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Evidence for a Structural Role for Acid-Fast Lipids in Oocyst Walls of Cryptosporidium, Toxoplasma, and Eimeria

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ABSTRACT Coccidia are protozoan parasites that cause significant human disease and are of major agricultural importance. Cryptosporidium spp. cause diarrhea in humans and animals, while Toxoplasma causes disseminated infections in fetuses and untreated AIDS patients. Eimeria is a major pathogen of commercial chickens. Oocysts, which are the infectious form of Cryptosporidium and Eimeria and one of two infectious forms of Toxoplasma (the other is tissue cysts in undercooked meat), have a multilayered wall. Recently we showed that the inner layer of the oocyst walls of Toxoplasma and Eimeria is a porous scaffold of fibers of β-1,3-glucan, which are also present in fungal walls but are absent from Cryptosporidium oocyst walls. Here we present evidence for a structural role for lipids in the oocyst walls of Cryptosporidium, Toxoplasma, and Eimeria. Briefly, oocyst walls of each organism label with acid-fast stains that bind to lipids in the walls of mycobacteria. Polyketide synthases similar to those that make mycobacterial wall lipids are abundant in oocysts of Toxoplasma and Eimeria and are predicted in Cryptosporidium. The outer layer of oocyst wall of Eimeria and the entire oocyst wall of Cryptosporidium are dissolved by organic solvents. Oocyst wall lipids are complex mixtures of triglycerides, some of which contain polyhydroxy fatty acyl chains like those present in plant cutin or elongated fatty acyl chains like mycolic acids. We propose a two-layered model of the oocyst wall (glucan and acid-fast wall lipids) that resembles the two-layered walls of mycobacteria (peptidoglycan and acid-fast lipids) and plants (cellulose and cutin).

IMPORTANCE Oocysts, which are essential for the fecal-oral spread of coccidia, have a wall that is thought responsible for their survival in the environment and for their transit through the stomach and small intestine. While oocyst walls of Toxoplasma and Eimeria are strengthened by a porous scaffold of fibrils of β-1,3-glucan and by proteins cross-linked by dityrosines, both are absent from walls of Cryptosporidium. We show here that all oocyst walls are acid fast, have a rigid bilayer, dissolve in organic solvents, and contain a complex set of triglycerides rich in polyhydroxy and long fatty acyl chains that might be synthesized by an abundant polyketide synthase. These results suggest the possibility that coccidia build a waxy coat of acid-fast lipids in the oocyst wall that makes them resistant to environmental stress.
plasma, Ziehl-Neelsen in Cryptosporidium best-characterized mycobacterial wall lipids are mycolic acids, (see Fig. S1 in the supplemental material) (12, 13). Among the presence of high-molecular-weight lipids that form a waxy coat following washing with hydrochloric acid in ethanol) due to the cell walls of mycobacteria are acid-fast (i.e., retain lipophilic dyes, Cryptosporidium mine the structural role, if any, of acid-fast lipids in oocyst walls of infections, such as virus (HIV), AIDS was diagnosed by the presence of opportunistic infections, such as Cryptosporidium, which was detected in stools (11). As background, the cell walls of mycobacteria are acid-fast (i.e., retain lipophilic dyes following washing with hydrochloric acid in ethanol) due to the presence of high-molecular-weight lipids that form a waxy coat (see Fig. S1 in the supplemental material) (12, 13). Among the best-characterized mycobacterial wall lipids are mycolic acids, which are synthesized in part by polyketide synthases (14). The plant cuticle on the surface of leaves and stems, which also labels with lipophilic dyes, is composed of wax esters and cutin (a polymer of glycerol and ω-hydroxy and mid-chain hydroxy fatty acids) (15).

We became interested in the lipid content of oocyst walls when we identified by mass spectrometry an extraordinarily abundant polyketide synthase (PKS1, also known as type 1 fatty acid synthase) in Toxoplasma and Eimeria oocysts, which resembles mycobacterial polyketide synthases. To explore the potential importance of acid-fast lipids in oocyst walls, we treated isolated walls with organic solvents, which made the walls fall apart. We analyzed released lipids with high-resolution and high-accuracy mass spectrometry. The most abundant oocyst wall lipids were triglycerides that have polyhydroxy fatty acyl chains like those of plant cutin but different than mycolic acids.

