Adaptation of *Cryptosporidium* oocysts to different excystation conditions

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SUMMARY

Within the genus *Cryptosporidium* 2 lineages have evolved, one adapted to the acid environment of the stomach and abomasum, the other comprising parasites that multiply in the small intestine. We tested whether the release of sporozoites from oocysts, a process known as excystation, is triggered by conditions which mimic the site of infection. Specifically, we exposed oocysts from gastric and intestinal *Cryptosporidium* species to acid conditions or to a neutral solution of taurocholic acid, at 37 °C. We found that oocysts from the gastric species *C. muris* and *C. andersoni* excysted in both conditions, whereas the intestinal species *C. parvum* and *C. hominis* did not respond to acid. When the effect of temperature alone was tested on *C. muris* and *C. parvum*, only oocysts from the former species excysted in significant numbers. Oocysts from intestinal species did not respond to temperature alone, nor to acidity. These observations are consistent with the need of gastric species to rapidly excyst and release the sporozoites upon ingestion, and indicate that *Cryptosporidium* oocysts have evolved to maximize delivery of sporozoites to the region of the gastro-intestinal tract where the parasite multiplies.

Key words: *Cryptosporidium* spp., gastro-intestinal tract, oocyst excystation, sporozoites.

INTRODUCTION

In the life-cycle of *Cryptosporidium* parasites, the infection initiates with the ingestion of oocysts and the release of infectious sporozoites into the gastro-intestinal (GI) tract. Consistent with the dissemination of oocysts in the environment, and with their characteristic longevity (Yang *et al*. 1996; Widmer *et al*. 1999; Jenkins *et al*. 2003), oocysts have evolved to maximize sporozoite survival and protect the sporozoites from the moment they are released from the intestinal epithelial cell, until they are ingested by a new host and reach the preferred organ of the GI tract. The precisely timed release of the 4 sporozoites contained in each oocyst is essential for parasite survival, as sporozoites are short lived and their mobility is limited. Depending on the species, sporozoites are released in the stomach or the small intestine. A small number of species, including *C. muris* and *C. andersoni*, multiply in the pits of the stomach and abomasum (Tyzzer, 1907; Lindsay *et al*. 2000). In contrast, the majority of *Cryptosporidium* species, including the most important agents of human cryptosporidiosis *C. parvum* and *C. hominis*, infect primarily the small intestine, with occasional involvement of the bile duct and colon.

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Whichever the evolutionary mechanism which has led to the partition of the genus in a gastric and intestinal clade, the mechanism controlling oocyst excystation must be under strong selection to maximize the probability of infection. Since the infectious dose can be fewer than 10 oocysts (Okhuysen *et al*. 1999), the precise release of sporozoites in the right organ of the GI tract is essential to parasite survival. Oocysts from gastric and intestinal species can be expected to have adapted to different requirements; oocysts from gastric species to promptly release the sporozoites when exposed to body temperature and to stomach acidity, whereas the trigger for intestinal species can be expected to be more complex, as the oocyst must transit through the stomach and reach the duodenum before excysting. Premature release of sporozoites adapted to the condition of the small intestine would cause the sporozoite to be inactivated in the stomach's acid environment.

The conditions which induce excystation of *C. parvum* oocysts have been extensively investigated (Fayer and Leek, 1984; Woodmansee, 1987; Campbell *et al*. 1992; Robertson *et al*. 1993; Kato *et al*. 2001). The research described in these publications was primarily driven by the need to optimize *in vitro* excystation to generate sporozoites for research, and to determine oocyst viability. Our motivation to study this process in different *Cryptosporidium* species was to improve our understanding of the evolution of different organ tropisms in this

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Table 1. *Cryptosporidium* isolates

Species	Organ tropism	Isolate	Propagation host	Oocyst purification	n[1]	Oocyst age (days)	% Excystation (S.D.) HCl	% Excystation (S.D.) TA[3]	Isolate reference
C. parvum	intestinal	P2	mouse[2]	Histodenz	1	12	0·5	14	Tanriverdi *et al.* (2007)
		MD/TU114	mouse[2]	Histodenz/IMS	3	20, 27, 34	0·2 (0·3)	22·3 (7·8)	Tanriverdi and Widmer (2006)
		VUZV-N	calf	Sheather's/sucrose	6	1, 1, 58	0	33·7 (3·3)	Unpublished
C. hominis		TU502	pig	Histodenz	1	27	0	16	Xu *et al.* (2004)
C. andersoni	gastric	JS6	calf	Histodenz/IMS	1	31	23·3[5]	39·0[5]	Unpublished
		VUZV-A	bull	Sheather's/sucrose	4	2, 58	94·7 (4·6)	94·2 (3·6)	Unpublished
C. muris		RN66	mouse[4]	Histodenz/IMS	3	20, 27, 34	87·7 (2·9)	41·0 (23·7)	Taylor *et al.* (1999)

