Cryptosporidium infections: molecular advances

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SUMMARY

Cryptosporidium host cell interaction remains fairly obscure compared with other apicomplexans such as *Plasmodium* or *Toxoplasma*. The reason for this is probably the inability of this parasite to complete its life cycle *in vitro* and the lack of a system to genetically modify *Cryptosporidium*. However, there is a substantial set of data about the molecules involved in attachment and invasion and about the host cell pathways involved in actin arrangement that are altered by the parasite. Here we summarize the recent advances in research on host cell infection regarding the excystation process, attachment and invasion, survival in the cell, egress and the available data on omics.

Key words: Cryptosporidium, Apicomplexa, host-parasite interaction, attachment, invasion, egress, mucin, actin, omics.

INTRODUCTION

Cryptosporidia are tiny, unicellular parasites that infect a wide variety of organisms. *Cryptosporidium parvum* is one of the most important causes of heavy, watery diarrhoea in calves leading to high economical losses. Calves can shed high numbers of oocysts (up to 10^{10} per animal) and thus they are one of the main sources of dispersal stages. Humans are mainly affected by *C. parvum* and *Cryptosporidium hominis* where they cause heavy self-curing diarrhoea. Nevertheless, *Cryptosporidium* is still an issue mainly due to the small oocysts which are difficult to detect in and to remove from drinking water resulting in several outbreaks in the last decades. They can also become a life-threatening disease in immunosuppressed patients. With the introduction of HAART against HIV *Cryptosporidium* has become less important in industrialized countries but is still a problem in third world countries, although a systematic evaluation of the current situation is missing. The phylogenetic classification of *Cryptosporidium* is still being debated as they share characteristics with apicomplexan coccidia as well as with apicomplexan gregarines (Barta and Thompson, 2006). Cryptosporidia possess an apical complex but have lost their apicoplast while retaining some of their genes. Like coccidia they attach to the cell surface and undergo gliding motility before they start to enter the cell. However, it seems that unlike coccidia they do not invade the cell actively but rather trigger the cell to embrace them with host cell-derived membrane. As a result cryptosporidia do not fully invade the cell but stay in an epicellular location. At the cell parasite interface *Cryptosporidium* forms an actin-rich disk, a feeder organelle that is thought to be responsible for nutrition intake, and a small channel funnelling into the host cell cytoplasm. Phylogenetic analyses of the SSU RNA place cryptosporidia as a separate branch next to the gregarines (Carreno *et al*. 1999). This evolutionary distance might explain why cryptosporidia are resistant to the common anti-coccidial drugs.

Cryptosporidium, in contrast to *Toxoplasma*, cannot continuously be propagated *in vitro* and all attempts to establish a continuous cell culture have failed so far. This might be the reason why there are no tools available for the genetic manipulation of these parasites although they are urgently needed to explore the host-parasite interaction. This review aims to summarize the progress in molecular research on host-parasite interaction in recent years. Most of the studies used *C. parvum* as a model and therefore the vast majority of the data refer to this parasite.

BIOLOGY OF *C. PARVUM*

The life cycle

Cryptosporidium parvum infects humans and a variety of animals but their main hosts are calves at the age of 7–12 days, where it causes watery, yellowish and foul-smelling diarrhoea. It is a common disease in calves, affecting up to 100% of the young animals. The calves become infected through oral uptake of the oocysts. Due to the temperature rise and the changed environment, four sporozoites excyst from each oocyst in the small intestine where they start to invade enterocytes. The host cell is triggered to embrace the parasite with membrane protrusions thereby forming a parasitophorous vacuole (PV). At the same time an actin disc is forming underneath the

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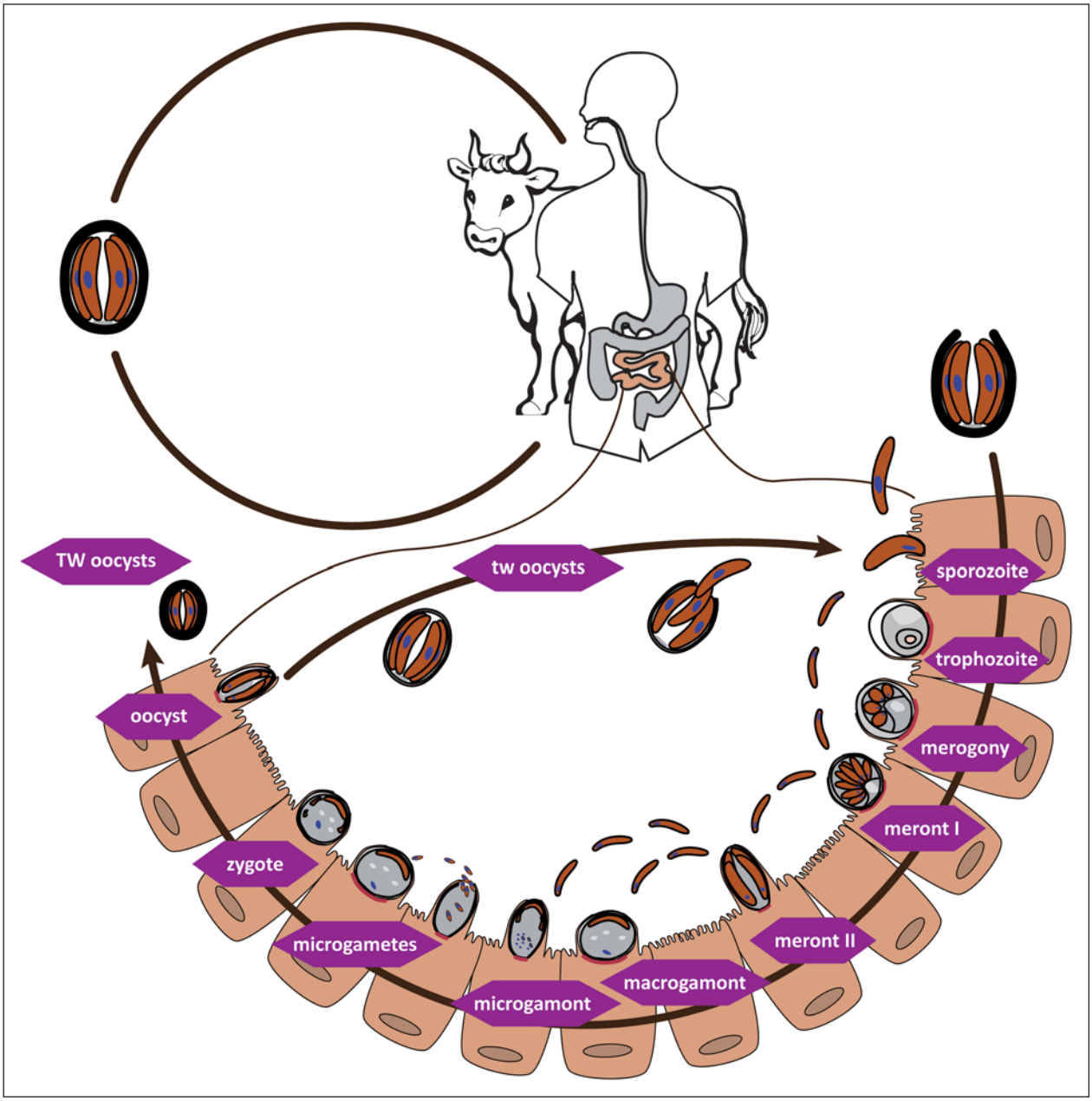


Fig. 1. Life cycle of *C. parvum*. Sporulated oocysts are released with the faeces containing four infectious sporozoites. The sporozoites excyst in the small intestine and start to invade enterocytes. During invasion the sporozoite is embraced by protrusions of the host cell forming the parasitophorous vacuole. Underneath the forming trophozoite emerges a host cell-derived actin disc (shown in red). After asexual replication a meront type 1 develops that contains 6–8 merozoites. Type 1 merozoites infect adjacent epithelial cells either developing to another trophozoite or into a type 2 meront. Merozoites originating from a type 2 meront are thought to start the sexual replication cycle. Upon infection of another cell they either develop to macrogamonts or microgamonts. Microgamonts then release microgametes that fertilize a macrogamont. The resulting diploid zygote differentiates into four haploid sporozoites and subsequently the oocyst wall is formed around them. Two forms of oocysts are described. Thin-walled oocysts are described to lead to autoinfection whereas the thick-walled oocysts are released with the faeces to infect new hosts. TW: thick-walled oocyst, tw: thin-walled oocyst.

attached parasite. The sporozoite then develops into a trophozoite that starts to replicate, leading to meronts with six to eight merozoites. Merozoites are released from the PV and start to infect circumjacent enterocytes to undergo another replication cycle resulting in meronts with four merozoites. Merozoites II are thought to be programmed to develop into gametocytes upon infection of further enterocytes. Fertilized macrogamonts develop to either thick- or thin-walled oocysts containing four sporozoites. Thin-walled oocysts are believed to cause auto-infection whereas thick-walled oocysts are released with the faeces to infect new hosts (see Fig. 1).