RESULTS
Oocyst walls of Cryptosporidium, Toxoplasma, and Eimeria all label with acid-fast stains. The oocyst walls of each parasite label with carbol-fuchsin, a lipophilic dye used for bright-field acid-fast stains (Kinyoun or Ziehl-Neelsen), and with auramine-O, a fluorescent acid-fast stain (Fig. 1A) (16). Developing Eimeria oocysts have acid-fast vesicles in their periphery (Fig. 1B). Sporocyst walls of Toxoplasma are acid-fast, while those of Eimeria are not. Instead acid-fast stains localize to “refractile bodies” of Eimeria sporozoites, an organelle of unknown function. The latter result suggests that acid-fast lipids are not an important component of sporocyst walls of Eimeria, which distinguishes this parasite from Toxoplasma. Plant cuticles also label with auramine-O (17). Additional acid-fast stains are shown in Fig. S1 in the supplemental material.

Polyketide synthases are among the most abundant proteins in oocysts of Toxoplasma and Eimeria. Coccidian parasites each have two predicted polyketide synthases that resemble those of mycobacteria (Fig. 2A) (10, 18, 19). In contrast, Plasmodium,
which is related to coccidian parasites but is not spread by the fecal-oral route, has no polyketide synthases (10). The coccidian polyketide synthases are very large, since each enzyme contains four modules (Toxoplasma and Eimeria) or three modules (Cryptosporidium) of catalytic domains. Each module contains six catalytic domains that add two carbons to the growing chain by a series of reactions that includes oxygenated intermediates (20). The PKS1 of Toxoplasma (encoded by the TGVEG_013030 gene) was very abundant in tryptic digests of oocyst proteins, as shown by 263 unique peptides and 36% sequence coverage (Fig. 2B). For comparison, the number of unique peptides and sequence coverage for the 10 most abundant cytosolic proteins of Toxoplasma are shown in Table S1 in the supplemental material. The PKS1 of Eimeria (encoded by the ETH_00015480 gene) showed 9% sequence coverage and 69 unique peptides. Mass spectrometry of Cryptosporidium proteins was not performed here. However, messenger RNAs of a Cryptosporidium polyketide synthases (type 1 fatty acid synthase encoded by cg3_2180) peak at 48 h of culture when oocyst walls are being made (21). Reverse transcription-PCR (RT-PCR) showed that oocysts of Toxoplasma and Eimeria express PKS1 and PKS2, as well as a 4′-phosphopantetheine transferase (PPTase), which is essential for PKS activity (see Fig. S1 in the supplemental material) (22).

Lipids appear to be an important component of the rigid bilayer present in the oocyst wall of Cryptosporidium. To explore further the possible role of acid-fast lipids in the structure of oocyst walls, we treated isolated walls with reagents that remove proteins or lipids. The oocyst wall of Cryptosporidium, which does not contain β-glucan, is simpler than the oocyst wall of Eimeria and so will be described first. Sonicated and washed walls of Cryptosporidium form scrolls that have a moderately electron-dense inner layer that is rich in glycoproteins (Fig. 3A) (7). There is also a rigid bilayer (as shown by scrolling) that is thicker than a cell membrane. Pronase, which digests proteins, removes the inner layer of the oocyst wall but leaves the rigid bilayer intact (Fig. 3B). Pronase-treated oocyst walls of Cryptosporidium remain acid-fast in a quantitative assay (Fig. 3D). In contrast, chloroform-methanol (2:1), which extracts lipids, completely disrupts the oocyst walls of Cryptosporidium and prevents acid-fast staining (Fig. 3C and D), while chymotrypsin, which degrades proteins, reduces acid-fast staining. Treatment with 1 N NaOH, which deproteinates yeast walls and Eimeria oocyst walls (see next section), dissolved Cryptosporidium oocyst walls (data not shown). These results suggest a simple, if incomplete, model of the Cryptosporidium oocyst wall, in which acid-fast lipids are likely an important component of the rigid bilayer, while glycoproteins are present in the inner layer (see Fig. 6) (7).

Organic solvents remove the outer layer of the oocyst wall of Eimeria. Because of issues of availability, these studies were performed with unsporulated oocysts of Eimeria from euthanized chickens rather than Toxoplasma from euthanized cats. Previously we have used Eimeria oocyst walls for transmission electron microscopic (TEM) studies of fibrils of β-1,3-glucan in the inner layer of the oocyst wall (8). The control for these studies was the wall of Saccharomyces, which is composed of a single layer that contains fibrils of β-1,3-glucan and chitin (Fig. 4A) (23). The wall of Saccharomyces, which does not contain lipids, is resistant to chloroform-methanol. In contrast, sodium hydroxide removes proteins from the Saccharomyces walls, so that only fibrils remain.

