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RESEARCH ARTICLE

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 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.

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Coccidian parasites make infectious walled oocysts that are spread by the fecal-oral route (1). *Toxoplasma gondii*, a zoonotic coccidian of worldwide distribution, makes oocysts with a double-layered wall that are shed by cats. Once shed in the environment, *Toxoplasma* makes a sporulated oocyst that contains two-walled sporocysts, each of which contains four sporozoites that infect humans and other warm-blooded animals (2). In immunocompetent persons, acute *Toxoplasma* infections are controlled, but the parasite remains within cysts in brain and muscle, which are not symptomatic. In contrast, *Toxoplasma* causes disseminated infections in fetuses and in AIDS patients who lack cellular immunity (3). *Eimeria* spp. are a large group of parasites infecting the gut that make oocysts and sporocysts similar to those of *Toxoplasma* (4). However, *Eimeria* is limited to a specific animal and specific region of the gut. For example, *Eimeria tenella* is confined to ceca of chickens, where it causes dysentery and costs billions of dollars worldwide (5).

Cryptosporidium parvum causes diarrhea in people and in livestock. Recently *Cryptosporidium* has been found to be among the four most important causes of moderate to severe diarrhea in children in the developing world (6). *Cryptosporidium* makes a different oocyst than those of *Toxoplasma* and *Eimeria*, which does not contain sporocysts and has a simpler wall (7).

We recently showed that the inner layer of the oocyst walls of *Toxoplasma* and *Eimeria* contains fibrils of β-1,3-glucan that form a porous scaffold (8). A parasite glucan hydrolase has a unique

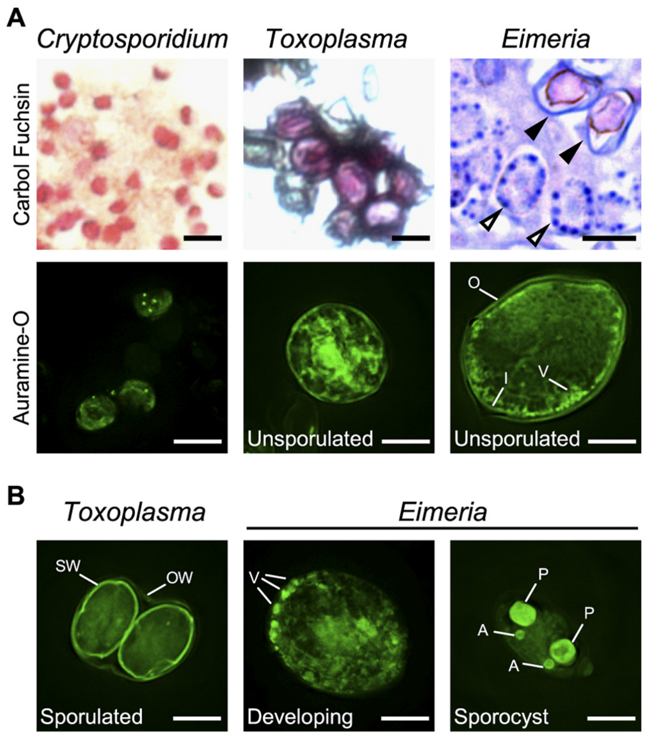

FIG 1 Oocyst walls of *Cryptosporidium*, *Toxoplasma*, and *Eimeria* label with acid-fast stains. (A) Bright-field (Kinyoun in *Cryptosporidium* and *Toxoplasma*, Ziehl-Neelsen in *Eimeria*) acid-fast stains bind to oocysts (11). Filled arrowheads mark mature *Eimeria* oocysts in ceca of infected chickens that stain red, while open arrowheads mark immature zygotes that do not stain red. The fluorescent acid-fast stain auramine-O stains *Cryptosporidium* and *Toxoplasma* oocysts, as well as the inner (I) and outer (O) layers of the *Eimeria* oocyst wall, as well as vesicles adjacent to the wall (V). (B) *Toxoplasma* oocyst walls (OW) and sporocyst walls (SW) are acid-fast with auramine-O. Auramine-O stains peripheral vesicles (V) in a developing oocyst of *Eimeria* and stains the anterior (A) and posterior (P) refractile bodies of two sporozoites. Black and white size bars represent 10 μm and 5 μm, respectively. Please see Fig. S1 in the supplemental material for additional data.

