and in proteasome/ubiquitin systems, respectively (41) (Table II). A number of enzymes associated with protein structural modification were also detected, *e.g.* protein-disulfide isomerase (N121), and Pre3p/proteasome regulatory subunit β type 6, nephrotoxic nephritis hydrolase fold (N122; Table II).

TABLE II

Data from a subset of 28 proteins from the total 303 C. parvum proteins identified during this investigation

Two proteins (N115 and N136) were not present in the GenBank™ database, thus their accession numbers refer to *C. parvum* predicted proteins (prefix cgd) in CryptoDB. ER, endoplasmic reticulum; EST, expression sequence tag; NTN, nephrotoxic nephritis.

N	ProtScore		Accession number	Protein name
	Unused	Total		
Cytoskeletal				
36	5.96	5.96	gi|20799249	Actin
277	2	2.01	gi|2522417	α-Tubulin
Undetermined function				
49	2.01	2.06	gi|66362692	Hypothetical protein cgd7_1270
Secreted				
65	5.6	5.6	gi|46227507	Predicted secreted protein, signal peptide
69	7.25	7.26	gi|46229704	CpCCp2; extracellular protein with a signal peptide, ricin, discoidin, NEC, LCCL, 2 levanase and an apicomplexan-specific cysteine-rich repeat (apicA repeat)
70	2.78	2.82	gi|46227948	CpCCp3, multidomain extracellular protein with a signal peptide and the following architecture: LH2, LCCLCCL, pentraxin, and 2×LCCL
73	2	2.01	gi|46227296	Signal peptide containing protein having a nucleoside-diphosphate kinase domain, transcript identified by EST
88	10.16	10.17	gi|4063042	GP900; mucin-like glycoprotein
109	4.02	4.02	gi|46228639	Zincin/aminopeptidase N-like metalloprotease
Surface and oocyst wall				
223	8.01	8.02	gi|30268708	Cp17 antigen precursor
224	2.48	2.49	gi|32398662	Oocyst wall protein precursor, possible
225	6.19	6.98	gi|46229286	CpCOWP1, oocyst wall protein with type I and type II cysteine-rich repeats
286	6.12	6.12	gi|8671728	15-kDa glycoprotein gp15
Metabolic enzymes				
98	6	6	gi|66357920	Enolase (2-phosphoglycerate dehydratase)
106	2	2.01	gi|66356424	Glycerol-3-phosphate dehydrogenase
110	2.01	2.02	gi|66475304	Pdr17p-like protein
124	24.2	24.2	gi|66360002	Pleckstrin homology (PH) domain-containing protein
236	11.7	11.7	gi|46227620	Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) (*C. parvum*)
Nucleic acid and protein synthesis, modification, and replication				
91	2	2.01	gi|32398899	Small GTP-binding protein Rab1a, probable
115	2	2	cgd2_1870	Guanine nucleotide-binding protein, putative
121	2	2.02	gi|46229649	Protein-disulfide isomerase, signal peptide plus ER retention motif
122	2.01	2.01	gi|66358282	Pre3p/proteasome regulatory subunit β type 6, NTN hydrolase fold
123	2.03	2.05	gi|46226970	Ubiquitin-activating enzyme E1 (UBA)
131	1.52	1.53	gi|46228920	Ribonucleotide reductase small subunit, duplicated adjacent gene
133	2.02	2.03	gi|66362450	Transcription regulator
136	2	2	cgd7_220	GTP-binding nuclear protein ran/tc4
137	4.02	4.03	gi|46228834	Eft2p GTPase; translation elongation factor 2 (EF-2)
264	1.7	1.7	gi|46228972	SEC14 domain-containing protein

DISCUSSION

The first and most extensive proteome maps for all the life cycle stages of an apicomplexan parasite were published in 2002 for *Plasmodium falciparum* (42). Proteome maps are available for the invasive stages of development of the other apicomplexan parasites including the sporozoite of *Eimeria tenella* as well as the tachyzoite of *T. gondii* and *Neospora caninum* (42). Our study is the first major proteomics investigation of any *Cryptosporidium* species and in particular of *C. parvum*, a major global human and animal pathogen. We examined the proteome of *C. parvum* oocysts at two important stages of its life cycle: the transmissive non-excysted oocyst and infective sporozoite stages. The identified proteins represent roughly 8% of the predicted proteome (19, 20). Together with the sequencing of the *C. parvum* (20) and *C. hominis* (19) genomes, our study identified several potential targets both for immunotherapy and chemotherapy. Because of the high sequence similarity between the *C. hominis* and *C. parvum* genomes (19), the major pathogenic *Cryptosporidium* species for humans (10, 11), it is conceivable that the development of an effective vaccine or a drug for one of these species would work on both of them. In the *C. parvum* life cycle, there are two motile stages, the sporozoite and the merozoite, which invade the host cell membrane (Fig. 1). Sporozoites initiate the infection process, slipping out of the oocyst and invading the epithelial cells of the small intestine (2). It is conceivable that sporozoites as well as merozoites are more susceptible to drugs and the host immune system because they are exposed in the intestinal lumen, whereas the other parasite stages are intracellular.