The outer layer of the Eimeria oocyst wall, which is relatively electron dense, has linear structures that extend from the bilayer to the external surface of the wall (Fig. 4B). The outer layer is uninterrupted, as shown by en face negative staining of intact oocysts, and so forms the permeability barrier in the oocyst wall. In contrast, the inner layer of the Eimeria oocyst wall, which is less electron dense, is composed of a porous scaffold of fibrils of β-1,3-glucan. The macrophage lectin dectin-1 binds to fibrils of β-1,3-glucan in the inner layer of the oocyst wall (Fig. 4C) (8, 24). Organic solvents disrupt the outer layer of the Eimeria oocyst wall and markedly reduce the acid-fast staining and UV fluorescence of dityrosines (Fig. 4B to D).

Treatment with sodium hydroxide, which extracts proteins and breaks ester bonds within triglycerides (see next section), disrupts the outer layer of the Eimeria oocyst wall that develops a “soap bubble” appearance by negative staining (Fig. 4C). Sodium hydroxide does not reduce dectin-1 binding or acid-fast staining, but it decreases dityrosine fluorescence (consistent with removal of proteins) (Fig. 4D). Together, these data suggest a model for the Eimeria oocyst wall in which the inner layer contains β-1,3-glucan like fungal walls, while the outer layer and the rigid bilayer contain acid-fast lipids like those of mycobacterial walls (Fig. 6). Because oocyst walls of Toxoplasma and Eimeria share common components, including proteins cross-linked with dityrosines, homologs of Cryptosporidium oocyst wall proteins, glucan hydrolases, β-1,3-
glucan, and acid-fast lipids (see next section), it is likely that this model also applies to oocyst walls of *Toxoplasma* (1, 2, 4, 8–11, 25). We do not presently have a model for sporocyst or tissue cyst walls of *Toxoplasma*.

**Triglycerides, many with polyhydroxy fatty acyl chains, are the most abundant lipids in oocyst walls.** High-resolution Fourier transform ion cyclotron resonance mass spectrometry, which has an accuracy of better than 1 part per million, allowed us to determine the elemental composition of lipids extracted with chloroform-methanol from oocyst walls (Fig. 5A; see Table S2 in the supplemental material) (26). For example, the chemical formula for the lipid with $[M + Na]^+ = 953.7419$ $m/z$ is $C_{57}H_{102}O_9$. *Cryptosporidium* oocyst wall lipids also include phosphatidylcholines, which may represent membrane contamination. Because the triglycerides vary in the lengths of the fatty acyl chains and their degrees of unsaturation and/or oxidation, oocyst wall lipids are a complex mix for each organism (Fig. 5B). The hydroxyl groups but not the double bonds can be localized by low-energy collision-induced dissociation (CID) of some of the triglycerides (26).

*Eimeria* triglycerides included numerous species with polyhydroxy acyl chains, while *Toxoplasma* and *Cryptosporidium* triglycerides included numerous species with longer fatty acyl chains (Fig. 5C and D; see Table S2 in the supplemental material). While it is not possible to estimate the relative abundance of each triglyceride in a complex mixture, multiple biological repeats of *Eimeria* lipids showed that triglycerides with polyhydroxy acyl chains, which contain 7 to 12 oxygens per triglyceride where glycerol contains six oxygens, are predominant in the higher-molecular-weight range. In the same way, *Cryptosporidium* triglycerides with elongated fatty acyl chains containing as many as 24 carbons are predominant in the higher-molecular-weight range. Triglycerides with polyhydroxy acyl chains and elongated fatty acyl chains are relatively less abundant in *Toxoplasma*.

Consistent with the presence of triglycerides in oocyst walls, mRNAs for diacylglycerol acyltransferases (DGAT1 and DGAT2) (27), as well as a putative acyl coenzyme A (acyl-CoA):cholesterol acyltransferase (ACAT), are expressed in *Eimeria* oocysts (see Fig. S1 in the supplemental material). While *Toxoplasma* tachyzoites (an asexual wall-less stage that can be propagated in vitro) make fatty acids with 14 to 26 carbons and zero to one carbon double bonds (28), they are missing the hydroxyl groups present in oocyst wall triglycerides. In contrast, fatty acyl chains containing multiple hydroxyl groups are present in cutin poly-