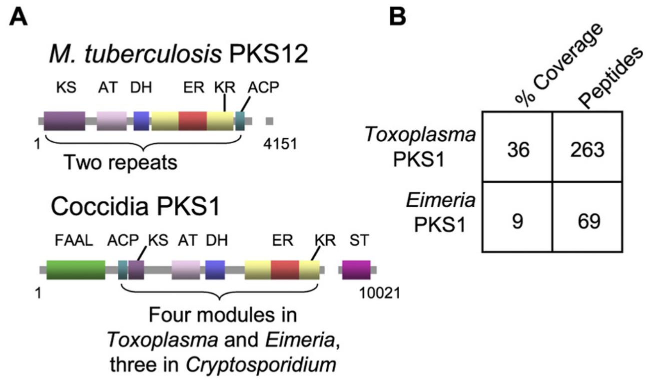

FIG 2 Polyketide synthases are extraordinarily abundant in oocysts of *Toxoplasma* and *Eimeria*. (A) Parasite polyketide synthases have domain structures like those of mycobacterial PKS12, except that the parasite enzymes may have three modules (*Cryptosporidium* PKS2 encoded by the cgd3_2180 gene) or four modules (*Toxoplasma* and *Eimeria* PKS1). Each module contains an acyl carrier protein (ACP), keto synthase (KS), acetyltransferase (AT), a hydroxyl dehydratase (DH), an enoyl reductase (ER), and a keto reductase (KR) (20). The fatty acyl-AMP ligase (FAAL) and sulfotransferase (ST) activate and release fatty acids, respectively (33). (B) Percentage of sequence coverage and number of unique tryptic peptides of PKS1 in mass spectrometry of unsporulated oocysts of *Toxoplasma* and *Eimeria*. Please see Fig. S1 and Table S1 in the supplemental material for additional data.

glucan-binding domain and is present in the inner layer of the oocyst wall. Echinocandins, which are inhibitors of fungal glucan synthases, arrest development of the *Eimeria* oocyst wall and inhibit release of oocysts into the intestinal lumen of chickens. The presence of the β-1,3-glucan fibrils can explain the strength but not the impermeability of oocyst walls. Dityrosines, which are present in tyrosine-rich oocyst wall proteins, may contribute to the impermeability of oocyst walls of *Toxoplasma* and *Eimeria*, but the oocyst wall of *Cryptosporidium* lacks dityrosines and is missing the scaffold of β-1,3-glucan (9, 10).

Prior to the identification of the human immunodeficiency virus (HIV), AIDS was diagnosed by the presence of opportunistic infections, such as *Cryptosporidium*, which was detected in stools by an acid-fast stains (Fig. 1A) (11). The goal here was to determine the structural role, if any, of acid-fast lipids in oocyst walls of *Cryptosporidium*, *Toxoplasma*, and *Eimeria*. 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 stain 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 cgd3_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-