[1] Number of replicate excystation experiments.
[2] C57BL/6, immunosuppressed with dexamethasone (Yang *et al.* 2000).
[3] Taurocholic acid.
[4] CD-1-Foxn1^{nu} nude (Charles River, Wilmington, Mass, USA).
[5] Mean of 3 counts of same excystation experiment.

genus, and the adaptation of pathogens to the host environment in general. Although not significant human or veterinary pathogens, *C. muris* and *C. andersoni* are among the few species which have adapted to the extreme environment of the stomach, and the study of these parasites is expected to uncover unique mechanisms of adaptation. This study was undertaken to test the assumption that *C. muris* and *C. andersoni* oocysts have adapted to excyst in response to elevated temperature and acid, whereas *C. parvum* and *C. hominis* oocysts do not respond to acidity.

MATERIALS AND METHODS

Parasites

Table 1 lists the *Cryptosporidium* isolates used in this study. Three *C. parvum*, 1 *C. hominis*, 2 *C. andersoni* and 1 *C. muris* isolate were used. MD/TU114 is an isolate obtained by co-infecting a mouse with 2 *C. parvum* isolates MD and TU114. P2 is a recombinant line derived from such a mixed infection which differs genetically from MD and TU114 (Tanriverdi *et al.* 2007). TU502 is a *C. hominis* reference isolate, which was used for sequencing the *C. hominis* genome (Xu *et al.* 2004) and has also been characterized with respect to host specificity (Akiyoshi *et al.* 2002). TU502 has been maintained by serial propagation in gnotobiotic piglets for over 5 years. *C. andersoni* isolate JS6 was isolated from a fecal sample collected in a pen which housed bulls and heifers aged approximately 6 months and 2 months, respectively, on a dairy farm in Waianae, Hawaii. *C. andersoni* VUZV-A was isolated from a 6-month-old Gascogne bull kept at the Institute of Animal Science, Prague, Czech Republic. VUZV-N was obtained from an experimentally infected 1-day-old Holstein calf kept at the same location. Oocysts of *C. muris* isolate RN66 (Taylor *et al.* 1999) were originally purchased from Waterborne Inc., New Orleans, Louisiana. The isolate has been maintained at TCSVM in Nu/Nu mice for over 6 years.

Oocyst purification

Different methods were used to purify the oocysts. In the first method, fecal samples were first fractionated on a 15%–25% step gradient of Histodenz (Sigma) as described (Widmer *et al.* 1998). Fractions containing the oocysts were aspirated from the interface between the two Histodenz solutions and oocysts further purified by immunomagnetic purification (Aureon Crypto Kit, Aureon Biosystems, Vienna, Austria). To avoid interfering with subsequent excystation assays, at completion of the IMS procedure, oocysts were released from the magnetic particles by a short incubation in 1 M NaCl instead of the recommended incubation in 0·1 M HCl. As

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indicated in Table 1, some oocyst samples were not subjected to IMS purification. Alternatively, feces were first homogenized in water and filtered through a nylon sieve to remove large debris. The resulting suspension was precipitated by low-speed centrifugation and the concentrated sample suspended in Sheather's sucrose solution to float the oocysts. The top layer containing the oocysts was recovered, the oocysts washed once in phosphate buffered saline (PBS) and loaded onto a sucrose gradient (Arrowood and Sterling, 1987). A fraction containing the oocysts was recovered and a final purification step on a gradient of Percoll was performed as described (Kuczynska and Shelton, 1999).

Species identification

For isolates maintained in the laboratory the species designation was known from prior analyses of the small subunit ribosomal RNA (SSU rRNA) sequence and/or the LIB13 species-specific PCR marker (Tanriverdi *et al*. 2003). This is the case for MD/TU114, P2 (Tanriverdi *et al*. 2007) and TU502 (Xu *et al*. 2004). Isolate RN66 (GenBank Accession AB089284) was confirmed as *C. muris* as part of an ongoing genome sequencing project (http://msc.tigr.org/c_muris/index.shtml). For JS6 a 484-base-pair portion sequenced on both strands was 99% similar to position 408–890 of the *C. andersoni* small subunit ribosomal RNA gene (Accession AB089285) and *C. muris* 'calf genotype' (Accession AF093496). The latter designation was used for *C. muris*-like oocysts isolated from bovine hosts prior to *C. andersoni* being named a separate species (Lindsay *et al*. 2000). The JS6 partial SSU rRNA sequence was deposited in GeneBank under Accession number EF432239. Confirming the classification of JS6 as *C. andersoni*, sequence similarity values between the JS6 partial SSU rRNA sequence and the homologous sequence from *C. muris*, *C. parvum* and other species was less than 99%. Isolate VUZV-A and VUZV-N were identified as *C. andersoni* and *C. parvum*, respectively, based on oocyst size, oocyst morphology, and the age of the bull (6 months and <7 days, respectively) from which they were isolated. Consistent with published dimensions for *C. andersoni* and *C. parvum* oocysts (Upton and Current, 1985; Lindsay *et al*. 2000), the mean oocyst size for VUZV-A was $7{\cdot}4 \pm 0{\cdot}4 \times 5{\cdot}6 \pm 0{\cdot}4\ \mu$m ($n=16$), and for VUZV-N $4{\cdot}8 \pm 0{\cdot}2 \times 4{\cdot}4 \pm 0{\cdot}1\ \mu$m ($n=25$).