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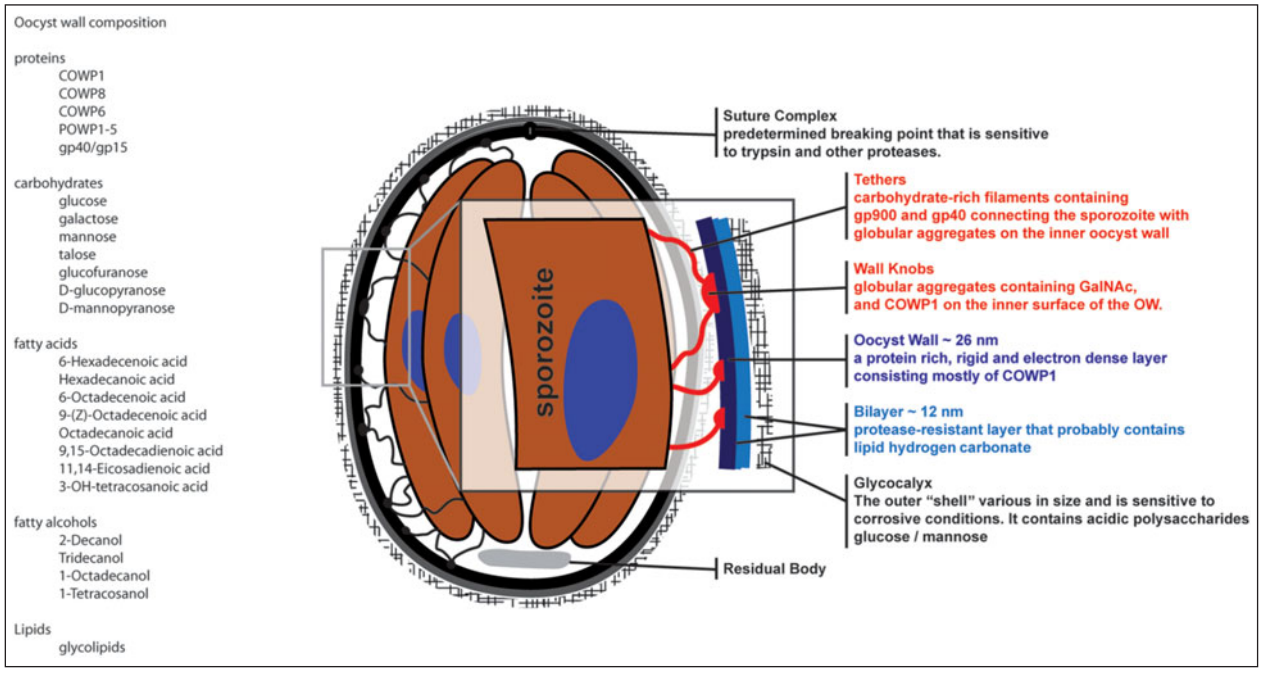


Fig. 2. Architecture and composition of the oocyst. Schematic drawing of the current structural model of the oocyst. The oocyst is covered by glycocalyx that alters the electrophoretic mobility and is shown to be immunogenic. The high resistance is provided by a lipid bilayer protecting against liquid intrusion and a protein rich layer that confers mechanical resilience. The sporozoites are connected to the OW by filaments that contain glycoproteins which play a role in host cell attachment and invasion. COWP: *Cryptosporidium* oocyst wall protein, GalNac: N-acetylgalactosamine, gp: glycoprotein, OW: oocyst wall.

The oocyst and the excystation process

In vitro studies revealed that *Cryptosporidium*, in contrast to other coccidia such as *Eimeria* or *Toxoplasma*, need only a single stimulus to start excystation (Fayer and Leek, 1984). This would explain why *Cryptosporidium* is able to settle in extra-intestinal locations such as the lungs of immunosuppressed hosts. However, there are several stimuli supporting the excystation of sporozoites. Firstly, temperature is a major factor. Low temperatures (4 °C) prevent hatching of sporozoites but just raising the temperature to 20 °C resulted in excystation rates of up to 13% and up to 91% when the temperature was 37 °C. Secondly, bile salts and digestive enzymes trigger the release of sporozoites (Reduker and Speer, 1985; Forney *et al*. 1996*a*). Finally, a corrosive treatment (NaOCl, HCl) supports excystation probably by facilitating the access of bile salts into the oocysts (Reduker and Speer, 1985; Robertson *et al*. 1993). However, there are publications reporting different outcomes of pretreatment of oocysts varying from a rather lethal impact on oocyst viability to amplifying effects on the excystation process. There are also reports on differences between the strains and isolates used (Robertson *et al*. 1993).

Upon activation of the sporozoites within the oocyst they start to produce a panel of proteases crucial for the excystation process. The overall protease activity in oocysts homogenates was shown to increase within the first hour of excystation (Forney *et al*. 1996*b*). Using different protease inhibitors it turned out that mainly serine and cystein proteases are expressed in activated oocysts. Blocking the activity of serine proteases by different inhibitors led to a strong although not complete inhibition of excystation emphasizing the importance of this class of proteases whereas the inhibition of cystein proteases did not lead to a significant inhibition of excystation (Forney *et al*. 1996*b*). Furthermore, an arginine aminopeptidase (RAP), expressed on the sporozoite surface, was shown to be involved in excystation (Okhuysen *et al*. 1996; Padda *et al*. 2002). Altogether, the data of these *in vitro* studies suggest that upon uptake of the oocysts the rise in temperature activates the sporozoites leading to the production of proteases and an increased motility. At the same time, the outer layer of the oocyst, consisting mostly of glycoproteins, is degraded by the corrosive environment of the stomach leading to higher permeability of the oocyst wall. The infiltrating bile salts promote the activation of the sporozoites. Digestive enzymes such as trypsin in the intestinal environment as well as proteases produced by the parasite start to degrade oocyst wall proteins, mostly in the area of the suture leading to the rupture of the suture (Reduker *et al*. 1985). The oocyst wall structure is not fully understood but supposed to be less complex than plant or fungal cell walls (Chatterjee *et al*. 2010) (see Fig. 2). Nevertheless, oocysts are highly resistant against chemical

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and environmental influences probably due to the layered structure of the oocyst wall consisting of a fragile glycocalyx, a lipid and glycoprotein layer. This protects against liquid intrusion as well as against mechanical stress. The inner proteinaceous layer mainly consists of *Cryptosporidium* oocyst wall protein (COWP) 1 and to a minor extent of COWP8. COWPs are a family (COWP1-9) of cysteine-rich proteins which, except for COWP1 and COWP8, are little characterized so far. The cysteines are thought to form disulphide bonds leading to the rigidity of the inner layer (Spano *et al*. 1997; Templeton *et al*. 2004*a*; Bushkin *et al*. 2013). The oocyst architecture is summarized in Fig. 2.

Attachment to the host cell

Attachment to the host cell is mediated by a variety of molecules that are expressed on the sporozoite surface and that can bind to host cell receptor or surface structures. The known molecules involved in attachment and/or invasion are summarized in Table 1 and Fig. 3. A genomic search for 32 widely conserved surface domains resulted in the finding of 51 proteins probably expressed on the zoite surface. Intriguingly many of them have a lineage-specific architecture different from *Plasmodium* and *Toxoplasma* indicating they have evolved separately. Many of the surface domains (such as Notch/Lin1, Neurexin-Callagen-domain) are only found in animals other than apicomplexans which hints towards a lateral transfer of genes of animal origin. This notion is supported by the presence of a previously detected protein O-linked glycosylation pathway in *Cryptosporidium* but not in *Plasmodium* that was found only in animals (Templeton *et al*. 2004*b*).

The first contact of the sporozoite is with the top mucus layer of epithelial cells. The mucus layer constitutes an efficient barrier against microbes. It reaches considerable thickness from 120 μm (small intestine) up to 800 μm (colon) (Atuma *et al*. 2001) which has to be penetrated by the sporozoite (3 μm). Whether *Cryptosporidium* utilizes proteases to digest the mucus as shown for *Entamoeba* (Moncada *et al*. 2003; Lidell *et al*. 2006), simply forces its way through the mucus by movement or uses other mechanisms is so far unclear.

Obviously *Cryptosporidium* expresses at least one lectin on the sporozoite surface that specifically binds to galactose-N-acetylgalactosamine (Gal/GalNAc) but not to other sugars (Chen and LaRusso, 2000). Preincubation with Gal/GalNAc or bovine submaxillary mucin (BSM) can prevent the attachment and/or invasion of cells *in vitro* (Joe *et al*. 1998; Chen and LaRusso, 2000; Hashim *et al*. 2006). Recently, a 30 kDa Gal/GalNAc lectin (p30) has been identified that is predominantly located in the apical region of sporozoites. Attachment inhibition assays with native and recombinant p30 revealed that p30 plays at least *in vitro* a substantial role in attachment. However, p30 is a soluble protein that lacks a GPI anchor or a transmembrane domain and is exocytosed during excystation leading to the question how it mediates binding. It was found out that p30 is associated with gp40 and gp900, two glycoproteins also involved in attachment, and therefore hypothesized that these molecules form an adhesion complex (Bhat *et al*. 2007).

The parasite lectins of course need Gal/GalNAc-protein counterparts on the host cell surface. Nelson and colleagues could show that *Cryptosporidium* leads to the formation of sphingolipid-enriched membrane microdomains (SEMs) on the host cell membrane that attract Gal/GalNAc epitope containing glycoproteins. Chemical inhibition of SEM formation as well as knock-down of acid-sphingomyelinase (ASM), which is important for the aggregation of the microdomains, reduced accumulation of Gal/GalNAc-glycoproteins and attachment and invasion of *C. parvum in vitro*. Moreover, recruitment and phosphorylation of PI-3K (phosphatidylinositol 3-kinase) and subsequent accumulation of cdc42 and actin was inhibited, indicating that SEMs might act as signal transducers for actin rearrangement (Nelson *et al*. 2006). Another well characterized attachment glycoprotein is the gp40/15 complex. Gp40 and gp15 originate from a 60 kDa (gp60) precursor that is proteolytically cleaved, probably by a subtilisin-like serine protease (CpSUB1) (Cevallos *et al*. 2000*a*; Strong *et al*. 2000; Wanyiri *et al*. 2007, 2009). Whereas gp15 is anchored in the membrane by a GPI anchor, gp40 is supposed to be a soluble protein (Cevallos *et al*. 2000*a*; O'Connor *et al*. 2003). Nevertheless, gp40 is shown to bind to cells in a dose-dependent and saturable manner and antibodies against gp40 reduce infection by up to 82% *in vitro* (Cevallos *et al*. 2000*a*). Furthermore, gp40 is shed in trails and co-localizes with gp15 indicating they might form a complex (O'Connor *et al*. 2007*b*). Gp40 is a highly glycosylated mucin-like protein (Cevallos *et al*. 2000*b*), a feature also exhibited by gp900 (Barnes *et al*. 1998). Gp900 is an immunodominant protein identified by antibodies from hyperimmune colostrums of cows (Petersen *et al*. 1992). It has a putative transmembrane domain indicating it might be anchored to the cell membrane upon release from the micronemes (Barnes *et al*. 1998). Prevention of gp900 binding to cells resulted in reduced infections. Interestingly, the structure and probably the attachment sites of gp900 as well seem to differ between *C. parvum* and *C. hominis*. Some antibodies raised against peptide and carbohydrate epitopes of either *C. parvum* or *C. hominis* gp900 turned out to be not cross-reactive between the two species. Hence, gp900 might be one molecule through which *Cryptosporidium* defines host specificity. The circumsporozoit-like glycoprotein (CSL) is shown to

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preferentially bind to epithelial cell lines but less to mesenchymal cells. CSL binding is mediated by an 85 kDa protein (designated as CSL-R) on the host cell. CSL binding was also confirmed in a bovine intestine indicating its relevance for the *in vivo* situation.