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One approach to the development of anticryptosporidial agents has been to identify sporozoite and merozoite surface antigens involved in recognition, attachment, and invasion of the host epithelial cells to block these interactions (42, 43). We detected a number of surface and extracellular proteins. Glycosylinositol phospholipids and glycosylphosphatidylinositol protein anchors are abundant in the surface membranes of a wide variety of parasitic protozoa and are increasingly recognized as important modulators of the immune function during infection (12). The immunodominant *C. parvum* 17-kDa surface antigen (N223; Table II) is glycosylphosphatidylinositol-anchored, and parasite surface glycosylinositol phospholipids are recognized by serum antibodies from infected patients (12). Furthermore this work also confirms the expression of antigenic proteins, namely Cpa135 (N64) and GP900 (N88) (Tables I and II, respectively), which in addition to inducing specific antibodies (40, 44) can also elicit a cellular Th1 response (45).

Importantly during oocyst excystation, we identified 26 proteins that demonstrated significant expression increases. Thus, future vaccine development strategies could potentially select for antigens blocking the recognition and the attachment to host cells. Increased ribosomal protein expression may be due to the requirement of a rapid activation protein synthesis after a variable period of quiescence at the oocyst stage. The regulation of the ribosome and of the associated proteins has been observed in other species of the phylum Apicomplexa, *e.g. E. tenella* (46–48). Heat shock proteins (HSPs) are probably important for stress tolerance in the host environment and for the folding of newly synthesized proteins. Previous studies have found that the expression of HSPs occurred during the development of apicomplexan parasites and that HSPs are interesting targets for the host immune system (49). The level of HSP70 expression in *T. gondii* has been found to be higher in mouse-virulent than in mouse-avirulent strains (50). Recently it has been reported that *E. tenella* HSP90 is essential for the invasion of the host cell and that its expression increases in sporozoites that secrete this protein (51). Interestingly we identified seven proteins that are unique for the genus *Cryptosporidium* and/or the phylum Apicomplexa (Table I). Five of these proteins have a signal peptide at their N terminus indicating that they are secreted proteins. These proteins probably play a specialized role in the invasion machinery of the parasite. Apart from its signal peptide, the cgd7_4280 protein (N51; Table I) is an apicomplexan protein that does not demonstrate identifiable traits. There are paralogs of this gene both in *C. parvum* and in *C. hominis* (hypothetical protein cgd7_1270 (N49; Table II) and hypothetical protein Chro.70152), suggesting that these proteins belong to a more extensive protein family. The cgd6_4460 (N46) protein belongs to an apicomplexan family sharing a peculiar architecture because they contain both a histone deacetylase-interacting domain and an armadillo repeat (ARM) domain. Histone deacetylases are involved in higher order chromatin assembly (52) and ARM proteins are involved in various processes, including intracellular signaling and cytoskeletal regulation (53).

Cpa135 has already been characterized; it is secreted by the apical complex prior to host cell invasion, and Cpa135-related proteins are a distinct family among the apicomplexan (LCCL) proteins (52). In accord with the present data, the expression of Cpa135 increases 4-fold within 30 min after excystation (52). On the whole, three proteins containing LCCL domains (including Cpa135) with peculiar domain architecture, *e.g.* CpCC2 (N69) and CpCC3 (N70), were detected. These proteins are composed of various adhesive motifs (ricin B, LCCL, and discoidin), suggesting that these proteins are involved in some adhesion process. They also have closely related homologs in *P. falciparum*, namely PfCCp1, PfCCp2, and PfCCp3 that are orthologs to Cpa135 (also named CpCCp1), CpCCp2, and CpCC3, respectively (52, 54). Interestingly our results demonstrated that these proteins are expressed simultaneously in both oocysts and free sporozoites, whereas all the *P. falciparum* orthologs have gametocyte stage-specific expression of these genes (54). This fact suggests that these proteins require a coordinate expression to exert their role.

This study is the first major proteomics investigation of its kind on any *Cryptosporidium* species. We ascertained the expression of many formerly putative proteins and the presence of several secreted or surface proteins in sporozoites that are restricted to the Apicomplexa or to the *Cryptosporidium* genus. These findings could favor further studies for the identification and role of specific molecules involved in motility, host recognition, and invasion. Interestingly three Apicomplexa-specific proteins and five *Cryptosporidium* sp.-specific proteins were augmented in excysted invasive sporozoites. These eight proteins represent promising targets for developing vaccines or chemotherapies that could block parasite entry into host cells. Therefore our study represents a substantial step forward toward increasing our understanding of *Cryptosporidium* sp. biology and the molecular mechanisms of parasite entry as well as the development of therapy to combat cryptosporidiosis.

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§§ To whom correspondence should be addressed. Tel.: 44-28-7032-3528; Fax: 44-28-7032-4375; E-mail: cj.lowery@ulster.ac.uk.

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