Excystation and numerical analysis

To measure excystation ratios oocysts were suspended in 0·8% taurocholic acid (TA) in PBS, pH 7·4, or 0·1 M HCl and incubated at 37 °C for 1 h. Following incubation, a portion of the oocyst suspension was loaded onto a haemocytometer and empty and intact oocysts present on the 1 mm^2 gridded area enumerated at 400× magnification. Excystation ratios were calculated as the fraction of empty plus partially excysted oocysts divided by the sum of empty and full oocysts. Empty oocysts present in the unexcysted oocyst sample were subtracted. For each isolate the number of independent replicate experiments is shown in Table 1.

RESULTS

The results of the excystation assays with oocysts from 4 *Cryptosporidium* species exposed to 2 excystation conditions are shown in Table 1. A total of 14 independent assays were performed. Except for *C. hominis*, each species was tested a minimum of 3 times in both excystation conditions. Excystation of *C. parvum* and *C. andersoni* oocysts were tested independently in 2 laboratories using different isolates. Oocysts from 1 *C. hominis* and 1 *C. muris* isolate were examined. In HCl, excystation of *C. parvum* and *C. hominis* oocysts was minimal, and the highest excystation ratio obtained was 0·5%. In taurocholic acid, *C. parvum* and *C. hominis* oocyst excystation rates ranged from 14% to 34%. *C. andersoni* and *C. muris* excysted well in both conditions. Clearly, oocysts of *C. muris* and *C. andersoni* excysted better in HCl than *C. parvum* and *C. hominis* oocysts, and also excysted slightly better than *C. hominis*/*C. parvum* in taurocholic acid (t-test, $P=0{\cdot}02$). Oocysts of the gastric species excysted equally well in both conditions (Mann-Whitney Rank Sum test, $P=0{\cdot}50$). *C. muris* oocysts appeared to respond better to acid than to taurocholic acid but the means of 3 experiments were not significantly different (t-test, $P=0{\cdot}09$).

To exclude the possibility that oocyst age may differentially affect excystation of oocysts of gastric and intestinal species, the mean oocyst age in both groups, measured from the day of excretion until the day the experiment was carried out (Table 1), was compared. The mean age for all gastric oocyst samples was 29 days ($n=6$, S.D. 21), whereas for intestinal species the mean age was 22 days ($n=8$, S.D. 21). The difference in age was statistically not significant (t-test, $P=0{\cdot}55$), indicating that age was not a factor in the relative response to HCl and TA.

The unexpected observation that *C. muris* and *C. andersoni* oocysts excysted well in HCl and in taurocholic acid suggested that temperature may be sufficient to induce excystation of oocysts from these species. We therefore compared the ability of *C. muris* and *C. parvum* oocysts to excyst at 37 °C in PBS. The excystation ratio for *C. muris* after a 1-h incubation at 37 °C was 38·3% (S.D. 3·4) as compared to 15·3% (S.D. 2·0) for oocysts maintained on ice. For *C. parvum* the ratios were very low at both temperatures, 2·7% (S.D. 2·5) at 37 °C, and 1·3%

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(S.D. 1·9) for the control oocysts kept on ice. This observation indicates that temperature is an important excystation trigger in *C. muris* but not in *C. parvum*.

DISCUSSION

The ability of *Cryptosporidium* species to infect different organs of the GI tract is a striking example of a pathogen's adaptation to colonizing different host environments. In this context, *C. muris* and *C. andersoni* are particularly interesting as not many pathogens have adapted to the harsh environment of the stomach. The comparison of the *C. muris* genome, currently being sequenced, with that of *C. parvum* (Abrahamsen *et al*. 2004) and *C. hominis* (Xu *et al*. 2004) will provide a detailed insight into how these genomes have been shaped by this adaptative process. The present study indicates that the ability of oocysts to respond to different environmental conditions has also been affected by this evolutionary process. Because of the short life-span of the sporozoites, delivery to the right location in the GI tract is critical for a successful infection. It would therefore be expected that the excystation phenotype is under strong selection.