There are a number of other molecules identified mostly due to their ability to inhibit invasion *in vitro* or by their immunodominant properties. They are summarized in Table 1.

Gliding motility

Gliding motility is a unique feature of Apicomplexa that was mainly studied in *Toxoplasma* and *Plasmodium*. The current model assumes that upon attachment micronemes release a panel of molecules that mediate attachment. One of these molecules is TRAP in *Plasmodium* or MIC2 in *Toxoplasma*. TRAP attaches via the TSP (Thrombospondin) and vWF-like A domains to the host cell and links the receptor through the cytoplasmic tail domain (CTD) with the motor complex. The CTD is coupled to an actin filament via an aldolase tetramer. Locomotion is created by myosin (MyoA) anchored to the inner membrane complex (IMC). It pushes the actin filament backwards thereby creating a force that drives the parasite forward. The TRAPs are thereby translocated to the posterior end of the zoites where they are cleaved within the transmembrane part by a rhomboid protease leaving trails of shed material behind them (reviewed in Baum *et al*. 2006; Morahan *et al*. 2009). However, there is not much research on gliding motility of *Cryptosporidium*, an apicomplexan parasite that does not have extra-intestinal stages and does not invade the host cell completely. Although it is considered that the machinery of gliding motility is highly conserved between Apicomplexa, there might be some variability. In *Cryptosporidium*, 12 TSP1 containing proteins have been identified *in silico*. In contrast to *Plasmodium*, CpTSPs bear Apple or Kringle domains, which are thought to replace the vWF domain. This might be due to the fact that *Cryptosporidium* attaches to epithelial cells instead of hepatocytes or erythrocytes and thereby facilitates different attachment molecules (Naitza *et al*. 1998; Spano *et al*. 1998; Deng *et al*. 2002). In *Cryptosporidium*, two TSPs, TRAP-C1 and TSP7 have the canonical CTD with the conserved tryptophan. TRAP-C1 is a single-copy gene with the most structural similarities to TgMIC2. Its expression (mRNA) varies during the life cycle peaking at 12 h and 48 h in the cell culture indicating that it is a highly regulated protein (Naitza *et al*. 1998; Deng *et al*. 2002). Infection of human volunteers with *C. parvum* revealed that TRAP-C1 is an immunogenic protein (Okhuysen *et al*. 2004). For TSP2-12 an expression profile has been generated showing different expression patterns for the different TSPs making it likely they fulfil different tasks during development (Deng *et al*. 2002). Nevertheless, *Cryptosporidium* relies on gliding motility shown by time-lapse microscopy and the inhibition of the conserved actin-myosin motor complex. Depolymerization of actin or inhibition of myosin by specific chemical drugs prevented movement and the active penetration of but not attachment to the host cell (Chen *et al*. 2004*c*; Wetzel *et al*. 2005).

Invasion of the host cell

Upon attachment to the cell and gliding around, the sporozoites somehow force their way into the cell. *Toxoplasma* sporozoites do this by forming a tight junction (moving junction) with the cell membrane through which they squeeze themselves into the nascent PV inside the host cell. This mechanism, which includes the release of the contents of the rhoptries, is well understood for *Toxoplasma* and *Plasmodium* (reviewed in Baum *et al*. 2008; Sibley, 2010). However, *Cryptosporidium* is different in so far as it does not fully invade the cell but rather stops half way through, which is called intracellular but extra-cytoplasmic. Moreover, histological and some molecular studies suggest an engulfment of the parasite rather than an active invasion.

Calcium is an important messenger

Most studies on the molecular processes during *Cryptosporidium* invasion address changes in the host cell rather than the processes in the sporozoites. From *Toxoplasma* and *Plasmodium* it is known that calcium is an important messenger that regulates many processes during invasion. The cytosolic calcium concentration orchestrates several transporters in the acidocalcisomes, the endoplasmatic reticulum, the mitochondria and the cell membrane (Moreno and Docampo, 2003). *Cryptosporidium* possesses a Ca^{2+}-ATPase located at the sporozoite apical and perinuclear regions (Zhu and Keithly, 1997). The function of this ATPase has not been identified yet and shows no homology with ATPases of the plasma membrane or the sarcoplasmic-endoplasmic reticulum of other parasitic protozoa (Moreno and Docampo, 2003). The essentiality of Ca^{2+} for the invasion of *Cryptosporidium* was proven by using the intracellular calcium chelator BAPTA-AM that prevented the apical organelle discharge, an effect that could be partially reverted by raising the intracellular calcium content (Chen *et al*. 2004*c*). The rise in intracellular calcium can be partially controlled by bile salts. While sporozoites show an immediate increase in intracellular calcium and degranulation upon excystation in the absence of bile salts, they show a delayed increase in calcium and less degranulation in the presence of bile salts or natrium

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Table 1. *Cryptosporidium* molecules possibly involved in host cell attachment and invasion

Molecule	Function/Characteristics	Structure	Localization	References
CP12	Immunodominant protective effect as DNA vaccine in mice	104 amino acids/12 kDa; N-glycosylation site at aa 58–61; casein kinase II phosphorylation site; two N-myristoylation sites; N-terminal signal peptide; transmembrane regions; no conserved domains and functional domains	Surface of sporozoite and oocyst	(Yao *et al*. 2007; Yu *et al*. 2010)
CP21	Immunodominant; protective effect as DNA vaccine in mice			(Yu *et al*. 2010)
CP2	Up-regulated expression during sexual development and during excystation; anti-CP2-Ab does not inhibit attachment but invasion	Predicted from cDNA: N-terminal signal peptide; multiple sites for N-glycosylation and phosphorylation; putative transmembrane domain	Localized on sporozoites and to a Much lesser extent on the PVM of trophozoites and type I meronts; at the periphery of amylopectin-like granules in sexual stages; in mature oocysts at on PVM and the sporozoite membrane	(O'Hara *et al*. 2004; Jenkins *et al*. 2011)
CP20	DNA vaccination of mice leads to specific antibody production, higher CD4+ count from spleen and reduced oocyst shedding	n.a.	Detected by Ab on surface of sporozoites and oocysts	(Xiao *et al*. 2011)
CP47	Binding of CP47 is Mn^{2+} dependent; was shown to compete with sporozoite membrane-associated proteins	n.a.	Localized in the apical region but not specifically for one of the organelles; binds to specific 57 kDa receptor on cell surface	(Nesterenko *et al*. 1999)
CPA135	CPA135 synthesis was up-regulated during the excystation process; after host-cell invasion, Cpa135 gene expression undetectable up to 48 h; expression started again at 72 h post-infection	1556 aa protein; common domains: ricin B and a LCCL motif; homology with CCP2 protein from *Plasmodium yoelii* and *Plasmodium berghei*	Localized at the apical pole; secreted by sporozoites during their gliding; supposed to be a microneme protein	(Tosini *et al*. 2004)
CPS-500	Polar glycolipid; treatment with certain glucosidases prevented recognition by antibody whereas treatment with proteases did not; anti-CPS-500 Ab partially effective against infection in neonatal mice	n.a.	Localized at the pellicle	(Riggs *et al*. 1989; Perryman *et al*. 1990)
CSL	CSP like reaction; antibody against CSL protects neonatal mice partially or fully against *C. parvum* infections; CSL has a 85 kDa (CSL-R) ligand on epithelial cells; CSL-R shows a higher expression level on epithelial cells than on mesenchymal cells which might determine cell specificity; CSL-R is expressed in calf ileum; CSL-R/CSL complex is rapidly internalized by Caco cells; after antibody-induced CSP-like reaction sporozoites fail to infect cells	app. 1300-kDa glycoprotein	Located at the apical complex of sporozoites and merozoites	(Riggs *et al*. 1994, 2001; Langer and Riggs, 1999; Schaefer *et al*. 2000; Langer *et al*. 2001)
P30	30 kDa Protein; binds Gal/GalNac; no transmembrane domain (probably soluble); interacts with GP900 and GP15/40 ->might form a complex with them to bind GalNac on cell surface	302 aa/app. 30 kDa glycoprotein; 22 aa N-terminal signal sequence; 6 N-glycosylation sites	p30 mostly localized in the apical region of sporozoites	(Bhat *et al*. 2007)