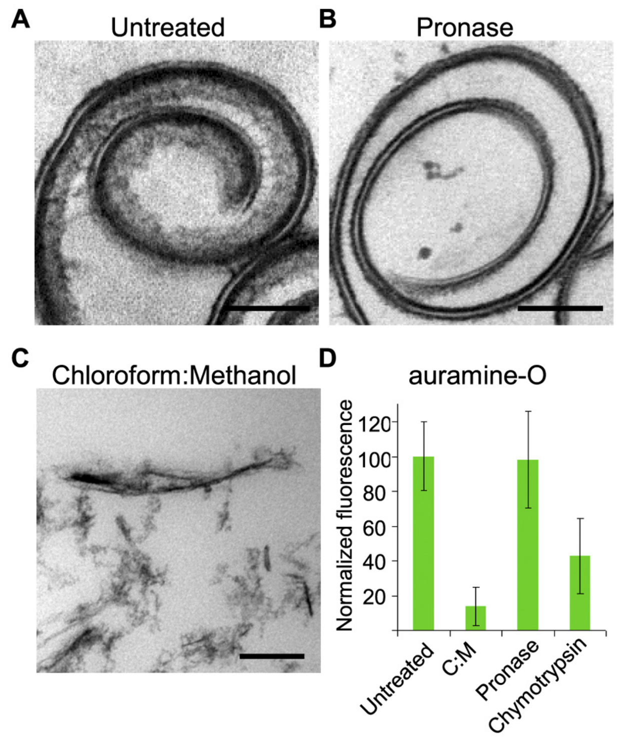

FIG 3 Lipids appear to be an important component of the rigid bilayer in the oocyst wall of *Cryptosporidium*. (A) Sonicated oocyst walls of *Cryptosporidium*, which curl into scrolls, have an outer bilayer and an inner glycoprotein layer (7). (B) The rigid bilayer, but not the protein layer, remains intact after treatment with pronase. (C) In contrast, oocyst walls are disrupted by exposure to organic solvents. Size bars represent 100 nm. (D) Acid-fast staining of oocyst walls with auramine-O, which was measured with a fluorimeter, is lost upon treatment with organic solvent, reduced with chymotrypsin, and retained with pronase. Error bars represent ±1 standard deviation from the mean in three experiments each performed in triplicate.