In addition to the need to respond to specific excystation conditions, oocysts must also be able to discriminate between the GI tract of the original host in which they were formed and the newly infected host. In contrast to oocysts from other coccidia, which sporulate upon release in the environment, *Cryptosporidium* oocysts are thought to be excreted fully sporulated (Lindsay *et al*. 2000; Upton and Current, 1985). The observation that oocysts of gastric species readily excyst at 37 °C is not consistent with the absence of a sporulation-like process, as in the absence of such a process, excystation would be expected to occur soon after oocyst formation and release from the epithelial cell, preventing the excretion of infectious oocysts. The apparent lack of specific excystation triggers other than temperature, raises the possibility of a maturation step analogous to sporulation occurring upon release of the oocysts into the environment. Microscopical observations (Lindsay *et al*. 2000; Upton and Current, 1985) indicate that such a process would not involve morphological changes which are readily apparent. Since elevated temperature by itself does not efficiently induce *C. parvum* oocysts to excyst, such a step would not be essential for intestinal species. While gastric species have apparently evolved methods to prevent excystation in the same host, intestinal species face the challenge of preventing premature release of the sporozoites after uptake by a new host, as excystation in the stomach would most likely lead to the immediate inactivation of the sporozoites. The observation that, regardless of conditions, *C. muris* and *C. andersoni* oocysts excyst in higher proportions than *C. parvum* and *C. hominis* oocysts is consistent with the need of oocysts from gastric species to quickly release the sporozoites following ingestion. Consistent with this view is the occasional presence of empty oocysts in preparations of *C. muris* oocysts. Temperature fluctuations during purification, and in particular during the various centrifugation steps, could be sufficient to initiate excystation in this species. This could also explain the presence of empty *C. muris* oocyst shells in samples that were not incubated at 37 °C. Since excystation of *C. parvum* and *C. hominis* requires a sequence of stimuli signalling arrival into the small intestine (Campbell *et al*. 1992), *in vitro* excystation conditions producing high excystation ratios are more difficult to reproduce. Among these stimuli, exposure to acid, which mimics the oocysts' travel through the stomach, has been shown to enhance excystation (Campbell *et al*. 1992; Kato *et al*. 2001; Robertson *et al*. 1993).

Because *Cryptosporidium* oocysts cannot be produced in culture in significant numbers, and the parasites cannot be cryopreserved, comparative studies involving multiple species are challenging. The excystation experiments presented here were performed over a period of over 2 years with oocysts obtained at different times from various sources, locations, and host species. Maintaining 4 species in the laboratory to produce oocysts for simultaneous excystation assays was not feasible and, given the inclusion of 2 gastric and 2 intestinal species, was not necessary. It was, however, essential to ascertain that no bias was introduced as a result of oocyst age, host species or oocyst purification method. The fact that excystation phenotypes of gastric and intestinal species were not affected by the propagation host indicates that the oocysts' response to specific stimuli is a stable phenotype. We were also able to rule out oocyst age as an explanation for the excystation results. Although oocyst age varied over a wide range, there was no significant difference in age between gastric and intestinal group. Similarly, centrifugal fractionation on Histodenz, an iodinated gradient medium, or sucrose and Percoll, did not impact excystation in a noticeable manner. Of particular concern was also avoiding exposing the oocysts to conditions known or suspected to promote excystation, such as bleach, acid and, of course, elevated temperature (Smith *et al*. 2005). For this reason the conventional procedure for releasing oocysts from the magnetic beads was modified by replacing HCl with a short incubation in 1 M NaCl. At no time during purification were oocysts exposed to bleach.

In spite of excystation and host cell invasion being the only phases of the *Cryptosporidium* life-cycle directly accessible to experimentation, our results show that our understanding of these processes remains superficial. The involvement of parasitic

proteases during excystation was postulated on the basis of the inhibitory effect of certain protease inhibitors on *C. parvum* excystation (Forney *et al.* 1996; Okhuysen *et al.* 1996). It is unknown how excystation signals are transmitted to the sporozoite and what other genes participate in excystation and sporozoite activation. The lack of methods for efficient *in vitro* cultivation and for genetic manipulation of *Cryptosporidium* parasite is at the root of our limited ability to directly test the role of candidate genes in controlling excystation and the parasite's life-cycle in general. Analyses of the oocyst's transcriptional response to excystation conditions will shed light on the molecular mechanisms leading to excystation.

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