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Lectins	galactose-N-acetylgalactosamine (Gal/GalNAc) is an important sugar on cell surfaces that mediates attachment of *Cryptosporidium* sp.; attachment via Gal/GalNAc of sporozoites could be inhibited by: Gal/GalNAc specific lectins (PMA), Gal/GalNAc, BSM endoglycosidase (O-glycosidase F) but not by N-glycosidase or neuraminidase; Gal/GalNAc mediated attachment was inhibited in the case of *C. parvum* but not in *C. hominis* and to HCT-8/primary bovine intestinal cells but not primary human intestinal epithelial cells indicating there is a species- and cell-specific difference	n.a.	n.a.	(Joe *et al*. 1994, 1998; Hashim *et al*. 2006; Nelson *et al*. 2006)
SEM ASM PI3K/Cdc42 Lipid rafts Gal/GalNAc	*Cryptosporidium* recruits sphingolipid-enriched membrane microdomains and activates acid-sphingomyelinase; disruption of known SEM components and associated SEM membrane aggregation decreases *C. parvum* attachment to and entry of cholangiocytes; recruitment of SEM components is associated with accumulation of Gal/GalNAc-associated membrane-binding molecules; recruitment of SEM components activates the PI-3K/Cdc42 pathway and leads to accumulation of actin infection sites indicating SEM could be involved in signal transduction	n.a.	n.a.	(Nelson *et al*. 2006)
GP40/15	Glycoproteins that have a predicted 60 kDa precursor that is proteolytically cleaved into a 15 and 40 kDa protein probably by a subtilisin-like serine protease (CpSUB1); the GP60 locus is a highly polymorphic cluster and can be divided in two allelic groups Ia-Id (C. hominis), II (*C. parvum*); GalNAc-specific lectins such as HPA, AIA, and MPA prevent cell attachment and invasion indicating GPs might bind via GalNAc to cells; moreover Nac could reduce binding of the mAb against GP15 but not glucose, mannose or galactose; immunization of mice with mAb against GP15 could reduce oocyst shedding by 67.5%; GP15/GP40 are shed during gliding	GP40: predicted N-terminal signal peptide, a polyserine domain, multiple predicted glycosylation sites, a single potential N-glycosylation site GP15: glycosylated, GPI anchor	GP15 is localized on the surface of sporozoites and merozoites; GP40 is localized in the apical region whereas GP15 is found on the complete surface; since GP40 does not have a transmembrane domain or GPI anchor it is proposed that GP15 and GP40 form a complex	(Tilley *et al*. 1991; Jenkins *et al*. 1993; Cevallos *et al*. 2000*a*, *b*; Strong *et al*. 2000; Sestak *et al*. 2002; O'Connor *et al*. 2003, 2007*a*, *b*; Wanyiri *et al*. 2009, 2007)
GP900	Ab against GP900 or subdomains reduced infection of cells in a concentration-dependent manner; GP900 mRNA levels peak at 14–26 h p.i. of cells; comparison of Ab reactivity between *C. parvum* and *C. hominis* against GP900 revealed that Ab did not cross-react between the species, indicating that morphological differences of surface molecules might account for species-specific infectivity	Mucin-like glycoprotein composed of distal cysteine-rich domains separated by polythreonine domains and a large membrane proximal N-glycosylated core region; deglycosylated protein app. 190 kDa	GP900 is stored in micronemes prior to appearance on the surface of invasive forms	(Petersen *et al*. 1992; Barnes *et al*. 1998; Sturbaum *et al*. 2003, 2008; Jakobi and Petry, 2006)

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Table 1. (*Cont.*)

Molecule	Function/Characteristics	Structure	Localization	References
P23	Identification of immunodominant P23 by mAb; passive immunization with mAb reduced infection in mice; hyperimmune serum against a recombinant part of P23 reduced oocyst shedding and diarrhoea of infected calves; immune response to P23 in IFN-γ ko mice: increased proliferation of splenocytes/ restimulated MLNs but unchanged cell composition; stimulated splenocytes from reconstituted mice do not produce IFN- γ in response to P23; splenocytes of infected mice (not reconstituted, not stimulated) showed increased mRNA levels for IL-2, IL-12, TNF-α compared with non-infected; stimulation with P23 of splenocytes from non-reconstituted mice led to an increase of IL-5 and decrease of IL-12 compared with medium control	111 aa protein; predicted N-glycosylation site	P23 was detected on the surface of sporozoites and merozoites	(Enriquez and Riggs, 1998; Perryman *et al*. 1999; Wyatt and Perryman, 2000; Bonafonte *et al*. 2000; Wyatt *et al*. 2000; Riggs *et al*. 2002; Takashima *et al*. 2003; Geriletu *et al*. 2011)
TRAP	*Cryptosporidium* possesses 12 TSP-related genes; all genes have putative signal peptide sequences, one or more TSP1-like domains plus additional extracellular protein modules such as Kringle, epidermal growth factor, and Apple domains; developmentally regulated: TRAPC1, CpTSP3 and CpTSP11 were expressed at high levels during both early and late stages of infection, whereas CpTSP2, CpTSP5, CpTSP6, CpTSP8 and CpTSP9 were maximally expressed during the late stages of infection; CpTSP4 was highly expressed solely at an early stage of infection; no von Willebrand factor in each of the 12 TSPs but other domains: Apple: TRAP-C1, TSP3-6 Kringle: TSP10 Notch/Lin: TSP2 TRAP-C1/CpTSP7 have an acidic nature and tryptophan residue in the CTD reminiscent of PfTRAP and TgMIC2; probably involved in gliding motility	depending on the different TSPs	TRAP-C1: Localized in the apical pole of sporozoites; probably stored in micronemes TSP8: stored in micronemes and translocated to the apical sporozoite surface upon contact with host cells	(Spano *et al*. 1998; Deng *et al*. 2002; Okhuysen *et al*. 2004; Putignani *et al*. 2008)
MUC1-7	Small mucin sequences were identified on chromosome 2 (CpMuc1-7); CpMucs are expressed throughout intracellular development; CpMuc4/CpMuc5: significant sequence divergence between *C. parvum* and *C. hominis* alleles, bind to fixed Caco cells, anti-CpMuc4 Ab inhibited infection *in vitro*	CpMuc4/5: probably proteolytically processed (multiple bands in WB with Cp lysate); predicted O-glycosylation	CpMuc4/5: Ab reacted with apical region of sporozoites	(O'Connor *et al*. 2009)

EF-1*α*	Identified by a mAb; mAb inhibited cell invasion; Identified as elongation factor 1 of *C. parvum* by MALDI-TOF MS	n.a.	Located in the apical region of sporozoites	(Matsubayashi *et al*. 2013)
CpClec	Mucin like glycoprotein with C-type lectin domain (CTLD); expression is probably developmentally regulated with a peak at 48 h p.i.	120 kDa protein; predicted: type 1 membrane protein, CTLD, O-glycosylated mucin-like domain, transmembrane domain, cytoplasmic tail containing a YXXφ sorting motif	Located on the apical surface, in dense granules and membranous structures of the parasite-cell interface	(Bhalchandra *et al*. 2013)
Cryptopain-1	Cryptospain-1: papain family enzyme; possibly involved in invasion	Cryptopain-1: transmembrane domain	Cryptopain-1: within oocyst, meronts	(Na *et al*. 2009; Kang *et al*. 2012; Ndao *et al*. 2013)
Cryptostatin	Cryptostatin: inhibitor of cysteine proteases	Cryptostatin: 178 aa, 18 kDa, 3 ICP motifs	Cryptostatin: diffuse within oocyst, meronts, (trophozoites)	

taurocholate, indicating a more controlled secretion (King *et al*. 2012). Knock-out studies in *Toxoplasma* revealed an essential function of calcium-dependent protein kinase (CDPK) 1 in the calcium-regulated exocytosis of micronemes (Lourido *et al*. 2010). CDPKs are protein kinases that have their ancestral origin in plants and are rare in animal cells, making them attractive targets for therapeutic intervention. *Cryptosporidium* contains seven CDPK family members which might be important in regulatory processes (Billker *et al*. 2009). Indeed, using a specific inhibitor for CpCDPK1, Murphy and colleagues demonstrated that CDPK1 is essential for cell invasion *in vitro* (Murphy *et al*. 2010). The importance of calcium signalling is also supported by a comparative analysis of eukaryotic genomes showing that compared with yeast (having a comparable number of protein-coding genes) *Cryptosporidium* and *Plasmodium* have a remarkably higher number of calcium-binding EF hand domains (Templeton *et al*. 2004*b*).

Formation of the PV and host cell actin rearrangement

Whatever drives the essential exocytosis of the apical organelles, the events following attachment and the start of internalization differ greatly from what is known from *Toxoplasma* or *Plasmodium*. Valigurová *et al*. performed extensive electron microscopy studies supporting the notion that *Cryptosporidium* is engulfed by the host cell. The authors suggested naming this developing compartment a parasitophorous sac (PS) instead of PV as there are considerable differences. The PS consists of a host cell membrane fold that can be incompletely fused thereby leaving openings to the environment. The membrane fold contains a thin layer of host cell cytoplasm. Between the host cell membrane fold and the parasite remains a small gap. In the apical region of the parasite an electron-dense band separates the cell cytoplasm from the modified part of the membrane fold (Valigurová *et al*. 2008). Another difference is the formation of a so-called feeder organelle at the parasite-host interface. This area of extensively folded membrane is thought to function as a site of substance exchange, which is supported by data showing the localization of a predicted ABC-transporter at this site (Perkins, 1999; Striepen *et al*. 2004). In other apicomplexan parasites the PV consists of one or more membranes that are highly decorated with parasite proteins that guarantee the exchange of nutrients and other molecules (Beyer, 2002; Spielmann *et al*. 2012). If this is the case in *Cryptosporidium* as well or if the feeder organelle is solely responsible for substance exchange remains to be elucidated. Early trophozoites of *Cryptosporidium* seem to have a direct connection to the host cell cytoplasm through a small channel that appears in transmission