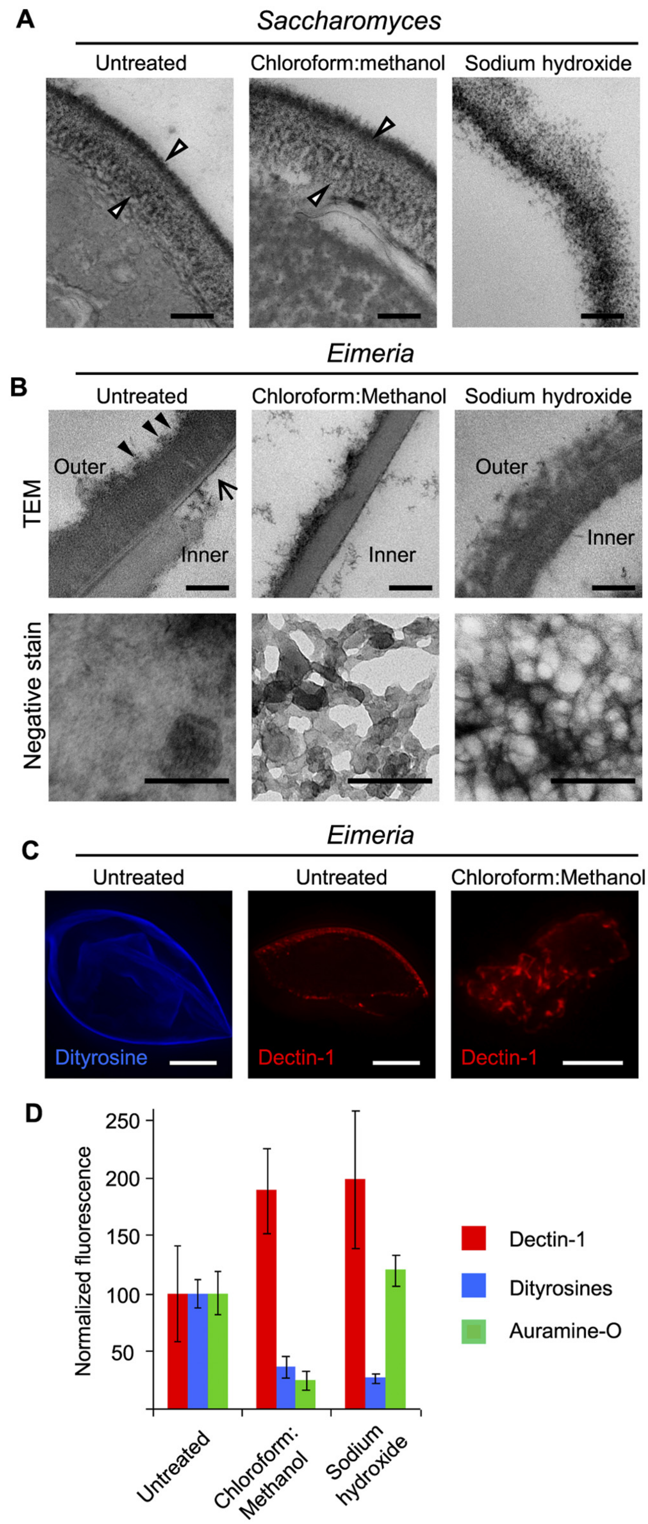

FIG 4 In contrast to fungal walls, the oocyst wall of *Eimeria* is very sensitive to organic solvents. (A) Transmission electron microscopy (TEM) shows that walls of *Saccharomyces cerevisiae*, which have a single layer (between the hollow arrowheads), remain relatively intact after treatment with chloroform-methanol. 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

(Continued)

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-

Figure Legend Continued

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.