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**FIG 4** In contrast to fungal walls, the oocyst wall of *Eimeria* is very sensitive to organic solvents. (A) Transmission electron microscopy (TEM) shows that walls of *Saccharomyces cerevisiae* (which have a single layer (between the hollow arrowheads), remain relatively intact after treatment with chloroform-methanol. In contrast, sodium hydroxide, which removes proteins, leaves behind only the mesh of fibrils of β-1,3-glucan and chitin. Size bars represent 100 nm. (B) TEM shows that oocyst walls of *Eimeria* have two layers sandwiched around a rigid bilayer. The outer layer, which contains linear structures (arrowheads), is removed with chloroform-methanol and is disrupted with sodium hydroxide. The inner layer, which may be fragmented by sonication (arrow), remains intact after chloroform-methanol treatment and has an extracted appearance after NaOH treatment. Negative stains show that the outer layer forms a continuous barrier that has a “soap bubble” appearance after treatment with sodium hydroxide. The inner layer is a porous scaffold of fibrils of β-1,3-glucan that is resistant to organic solvents (8). Size bars represent 100 nm. (C) Broken, washed oocyst walls contain dityrosines that autofluoresce in UV and glucan fibrils that bind dectin-1. After chloroform-methanol treatment, dectin-1 binds in a punctate manner to oocyst walls. Size bars represent 5 μm. (D) Fluorometric measurements show that chloroform-methanol removes acid-fast lipids and dityrosines (cross-linked proteins) from oocyst walls and exposes glucan fibrils that bind dectin-1. Sodium hydroxide removes proteins and dityrosines but leaves β-glucan and acid-fast lipids intact. Error bars represent ±1 standard deviation from the mean in three experiments performed in triplicate. Please see Fig. S2 in the supplemental material for additional data.

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(Continued)
mers in the plant cuticle (15). Finally, although oocyst walls are acid-fast and oocysts strongly express a polyketide synthase, we did not identify lipids that resemble mycolic acids.

**DISCUSSION**

These observations suggest structural roles for lipids in parasite walls and appear to broaden our understanding of what lipids make walls acid-fast (Fig. 6). The evidence for the importance of triglycerides in the oocyst walls of coccidian parasites includes the following. The oocyst walls of *Cryptosporidium*, *Toxoplasma*, and *Eimeria* are each acid-fast. The oocyst walls of *Cryptosporidium* and *Eimeria* fall apart when treated with organic solvents. Each oocyst wall contains a rigid bilayer that is reminiscent of the outer membrane of mycobacteria (13). By far the most abundant lipids in extracts of the oocyst walls of all three parasites are triglycerides, which contain fatty acyl chains that vary in length and in the degree of unsaturation and hydroxylation. *Cryptosporidium* lipids also include some phosphatidylcholines (blue), which are lower molecular weight and have an even-numbered m/z. (B) The complexity of the lipids extracted from *Eimeria* oocyst walls is shown by a close-up view of lipids with an m/z from 900 to 930. Peaks with even-unnumbered masses, which are marked with asterisks, are the results of naturally occurring isotopes of carbon (13C) and hydrogen (1H) present within the triglycerides. (C) CID fragmentation of an *Eimeria* triglyceride with [M + Na]⁺ m/z 953.7419 and a chemical composition of C₅₇H₁₀₂O₉ localizes hydroxyls in acyl chains. Fragments that prove structures are shown with abbreviated masses. The blue double arrow represents the loss of a hydroxyl group. The locations of the carbon double bonds cannot be determined by CID fragmentation. Unassigned m/z values come from two isomers, one of which contains an acyl chain with three hydroxyl groups. (D) CID fragmentation of a *Cryptosporidium* triglyceride with [M + Na]⁺ m/z 927.7417 and a chemical composition of C₅₉H₁₀₀O₆ reveals the presence of one acyl chain with 20 carbons and 4 double bonds. CID fragmentation of an isomer of this triglyceride has one acyl chain with 22 carbons and 5 double bonds.

Because the gene knockout methodology is not available (29), we are unable to prove the link between the abundant polyketide synthase identified by mass spectrometry in *Toxoplasma* and *Eimeria* and the triglycerides extracted from the oocyst walls. Because we were unable to extract lipids from oocyst walls without killing the parasites inside, we were unable to prove that lipids are essential for the impermeability of the oocyst wall and for pathogenicity. The two-layered oocyst walls of *Cryptosporidium* (glycoproteins and acid-fast lipids) and *Toxoplasma* and *Eimeria* (glucan and acid-fast lipids), if this is the case, resemble two-layered walls of mycobacteria (peptidoglycan and acid-fast lipids) and plant cuticles (cellulose and waxes/cutin) (Fig. 6). In addition to chitin and proteins, nematode eggs contain an inner layer rich in lipids (30). Because coccidia, mycobacteria, and plants are deeply divergent, the use of lipid coats to protect these organisms from environmental challenges appears to be the result of convergent evolution. In contrast, walls of fungi and of other parasites