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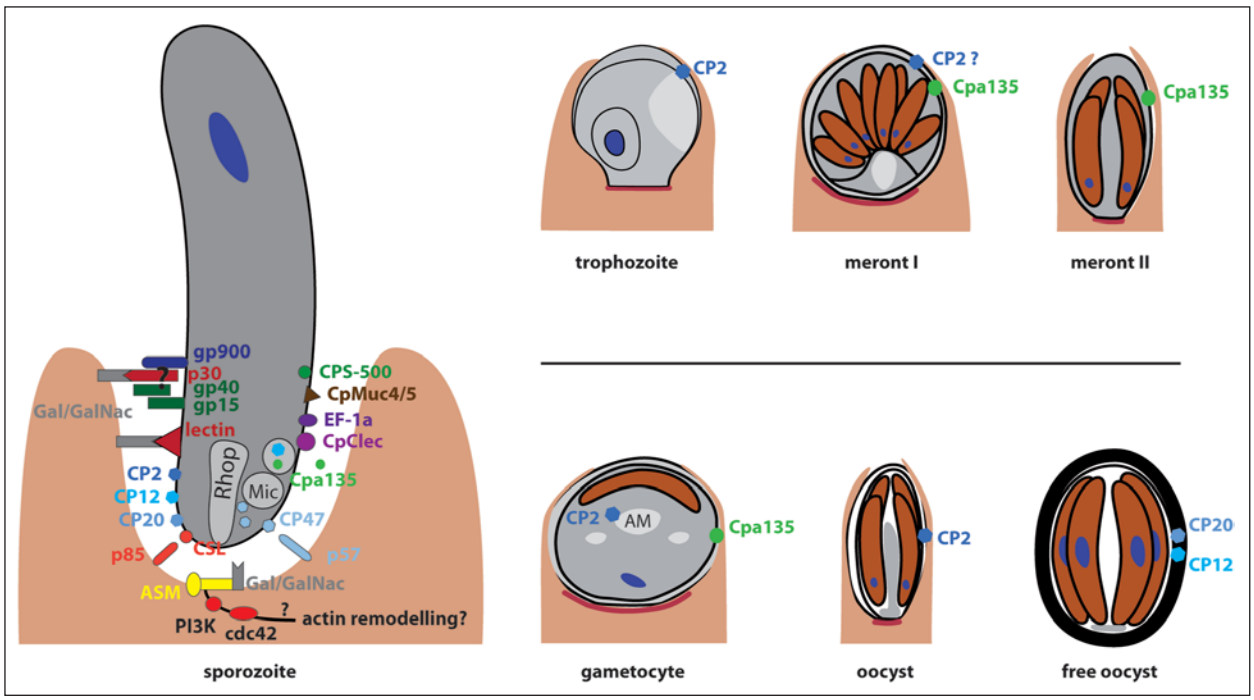


Fig. 3. Molecules involved in *Cryptosporidium* attachment and invasion of the host cell. Most data are based on studies with *C. parvum*. Some molecules were also detected in other developmental stages than the sporozoite. They might have additional functions and/or be involved in merozoite invasion.

electron microscopy (TEM) pictures (Huang *et al.* 2004*b*; Valigurová *et al.* 2008). It is likely that *Cryptosporidium* secretes molecules into the host cell through this tunnel connection to manipulate the host cell. The export of parasite-derived molecules to the parasite-host interface has been proven by immunogold staining (Huang *et al.* 2004*b*). The host cell undergoes a massive reorganization during the infection. How this reorganization is triggered by the parasite is hardly understood. However, two mechanisms through which the host cell might be forced to produce the epithelial protrusions that embrace the parasite have been proposed (see also Fig. 4). One is based on the observation that *Cryptosporidium* attracts the water channel aquaporin 1 (AQP1) in combination with a Na^+/glucose cotransporter (SGLT1) at the site of infection. The uptake of glucose goes along with a water influx that might support the increase of volume of the protrusions. Inhibition of SGLT1 and AQP1 reduced infectivity of *C. parvum* in cholangiocytes (Chen *et al.* 2005). SGLT1 aggregation is also shown to be dependent on myosin IIB of cholangiocytes (O'Hara *et al.* 2010). The second mechanism is based on the massive actin rearrangement that has been found in infected cells. Actin rearrangement might be the leading force that creates the membrane envelope around the parasite but at the same time it leads to the formation of a solid actin disc underneath the attachment site. Recently integrin $\alpha 2$ (ITGA2) and integrin ß1 (ITGB1) have been identified as potential receptors that transduce the parasite signal into the host cell (Zhang *et al.* 2012*b*). Intriguingly, integrins are key players controlling the dynamics and structures of actin-based processes in lamellipodia and filopodia in cells (Martin *et al.* 2002). Several authors studied the mediators involved in actin signalling on the host cell site. Notably, the signalling cascade very much resembles the signalling pathway found in the formation of lamellipodia/filopodia suggesting *Cryptosporidium* utilizes the same signal cascades. It was shown that *Cryptosporidium* uses the PI3K/Cdc42/WASP (Phosphatidylinositide 3-kinases/Cell division control protein 42/Wiskott–Aldrich Syndrome Protein) pathway to activate the Arp2/3 (Actin-Related Proteins) complex which leads to the branching of the actin filaments (Forney *et al.* 1999; Chen *et al.* 2004*b*). The involvement of further mediators of the Rho (Ras homologue gene) family (RhoA, Rac1) was ruled out indicating that only the Cdc42 pathway is utilized by *Cryptosporidium* (Chen *et al.* 2004*b*). This notion was supported by the finding that the downstream effectors of Cdc42, N-WASP and p34-Arc also accumulate at the host-cell parasite interface (Chen *et al.* 2004*a*). Nevertheless, inhibition of Cdc42 reduced *Cryptosporidium* invasion only up to 80% but never blocked it completely, making it likely that an alternative pathway exists. Indeed, *Cryptosporidium* induces the accumulation of the tyrosin kinase Src and a subsequent phosphorylation of cortactin, which in its activated stage recruits the Arp2/3 complex to open up another potential signalling cascade (Chen *et al.* 2003). Most recently, the involvement of calpain in

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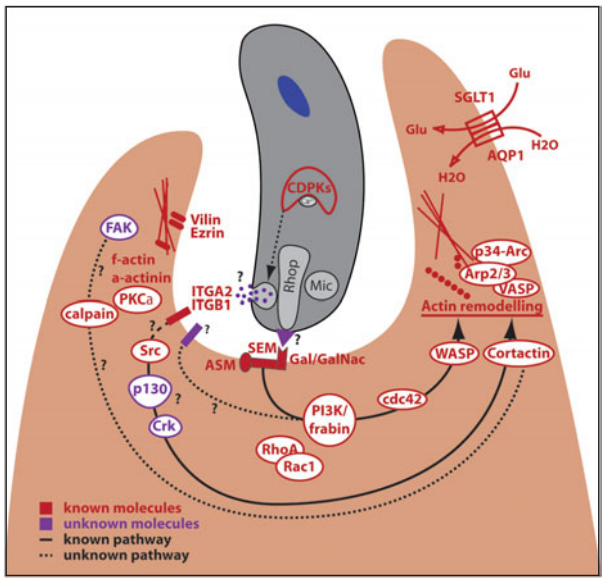


Fig. 4. Actin rearrangement and protrusion formation in the host cell induced by *Cryptosporidium*. Upon attachment *Cryptosporidium* triggers the host cell to undergo massive structural changes. During invasion microneme and rhoptry content are released. The transmitters and receptors are fairly unknown. Two possible receptors that might transduce a signal into the cell have been identified. Sphingolipid-enriched membrane microdomains in association with GalNac containing glycoproteins accumulate at the site of infection. In conjunction with likewise attracted PI3K and cdc42 this signal cascade might be how *Cryptosporidium* induces actin remodelling. Furthermore integrins (ITGA2/ITGB1) could pass a signal via Src kinase although this link has not been established. The activation of the PI3K/cdc42/N-WASP pathway leads in turn to the activation of the Arp2/3 complex and subsequently to the elongation and branching of actin filaments and formation of protrusions. The enlargement of the protrusions is supported by an increase in volume as a result of the attraction of the water channel aquaporin (AQP1). Several other molecules involved in actin rearrangement have been identified but the link between them has yet to be established. AQP: Aquaporin, Arp: actin releated proteins, ASM: acid-sphingomyelinase, ITGA: integrin α, ITGB: integrin β, cdc: cell division control protein, CDPK: calcium dependent protein kinase, FAK: focal adhesion kinase, Gal/GalNac: galactose-N-acetylgalactosamine, PI3K: phosphatidylinositol 3-kinase, PKC: protein kinase C, MIC: microneme, N-WASP: neural Wiskott–Aldrich syndrome protein, Rac: Ras-related C3 botulinum toxin substrate, RhoA: Ras homologue gene family, member A, Rhop: rhoptry, SEM: sphingolipid-enriched membrane microdomains, SGLT: sodium-glucose linked transporter, VASP: vasodilator-stimulated phosphoprotein.

actin plaque formation was demonstrated. Vital in contrast to heat-inactivated sporozoites could induce autolytic activation of calpains. Inhibition of calpain 1 and 2 led to reduced infectivity and diminished actin plaque formation (Perez-Cordon *et al*. 2011).

Calpains are a family of calcium-controlled cystein proteases shown to interact with many molecules involved in actin remodelling and are suggested to regulate membrane protrusions (Franco and Huttenlocher, 2005; Lebart and Benyamin, 2006). Another kinase that might be involved in the regulation of actin rearrangement is protein kinase Cα (PKCα), which has been shown to regulate actin condensation during *E. coli* invasion. Inhibition of PKCs in the *Cryptosporidium in vitro* model with different inhibitors reduced the number of infected cells (Hashim *et al*. 2006). However, PKCs regulate a diverse array of cell functions and might inhibit *Cryptosporidium* invasion through alternative pathways. The hypothesis of actin-driven envelopment is supported by the accumulation of villin and ezrin, two molecules that link F-actin to the membrane of microvilli at the PVM although F-actin was only found in rather small amounts at this site (Bonnin *et al*. 1999). Further enzymes that might be involved in host cell invasion are phospholipase (PLA) family members. For *Toxoplasma* it was demonstrated that cytosolic PLA_2 and the secreted form are necessary for infection and it was speculated that PLA might alter the host cell membrane fluidity (Bonhomme *et al*. 1999). Similar results were obtained when *C. parvum* sporozoites were incubated with the PLA inhibitor p-BPB or an anti-PLA_2 antibody and then used to infect HT-29 or Caco-2 cells. p-BPB as well as the antibody reduced infection in a dose-dependent manner when sporozoites were pre-treated but not when host cells were pre-treated with p-BPB (Pollok *et al*. 2003). However, the same publication describes exogenous PLA_2 isolated from cobra venom as supportive for the infection with *C. parvum in vitro*, whereas a recent publication showed the opposite effect using honey bee-derived PLA_2 (Carryn *et al*. 2012). This might be attributed to the diverse array of functions fulfilled by the PLA_2 family.