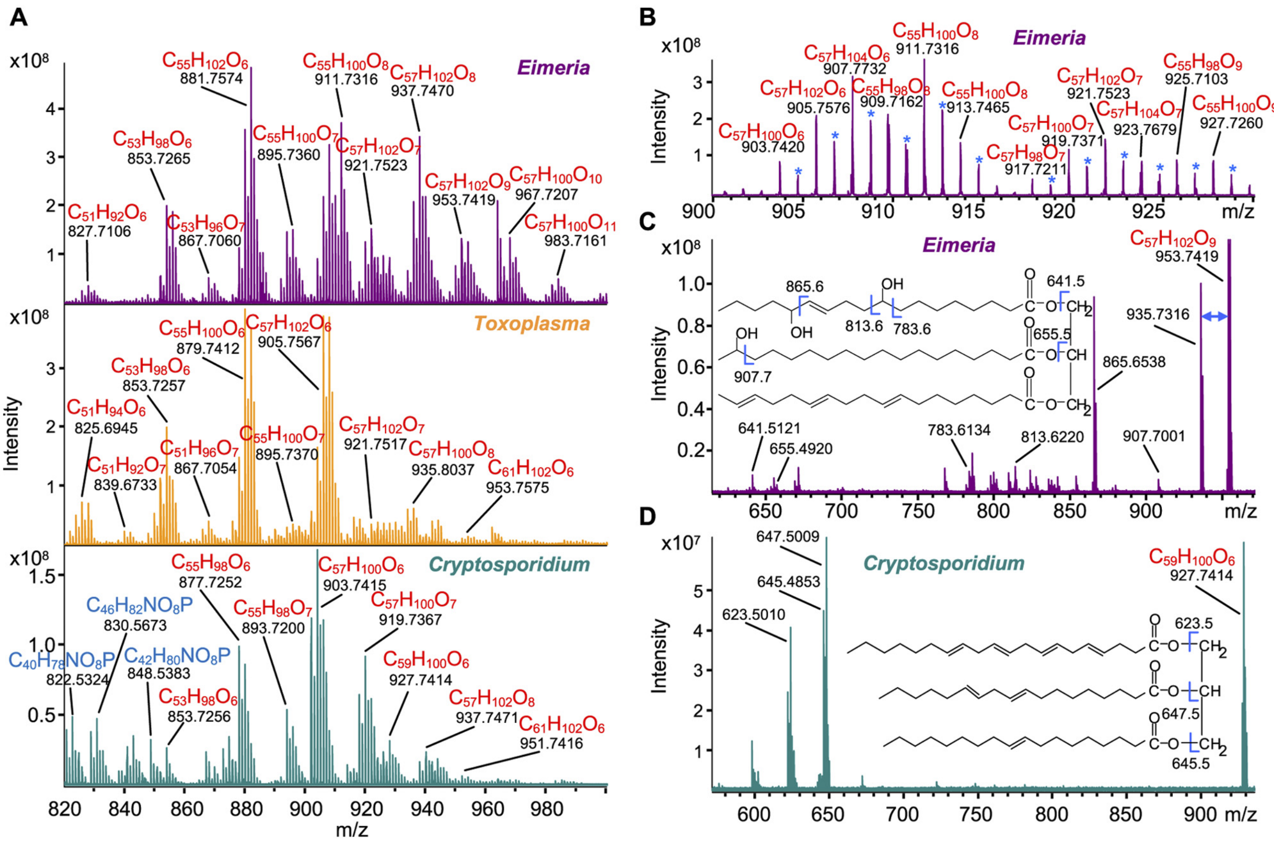

FIG 5 Triglycerides are the most abundant lipids in chloroform-methanol extracts of oocyst walls. (A) High-accuracy and high-resolution mass spectrometry makes it possible to determine the *m/z* and assign the chemical composition to the complex set of lipids extracted from oocyst walls of *Cryptosporidium*, *Toxoplasma*, and *Eimeria*. A complete list of lipids is given in Table S2 in the supplemental material. Triglycerides (red) vary in the length of the fatty acyl chains and in their 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-numbered masses, which are marked with asterisks, are the results of naturally occurring isotopes of carbon (^{13}C) and hydrogen (^{2}H) present within the triglycerides. (C) CID fragmentation of an *Eimeria* triglyceride with $[M + Na]^+$ *m/z* 953.7419 and a chemical composition of $C_{57}H_{102}O_9$ 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 $C_{59}H_{100}O_6$ 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.

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/or oxidation. At least 250 species of triglycerides are made by mycobacteria and may contribute to the acid-fast walls (12). Previously triglycerides have been considered only as storage lipids in *Toxoplasma* tachyzoites, parallel to their role in host cells (27).

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

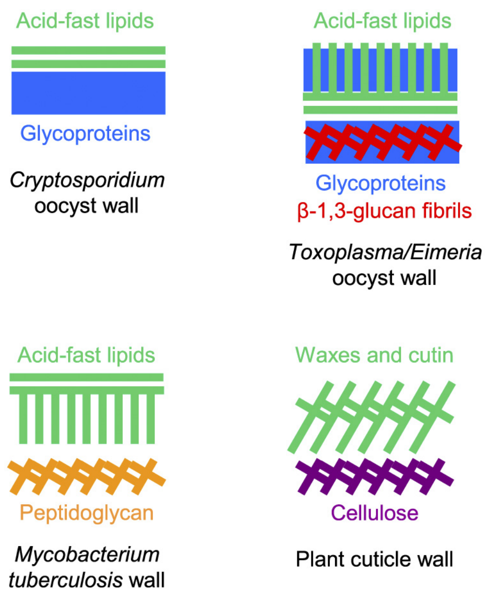

FIG 6 Coccidia, mycobacteria, and plants, which are deeply divergent organisms, each have a lipid-rich coat that makes them resistant to environmental stress. The rigid bilayer of the *Cryptosporidium* oocyst wall is composed of acid-fast lipids, while glycoproteins, in particular Cys- and His-rich oocyst 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. Glycoproteins, which include homologs of *Cryptosporidium* OWPs as well as Tyr-rich proteins that form dityrosines, are also present in oocyst walls (9, 10, 25). Mycobacteria have an inner layer of peptidoglycan and an outer layer of acid-fast lipids. Finally, plant leaves and stems have a cuticle composed of cellulose (inner layer) and waxes and cutin (outer layer). Proteins have been left out of the models of the mycobacteria and plant walls.

transmitted by the fecal-oral route (e.g., *Entamoeba* and *Giardia*) are missing the lipid layer (31). Finally, these results may help explain why *Eimeria* oocysts are destroyed *in vitro* by essential oils (32).

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, respectively, using previously described methods (8). *Eimeria* oocysts at various stages of development were prepared from homogenized ceca by centrifugation in the absence of high salt. Oocysts of *Eimeria* and *Toxoplasma* were sporulated by incubation for 48 to 72 h at 30°C. Oocysts of *Cryptosporidium parvum* (Iowa strain), which had been passaged through newborn calves, were purchased from Bunch Grass Farm, Dury, ID.