![Image](https://example.com/image.png)
Unsporulated oocysts of Animal Care and Use Committees at Boston University and at the USDA. Parasites and animals. MATERIALS AND METHODS (32). are missing the lipid layer (31). Finally, these results may help transmitted by the fecal-oral route (e.g., Coccidia, mycobacteria, and plants, which are deeply divergent organ-

FIG 6 Coccidia, mycobacteria, and plants, which are deeply divergent organisms, each have a lipid-rich coat that makes them resistant to environmental stress. The rigid bilayer of the Cryptosporidium oocyst wall is composed of acid-fast lipids, while glycoproteins, in particular Cys- and His-rich oocyst wall proteins (OWPs), are present in the inner layer (1, 7). Acid-fast lipids are present in the rigid bilayer and in the outer layer of the oocyst wall of Toxoplasma and Eimeria, while fibrils of β-1,3-glucan are in the inner layer. Glyco-

MATERIALS AND METHODS Parasites and animals. All animal work was approved by Institutional Animal Care and Use Committees at Boston University and at the USDA. Unsporulated oocysts of Eimeria tenella and Toxoplasma gondii (VEG and ME49 strains) were prepared from infected chickens and cats, respecti-

Acid-fast staining and fluoresce microscopy. Oocysts were washed extensively in phosphate-buffered saline (PBS) and applied to glass slides, which were then heat fixed. Alternatively, cryosections of ceca of infected chickens with Eimeria were applied to glass slides. For bright-field acid-fast stains, slides were incubated in carbol-fuchsin for 45 min at room temperature (Kinyoun method), washed, and destained with 3% HCl in ethanol for 5 s (11). Mycobacterium smegmatis, a gift of Eric Rubin of the Harvard School of Public Health, was a positive control, while Saccharomyces cerevisiae was a negative control. Histology slides were acid-fast stained by the Ziehl-Neelsen method, using methylene blue as a counterstain. For fluorescent acid-fast stains, heat-fixed slides were stained with auramine-O (Polysciences kit 24665) for 30 min at room temperature and destained in ethanol-HCl solution for 30 s at room tem-

perature (16). Slides were examined with a DeltaVision deconvolving mi-

Electron microscopy of oocysts treated with proteases and organic solvents. Oocysts of Cryptosporidium were washed and broken with glass beads, and walls were isolated as previously described (8). Walls were left untreated, extracted in chloroform-methanol (2:1) for 3 h, or treated with 10 μg/ml promace or 1 mg/ml chymotrypsin, for 3 h at 37°C. Sonicated treated or untreated Cryptosporidium oocyst walls were washed in PBS, fixed in aldehyde containing ruthenium red, and prepared for transmis-

Quantitative fluorescence assays. Treated and untreated broken oocyst walls of Cryptosporidium in PBS were pipetted into wells of black 96-well plates (Greiner Bio-One), left to dry overnight at 37°C, heat fixed, and acid-fast stained with auramine-O. Auramine-O acid-fastness of trip-

Mass spectrometry of oocyst proteins. Sporulated and unsporulated oocysts of Toxoplasma (VEG strain) and Eimeria (1 to 2 million oocysts each) were extensively washed and broken with glass beads. Oocyst pro-

RT-PCR of oocyst mRNAs. RNA was extracted from Toxoplasma (ME49 strain) and Eimeria unsporulated and sporulated oocysts using PureLink RNA minikit (Life Technologies) by breaking the oocysts with glass beads in the extraction buffer. Reverse transcription-polymerase chain reactions (RT-PCR) were performed using SuperScript III kit (Life Technologies) with 30 ng of total RNA per sample, according to the man-

Toxoplasma primers were to the PKS1 (TGME49_294820), PKS2 (TGME49_204560), PPTase (TGME49_214440), and actin (TGME49_209030) genes (shown in Table S3 in the supplemental mate-

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Temperature and destained in ethanol-HCl solution for 30 s at room temper-

FIG 6 Coccidia, mycobacteria, and plants, which are deeply divergent organ-

Acid-fast lipids

Glycoproteins

Cryptosporidium oocyst wall

β-1,3-glucan fibrils

Toxoplasma/Eimeria oocyst wall

Acid-fast lipids

Waxes and cutin

Mycobacterium tuberculosis wall

Peptidoglycan

Cellulose

Plant cuticle wall

Acid-fast lipids
Acid-Fast Lipids in Oocyst Walls

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org.

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