Survival in the host cell

After *Cryptosporidium* has settled down in its niche the parasite faces new challenges. First, it has to obtain nutrients, second, make sure the host cell is not trying to eliminate it and third, it has to escape the immune response. We will focus on the interaction between parasite and host cell in the following paragraphs.

Nutrition

Cryptosporidium has a comparatively small genome that also lacks considerable parts of the metabolic machinery constraining the parasite to acquire nutrients from its host cell. Consequently, the *C. parvum* genome contains at least 69

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Table 2. Experimentally addressed molecules involved in the metabolism of *Cryptosporidium*

Molecule	Function	References
Carbohydrate metabolism		
PK	Pyruvate kinase: catalyses the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP	(Cook *et al*. 2012)
Lipid metabolism		
FASI	Fatty acid synthetase I: elongation of fatty acid chains on an activated acyl carrier protein (ACP)	(Zhu *et al*. 2002, 2004)
SFP-PPT	Surfactin production element – phosphopantetheinyl transferase (SFP-PPT): activates ACP by the transfer of a 4′-phosphopantetheine from CoA to ACP	(Cai *et al*. 2005)
PKS	Polyketide synthetase class I: Production of polyketides	(Zhu *et al*. 2002; Fritzler and Zhu, 2007)
LCE	Long-chain fatty acid enlongase: catalyses elongation of preferentially myristoyl-CoA and palmitoyl-CoA	(Fritzler *et al*. 2007)
ACS	Fatty acyl synthase: activates fatty acids; supposed role in lipid trafficking and import	(Zhu *et al*. 2002, 2004; Camero *et al*. 2003)
ACBP1	Acyl-CoA-binding protein: storage or transport of fatty acids	(Zeng *et al*. 2006)
ACBP	Ankyrin repeat-containing acyl-CoA binding protein: mediates incorporation of activated acyl-CoA in metabolic pathways	(Zeng *et al*. 2006)
ORP	Oxysterol binding protein related proteins: suggested to be responsible for lipid trafficking between host cell und parasite	(Zeng and Zhu, 2006)
ACCase	Acetyl-CoA carboxylase: *in silico* identification within the genome; converts acetyl-CoA to malonyl-CoA	(Zhu, 2004)
Amino acids/polyamine metabolism		
ADC	Arginine decarboxylase: plant derived; converts arginine to putrescine	(Keithly *et al*. 1997)
SSAT	Spermidine/spermine N1-acetyltransferase: converts spermine and spermidine to lower polyamines	(Yarlett *et al*. 2007)
SAHH	S-adenosylhomocysteine hydrolase: catabolizes S-adenosylhomocysteine into adenosine and L-homocysteine	(Ctrnáctá *et al*. 2007)
Nucleotide metabolism		
DHFR	Dihydrofolate reductase: reduces dihydrofolic acid to tetrahydrofolic acid	(Anderson, 2005; Doan *et al*. 2007; Martucci *et al*. 2008)
IMPDH	Inosine 5′-monophosphate dehydrogenase: converts AMP into GMP	(Umejiego *et al*. 2004)
AK	Adenosine kinase: converts adenosine to AMP	(Galazka *et al*. 2006)

transporters for different substrates (Abrahamsen *et al*. 2004). Direct acquisition of nutrients from the cytosol is hindered by the PV making it necessary to integrate appropriate transporters into the PV membrane, a strategy that is for example described for *Plasmodium* (Spielmann *et al*. 2012). *Cryptosporidium* differs in that it does not reside in an intracellular PV but in an epicellular location, limiting the contact area to the cytosol. This might be partially reversed by the feeder organelle that is thought to be responsible for the uptake of nutrients through increasing the contact area due to multiple membrane folds. However, ultimate proof of such a material transfer by the feeder organelle and involved transporters is still missing. The epicellular location of the parasite results in an exposure to the gut lumen that could be another source of nutrients. The zoites might directly endocytose material from the intestinal lumen if they are only partially embraced. Otherwise they need to facilitate transporters that ensure an active transport through the PV membrane.

Cholesterol is an essential lipid in membranes of eukaryotic cells that controls the membrane fluidity. *Cryptosporidium* is not able to *de novo* synthesize cholesterol and therefore relies on host cell-derived cholesterol. Ehrenman and co-workers showed that *Cryptosporidium* is auxotroph for plasma Low Density Lipoprotein (LDL) but not High Density Lipoprotein (HDL) and that it derives its cholesterol from these proteins. Moreover, the cholesterol incorporated by *Cryptosporidium* did not originate from *de novo* synthesis of the host cell but from micelles imported via the NPC1L1 transporter into the cell (Ehrenman *et al*. 2013). The way in which it enters the parasite, however, remains elusive.

Fatty acids are produced by fatty acid synthetase (FAS) I (common in mammals) or FAS II (common in bacteria). FAS I was found in *C. parvum* but its preferred substrate is a C16 palmitic acid and is therefore not involved in *de novo* synthesis of parasite fatty acids (Zhu *et al*. 2004). Since *Cryptosporidium* lacks the FAS II (which is for

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example present in *Toxoplasma*) it was hypothesized that *Cryptosporidium* is generally not able to use *de novo* synthesis of fatty acids and instead relies on scavenging fatty acids from the host cell (Zhu, 2004). Just recently, it has been demonstrated that polyketide synthetase (PKS) 1 of *Toxoplasma* and *Eimeria* are highly abundant in oocysts and therefore proposed to play a crucial role in oocyst wall lipid synthesis. Similarly, FAS I of *C. parvum* is highly expressed during late time points in cell culture when the oocyst walls are made (Bushkin *et al*. 2013). *Cryptosporidium parvum* possesses a PKS class I. The function of the PKS for *Cryptosporidium* is so far unknown but it was speculated that the produced polyketides might function to inhibit bacteria within the niche of *Cryptosporidium* (Zhu *et al*. 2002). A few other enzymes involved in fatty acid metabolism have been identified and experimentally addressed (see Table 2). Since the genome of *Cryptosporidium* lacks any enzymes for ß-oxidation, fatty acids obviously cannot be used as an energy source. The *C. parvum* genome also lacks all genes of the Krebs cycle and most of the important genes for the respiratory chain of mitochondria. *Cryptosporidium parvum* is capable of catabolizing mono-sugars and can synthesize, store and catabolize polysaccharides indicating that it predominantly relies on glycolysis (Abrahamsen *et al*. 2004). However, the enzymes involved in the carbohydrate metabolism identified in the genome of *C. parvum* have not been studied biochemically.

Cryptosporidium is capable of interconversion of several amino acids but not of *de novo* synthesis (Rider and Zhu, 2010) and seems to have a large set of 11 amino acid transporters (Abrahamsen *et al*. 2004). Biochemical approaches identified a plant-derived enzyme that converts arginine into polyamines. Polyamines such as spermidine and spermine can interact with negatively charged molecules such as DNA, RNA, phospholipids and acidic proteins and modulate various cellular functions. In *C. parvum* the conversion was accomplished by a plant-derived arginine decarboxylase (ADC) instead of an ornithine decarboxylase (ODC) that is utilized by mammalian cells (Keithly *et al*. 1997). Furthermore, *Cryptosporidium* is able to convert scavenged spermidine and spermine to lower polyamines by a spermidine/spermine N1-acetyltransferase (SSAT) (Yarlett *et al*. 2007). Very recently, it was demonstrated that *C. parvum* also induces the human SSAT that in turn provides acetylspermine that can be scavenged by the parasite (Morada *et al*. 2013). Nucleotides are another important class of molecules. The thymine synthesis pathway has become an important antimalarial drug target by inhibiting dihydrofolate reductase (DHFR). Interestingly, *Cryptosporidium* is resistant against pyrimethamine treatment targeting DHFR. Crystal structures of *C. hominis* DHFR and a comparison to resistant and non-resistant *Plasmodium* DHFR revealed three amino acid positions that might confer resistance (Anderson, 2005). *Cryptosporidium parvum* has a greatly simplified metabolism of nucleotides that mainly relies on salvage and interconversion (Abrahamsen *et al*. 2004).

All in all, *Cryptosporidium* has a highly streamlined metabolism. The metabolic pathways have been mainly identified by analysing genomic data. Only a few biochemical approaches have been made, which are summarized in Table 2.