Acid-fast staining and fluorescence 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 chickens infected 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 temperature (16). Slides were examined with a DeltaVision deconvolving microscope (Applied Precision, Issaquah, WA), using the filters for fluorescein. Images were taken at 100× primary magnification and deconvolved using Applied Precision's softWoRx software. Broken oocysts of *Toxoplasma* and *Eimeria* were incubated with Alexa Fluor-labeled dectin-1, as previously described (8). Dityrosine autofluorescence of oocysts of *Toxoplasma* and *Eimeria* was observed in the UV channel and photographed.

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 pronase or 1 mg/ml chymotrypsin, for 3 h at 37°C. Sonicated treated or untreated *Cryptosporidium* oocyst walls were washed in PBS, fixed in aldehydes containing ruthenium red, and prepared for transmission electron microscopy (TEM), as previously described (8). Unsporulated oocyst walls of *Toxoplasma* and *Eimeria* were broken with glass beads, isolated by centrifugation, and deproteinated with 1 N sodium hydroxide for 60 min at 80°C. Alternatively, pelleted broken walls of *Toxoplasma* and *Eimeria* were extracted with 50 volumes of chloroform-methanol (2:1) or hexane isomers overnight at room temperature. As a control, intact *Saccharomyces* cells were treated with chloroform-methanol or sodium hydroxide. Treated and untreated walls of the parasites and fungi were prepared for TEM and negative staining, as previously described (8).

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 triplicate samples of oocyst walls was measured with a fluorimeter using 410-nm excitation and 500-nm emission wavelengths, and the experiment was repeated 3 times. For quantitation of binding of auramine-O, dectin-1, and UV autofluorescence, treated and untreated *Eimeria* walls were fixed to 96-well plates and stained or labeled, and fluorescence was measured for auramine-O using methods described above. The excitation/emission wavelengths were 495/519 nm for Alexa Fluor 488-labeled dectin-1 and 360/457 nm for autofluorescence.

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 proteins were extracted by breaking unsporulated oocysts in 2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} with complete protease inhibitor cocktail lacking EDTA (Roche). Tryptic peptides were prepared and analyzed with the LTQ-Orbitrap Discovery ETD hybrid tandem mass spectrometer (Thermo-Fisher Scientific, Inc., Waltham, MA), as previously described (21). The predicted proteins of *Toxoplasma* and *Eimeria* at EupathDB and Mascot were used to identify tryptic peptides, the bulk of which will be reported elsewhere. Data acquisition and analysis were performed with XCalibur software (Thermo, Fisher Scientific). In Table S1 in the supplemental material, the number of unique peptides and percentage of coverage are shown for the 10 most abundant cytosolic proteins of *Toxoplasma* oocysts.

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 manufacturer's instructions. Primers were designed to produce products that span several exons to distinguish RNA from potential DNA products. *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 material) (10). *Eimeria* primers were to the PKS1 (ETH_00015480), PKS2 (ETH_00005790), PPTase (ETH_00040195), DGAT1 (ETH_00032635), DGAT2 (ETH_00034355), ACAT (ETH_00032235), and actin

(ETH_00009555) genes (see Table S3). Products were analyzed on agarose gels with ethidium staining. There was no attempt at quantitation.

Extraction of lipids from oocyst walls and analysis with high-resolution and high-accuracy mass spectrometry. Oocysts were broken using glass beads in a bead beater and washed extensively in PBS and high-performance liquid chromatography (HPLC)-grade water. Oocyst walls were dried, extracted in 2:1 chloroform-methanol overnight, and centrifuged to remove insoluble material. Extracted wall lipids were analyzed with a 12-T solariX hybrid Qq-FTICR mass spectrometer (Bruker Daltonics, Billerica, MA) (26). The collision voltage was varied between 18 V and 30 V for fragmentation of the selected triglycerides, and argon was used as the collision gas. DataAnalysis 4.0 (Bruker Daltonics) was used for data analysis. The lipids were manually identified by use of elemental composition and CID fragmentation patterns. Six biological replicates of *Eimeria* lipids, four of *Toxoplasma*, and three of *Cryptosporidium* were examined by mass spectrometry.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00387-13/-/DCSupplemental.

Figure S1, TIF file, 3.5 MB.
Figure S2, TIF file, 2.7 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.

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