Apoptosis

Programmed cell death is well known to serve as a defence mechanism against microbial pathogens. Consequently, pathogens are under evolutionary pressure to regulate apoptosis in a way that promotes their survival either by inducing apoptosis to eliminate e.g. immune cells or by preventing apoptosis to keep the intracellular niche intact (Lamkanfi and Dixit, 2010). Protozoan parasites are no exception in this regard and are shown to modulate the host cell apoptosis pathways (Lüder *et al*. 2001). The first observation that *Cryptosporidium* might induce apoptosis was published by Chen *et al*. who reported on DNA condensation and nucleus fragmentation of infected cells *in vitro* (Chen *et al*. 1998). Induction of apoptosis by *Cryptosporidium* depends on caspases and the extracellular death receptor (FasR) because blockage of these pathways by inhibitors or antibodies prevents apoptosis (Chen *et al*. 1999; Ojcius *et al*. 1999). As a consequence of *Cryptosporidium* infection, cells up-regulate FasR and Fas ligand (FalL) expression. Co-culture experiments demonstrated that FasL-sensitive Jurkat T cells undergo apoptosis when co-cultured with infected epithelial cells, indicating an autocrine and paracrine activation of apoptosis. Increased amounts of soluble FasL (mediating paracrine activation) could be detected in supernatants of infected cells by western blot, whereas other apoptosis-associated mediators such as IL-1β, TNF-α, and TGF-β remained unchanged (Chen *et al*. 1999). Unchanged levels of TNF-α and IFN-γ were also reported by Gong *et al*. They demonstrated the up-regulation of the programmed cell death ligand 1 (PD-L1, B7-H1) in infected epithelial cells while the expression of the negative regulator of PD-L1 miR-513 was reduced. Among other things, PD-L1 is involved in the homoeostasis of activated T cells by inducing apoptosis. Co-cultivation of infected epithelial cells with activated T cells led to the induction of apoptosis in the latter. This might enable the parasite to actively modulate the immune response of the host (Gong *et al*. 2010). In contrast another publication reported on the absence of paracrine activation as it was found that most cells undergoing apoptosis had to be directly infected with *Cryptosporidium* (McCole *et al*. 2000).

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Interestingly, these authors also found NF-κB-dependent inhibition of apoptosis by *Cryptosporidium*. Moreover, *Cryptosporidium* infections reduced the number of apoptotic cells if cells were treated with apoptosis-inducing agents in comparison with non-infected cells (McCole *et al*. 2000). Using an *ex vivo* piglet model, Foster *et al*. suggested that the modulation of apoptosis in *C. parvum*-infected animals is rather a retention mechanism of the host than an active modulation by the parasite. *Ex vivo* samples of the ileum of infected piglets were positive for cleaved (activated) caspase-3 and showed increased enterocyte shedding predominantly at the tip of the villi, indicating an activation of the apoptotic pathway. The finding that the inhibitors of apoptosis XIAD and survivin were up-regulated in the epithelium of infected animals together with the finding that inhibition of XIAD or the proteasome could revert the anti-apoptotic effect demonstrated that apoptosis is inhibited in infected cells. Moreover, NF-κB, as a negative regulator of apoptosis, was up-regulated in cells of infected animals. Within the villous epithelium there was no difference in NF-κB activation between infected and non-infected enterocytes but cells in the process of shedding appeared NF-κB negative. This would be in agreement with the finding that most apoptotic cells were detected rather in the intestinal lumen than in the epithelial layer of the villi. The results led to the hypothesis that the host itself represses apoptosis of enterocytes in order to maintain the epithelial barrier function until the enterocytes are shed and undergo apoptosis (Foster *et al*. 2012). An induction of apoptosis has also been shown in an *in vivo* mouse model (Sasahara *et al*. 2003) but whether apoptosis is actively altered by *Cryptosporidium*, by the epithelial cell itself or a combination of both, and how far this favours the parasite or the host remains obscure. If and to what extent *Cryptosporidium* intervenes with the host cell apoptotic pathway can probably only be comprehensively answered through the characterization and inhibition of defined parasite-derived molecules involved in apoptosis modulation.

Egress from the host cell

Many studies on parasite host-cell egress have been conducted for *Toxoplasma* and *Plasmodium* identifying the different strategies of and many molecules involved in parasite egress (reviewed in Roiko and Carruthers, 2009; Sibley, 2010). Egress from the host cell is an essential step to complete the life cycle. Unlike *Toxoplasma* and *Plasmodium*, *Cryptosporidium* does not reside in an intracellular PV and might therefore utilize other escape strategies. *Toxoplasma* and *Plasmodium* utilize various proteases and pore-forming proteins (PFP) to escape from the host cell. PFPs have been identified in many apicomplexan parasites except *Cryptosporidium* (Kafsack *et al*. 2009). This indicates that *Cryptosporidium* either uses different proteins to disrupt the PVM or relies on a mechanic disruption. *Cryptosporidium* might utilize the apoptotic pathways of the host cell. Although it is not entirely clear if *Cryptosporidium* alters the apoptotic pathways, current research shows that apoptosis is induced and regulated in infected enterocytes. The studies of Foster *et al*. demonstrated that apoptosis is induced in enterocytes but dampened until they reach the tip of the villus. This might be a mechanism with which the host ensures the integrity of the epithelium, but it might also be used by the parasite to escape from the host cell at a certain point in its life cycle progression. A family of proteases that could be involved in egress are the calpains. Calpains are involved in cell migration and thought to destabilize the adhesion to the extracellular matrix (ECM) (mainly calpain 1 and 2) (Franco and Huttenlocher, 2005). Just recently it was demonstrated that *Plasmodium* and *Toxoplasma* exploit host cell calpain 1 to egress from their respective host cells (Chandramohanadas *et al*. 2009). One role of calpain during invasion of *Cryptosporidium* has been shown (Perez-Cordon *et al*. 2011) but a possible function in egress still needs to be investigated. There are no experimental data on the egress of *Cryptosporidium* and as such the discussed points are hypothetical.

Merogony/gamogony

Although we know details on sporozoites, particularly attachment and invasion, much less is known about the molecular processes following cell invasion. There are hardly any molecular data on merogony, gamogony and oocyst formation. *Eimeria* gametocyte antigens (GAM proteins), some of which are key components of the oocyst wall, have been studied and used to generate the first subunit vaccine against *Eimeria* in broilers (Belli *et al*. 2002; Sharman *et al*. 2010). The example of *Eimeria* might emphasize that understanding of the molecular aspects of the entire life cycle is not only of academic interest but could ultimately lead to the development of treatment strategies.

OMICS

Genome

The full description of the genomes of both *C. parvum* and *C. hominis* was an important step to uncover the biology of *Cryptosporidium* (Abrahamsen *et al*. 2004; Xu *et al*. 2004). It immediately became clear that both species rely on an extremely streamlined metabolism. The inability to synthesize many indispensible molecules *de novo* hinges the parasite on its host, but the lack of certain pathways, especially the apicoplast-associated pathway, also makes it

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resistant to common treatments against protozoan parasites (Abrahamsen *et al*. 2004). Both genomes consist of eight chromosomes of less than 10 Mb. A plastid or mitochondrial genome as found in other apicomplexans such as *Plasmodium* is missing although some reminiscent genes have been introduced into the parasite genome. The genomes somehow look like a mosaic since they contain many genes that are most likely of bacterial or plant origin, introduced by lateral gene transfer. In the genome study of *C. parvum*, 15 plant- and 14 bacteria-derived genes were identified (Abrahamsen *et al*. 2004). Huang *et al*. analysed the *C. parvum* genome in more detail and found as much as 783 high probability ($<10^{-7}$) hits for plant- or bacteria-like genes. Using these genes for a more stringent analysis resulted in 31 genes of which some have been experimentally validated as originating from plants or bacteria (Huang *et al*. 2004*a*). The small genome size is a result of a reduced repertoire of metabolic genes and the absence of variant surface antigen families. Moreover, there are only a few introns (5% in *C. parvum*, 5–20% in *C. hominis*) and the intergenic regions are comparatively small, a feature *Cryptosporidium* shares with *Theileria* but that separates it from other apicomplexans (mean intergenic length: *C. parvum* – 566 bp, *Theileria parva* – 405 bp, *Plasmodium falciparum* – 1694 bp) (Abrahamsen *et al*. 2004; Xu *et al*. 2004; Gardner *et al*. 2005). The genomes of *C. parvum* and *C. hominis* are nearly identical and Xu *et al*. therefore concluded that phenotypic differences are due to polymorphism and gene regulatory variations. This notion is supported by a study that analysed the presence of ten genetic loci which were thought to be species specific for either *C. parvum* or *C. hominis*. Nine out of ten genes were present in both genomes indicating the difference in sequence of 3–5% between *C. parvum* and *C. hominis* is likely due to technical reasons (gaps, missing annotations). However, the authors found several single nucleotide polymorphisms (SNP) which appeared to be species specific (Bouzid *et al*. 2010). Moreover, the same group identified a new family of telomeric encoded proteins in *C. parvum* and *C. hominis* that show sufficient sequence diversity for epidemiological analysis and might be mediators of host specificity (Bouzid *et al*. 2013*a*, *b*). Blast searches revealed several microneme- and rhoptry-associated proteins that show homologies to organelle proteins from other apicomplexans. Of the five homologues found in the *C. hominis* genome three are already described for *C. parvum* (gp900, TRAP-C1, Cpa135), whereas the six rhoptry protein homologues found have not been studied in *Cryptosporidium*. Interestingly, the rhoptry protein orthologues p235-E5 and p235-E2 of *Plasmodium yeolii* seem to have many copies within the *C. hominis* genome (Xu *et al*. 2004). The p235 gene family (reticulocyte-binding homologue family in *P. falciparum*) is shown to be involved in red blood cell binding, but the function in *Cryptosporidium* needs further investigation (Grüner *et al*. 2004).

Genomic data have also been used to look for intra- and interspecies differences in order to find genes that can be used to delineate species, genotypes and subtypes and to identify genes that account for host specificity. Single locus genotyping of the gp60 locus is commonly used to discriminate between *C. parvum* and *C. hominis* and between anthroponotic and zoonotic *C. parvum*. A recent comparison of single- and multilocus genotyping, however, revealed that single locus genotyping of gp60 is not a valid surrogate for multilocus genotyping (Widmer and Lee, 2010). A suggested approach to delineate species is to look for species-specific SNPs (Bouzid *et al*. 2010). A comparison of an anthroponotic and a zoonotic genome of *C. parvum* revealed over 12 000 SNPs. Highly diverged regions (> 5 SNPs/kb) were mainly found at the end of the chromosomes. Interestingly, a three-way comparison between *C. hominis* and anthroponotic and zoonotic *C. parvum* resulted in a panel of genes that showed a higher similarity between the human parasite *C. hominis* and the anthroponotic *C. parvum* than between anthroponotic and zoonotic *C. parvum*. This might indicate that these genes account for host specificity (Widmer *et al*. 2012).

The phylogenetic position of *Cryptosporidium* is still under debate. There are reasonable arguments that place *Cryptosporidium* together with the *Gregarina* at the early branch of the apicomplexan clade (Barta and Thompson, 2006). Just recently, the genome of *Ascogregarina taiwanensis* was partially sequenced. The phylogenetic analysis of 66 gene fragments of 14 alveolata supported the hypothesis that *Cryptosporidium* is more closely related to *Gregarina* then to *Coccidia* (Templeton *et al*. 2010).

The decoding of the genome of *Cryptosporidium* gives direct access to gene sequences making it theoretically easy to implement functional gene studies. Unfortunately, *Cryptosporidium* lacks some key components of the RNAi pathway excluding the knock-down strategy to elucidate gene functions (Fayer and Xiao, 2008). Genetic manipulation by introducing genes of interest to perform knock-out or knock-in studies or to create stable reporter lines would be the optimal solution but so far such tools are still missing. Nevertheless, Li and colleagues reported on the transient transfection of *C. parvum* with a gfp reporter construct presenting the possibility to genetically manipulate these parasites (Li *et al*. 2009).

Transcriptome

Zhang *et al*. developed the first microarray covering all predicted open reading frames (ORF) in the

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Table 3. Literature published on various omics studies on *Cryptosporidium*

Omics	Description	Reference
cryptoDB	Publicly available database that aims to organize the omics data	http://cryptodb.org (Puiu *et al*. 2004; Heiges *et al*. 2006)
Genomics	The genome sequence of *C. parvum* The genome sequence of *C. hominis* The genome sequence of *C. muris* Genome survey sequences of *C. parvum*	(Abrahamsen *et al*. 2004) (Xu *et al*. 2004) http://cryptodb.org (Strong and Nelson, 2000)
Transcriptomics	Analysis of global gene regulation in *C. parvum in vitro* development The transcriptome of *C. parvum* oocysts (microarray) The transcriptome during *C. parvum in vitro* development (qPCR) Full length cDNA library of *C. parvum* Expressed sequence tags of *C. parvum* sporozoites	(Oberstaller *et al*. 2013) (Zhang *et al*. 2012*a*) (Mauzy *et al*. 2012) (Yamagishi *et al*. 2011) (Strong and Nelson, 2000)
Proteomics	LC-MS/MS/MudPit analysis of excysted *C. parvum* sporozoites LC-MS/MS analysis of unexcysted and excysted *C. parvum* sporozoites LC-MS/MS analysis of excysted *C. parvum* sporozoites	(Sanderson *et al*. 2008) (Snelling *et al*. 2007) (Siddiki, 2013)
Kinomics	*In silico* identification and characterization of 73 kinases	(Artz *et al*. 2011)

genome of *C. parvum*. Using this microarray, they discovered the gene expression pattern of *C. parvum* oocysts (low metabolic activity) in untreated compared with UV-light treated parasites. In untreated oocysts 1924 genes (51%) turned out to be expressed, among them genes for enzymes (25%), RNA metabolism (14·3%), ribosome biogenesis (13·8%) and gene expression (12·8%). A substantial number of the expressed genes are devoted to protein recycling as a result of unobtainable host nutrients and the inability of *Cryptosporidium* to *de novo* synthesize proteins. Interestingly, glycolysis as the source for energy and carbons seems to be regulated differentially in different developmental stages. The microarray analysis revealed a high expression of lactate dehydrogenase (LDH) whereas acetate-CoA ligase (AceCL1) and alcohol dehydrogenase (ADH) were not expressed in oocysts. Further investigations using qPCR showed that LDH is expressed in oocysts and sporozoites but only at low levels in intracellular stages. AceCL1 and ADH showed the opposite expression pattern with highest expression levels at late intracellular stages. Thus *Cryptosporidium* seems to metabolize pyruvate to lactate in oocysts and sporozoites and to ethanol and acetate in intracellular stages (Zhang *et al*. 2012*a*).

Another study utilized qPCR to detect the transcription level for 3302 genes of the *C. parvum* protein-coding genome within a 72 h *in vitro* infection course. Analysing the expression pattern revealed that the analysed genes aggregate in nine clusters with different expression profiles during the 72 h infection course. However, since *Cryptosporidium* does not develop uniformly in cell cultures, only the measuring points within the first 24 h represent more or less the same developmental stages (zoite, trophozoite, meront). After the first replication round, parts of the parasites do not develop further, leading to a mixture of zoites, trophozoites and meronts at the times of analysis. Analysis of the expression pattern of the first 24 h led to only six expression profiles. Interestingly, excysted sporozoites (2 h after excystation) showed the lowest number of expressed genes indicating that sporozoites are already packed with the necessary proteins for attachment and invasion of the host cell. Although merozoites, like sporozoites, invade enterocytes the expression profile shows that these two zoites are biochemically distinct (Mauzy *et al*. 2012).

A computational approach using different algorithms to identify overrepresented motifs of potential transcription factor binding sites upstream of genes within a set of 3·281 transcripts resulted in 25 overrepresented motifs. Some of these motifs belong to known transcription factor families such as AP2, C-box-like or E2F, whereas others were not assignable. Most interestingly, the transcriptome could be clustered in 200 groups of genes that were expressed at different points of time (2, 6, 12, 24, 36, 48, 72 h). These groups were then analysed for the occurrence of potential transcription factor binding sites leading to sub-clusters of probably cis-regulated genes and a first map of global gene regulation in *C. parvum* (Oberstaller *et al*. 2013).

Proteome

The difficulty in separating the epicellular stages of *Cryptosporidium* from their host cells is also reflected in the scarce proteome data. So far, three studies exploring the protein repertoire of *C. parvum* have been undertaken. All are targeting the sporozoite/oocyst as a stage that can be obtained in high purity in contrast to the epicellular stages.

Sanderson and colleagues identified 1237 non-redundant proteins by liquid chromatography with

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tandem mass spectrometry detection (LC-MS/MS) and multidimensional protein identification technology (MudPit) analysis. Among them 18 proteins were of mitochondrial origin supporting the hypothesis of a remnant mitochondrion. Micronemal and rhoptry proteins are thought to have a key function in invasion. A search for orthologues in *Toxoplasma gondii* and proteins containing microneme-associated domains revealed 24 potential micronemal proteins of which 14 could be detected on the protein level. Of 38 rhoptry orthologues present in the *T. gondii* and *P. falciparum* genome 12 were identified in the protein content of sporozoites. Only one dense granules protein, an orthologue to *T. gondii* subtilisin, was identified (Sanderson *et al*. 2008). The low abundance of dense granule-associated orthologues was also seen on the genomic level in *C. hominis*, which is believed to be the result of an early separation and fast diversification of these genes (Xu *et al*. 2004). However, *Cryptosporidium* apparently possesses many mucin like and other surface proteins of which 51 could be identified as proteins highlighting the importance of these molecules for invasion. In total, the study identified many molecules of unknown function that might give new information about the molecular mechanisms of *Cryptosporidium* host cell invasion (Sanderson *et al*. 2008).

An overview of the omic literature can be found in Table 3.

CONCLUDING REMARKS

Cryptosporidium is one of the most resistant protozoan parasites. Few drugs (nitazoxanid, paromomycin, halofuginone) have been found to be effective against *Cryptosporidium*, and these have only partial effects. None of the common treatments against protozoan parasites are effective against *Cryptosporidium*. Moreover, the small oocysts are highly resistant against most disinfectants. The rigidity of the oocyst is due to the multilayered structure of the oocyst wall protecting the sporozoites from mechanical, environmental and chemical influences. Further insights into the structure and chemical composition of the oocyst might give us the opportunity to develop more efficient disinfectants. Upon infection of the host, the parasite expresses many molecules on the sporozoite surface that mediate attachment and probably invasion. However, no data are available about the signalling events taking place in the parasite. Calcium is probably one of the important second messengers that are responsible for signal transduction, but only a few kinases and transporters have been addressed so far. CDPKs might be responsible for microneme exocytosis as shown for *Toxoplasma* but there is no proof yet. The application of experimental results from other coccidian parasites to *Cryptosporidium* is not possible in many respects because of the substantial peculiarities of *Cryptosporidium*. *Cryptosporidium* is special in that it is embraced by the host cell instead of invading it, that it stays in an epicellular location and that it induces tremendous actin rearrangement in the cell. This shows that *Cryptosporidium* utilizes other infection strategies than, for example, *Toxoplasma* or *Eimeria*. This notion is supported by the analysis of the genomic data of *C. parvum* and *C. hominis* showing a highly streamlined metabolism that lacks many pathways found in other apicomplexans and depends on the import of essential nutrients from the host. Moreover, *C. parvum* expresses several potentially microneme-associated proteins of which several have no orthologues to proteins of other apicomplexans. Thus, *Cryptosporidium* probably releases unique molecules from the micronemes addressing different receptors/pathways in the target cell. Although we have some knowledge about the events within the target cell upon sporozoite attachment, many question marks remain. They include questions about the mediators released by the parasite, the receptors addressed, how nutrients are imported and what the molecular basis of egress is as well as questions concerning gametogenesis and oocyst formation. The publication of three *Cryptosporidium* genomes was a milestone giving insight into the organization and function of these parasites. However, the translation from *in silico* to *in vitro*/*in vivo* remains difficult. The lack of methods that allow full reproduction of the life cycle *in vitro* and a missing toolbox for genetic manipulation are major drawbacks. Moreover, it is not currently possible to store *Cryptosporidium* for a long period of time. Consequently, preservation of isolates or modified parasites relies on a constant passage of the parasites. Thus, the future challenges will be to improve *in vitro* systems and/or to establish alternative models such as *Cryptosporidium baileyi* or *Cryptosporidium wrairi* and to develop a transfection system for *Cryptosporidium*.

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