Stage-Specific De Novo Synthesis of Very-Long-Chain Dihydroceramides Confers Dormancy to Entamoeba Parasites

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ABSTRACT Amoebiasis is a parasitic disease caused by Entamoeba histolytica infection and is a serious public health problem worldwide due to ill-prepared preventive measures as well as its high morbidity and mortality rates. Amoebiasis transmission is solely mediated by cysts. Cysts are produced by the differentiation of proliferative trophozoites in a process termed “encystation.” Entamoeba encystation is a fundamental cell differentiation process and proceeds with substantial changes in cell metabolites, components, and morphology, which occur sequentially in an orchestrated manner. Lipids are plausibly among these metabolites that function as key factors for encystation. However, a comprehensive lipid analysis has not been reported, and the involved lipid metabolic pathways remain largely unknown. Here, we exploited the state-of-the-art untargeted lipidomics and characterized 339 molecules of 17 lipid subclasses. Of these, dihydroceramide (Cer-NDS) was found to be among the most induced lipid species during encystation. Notably, in encysting cells, amounts of Cer-NDS containing very long N-acyl chains (≥26 carbon) were more than 30-fold induced as the terminal product of a de novo metabolic pathway. We also identified three ceramide synthase genes responsible for producing the very-long-chain Cer-NDS molecules. These genes were upregulated during encystation. Furthermore, these ceramide species were shown to be indispensable for generating membrane impermeability, a prerequisite for becoming dormant cyst that shows resistance to environmental assault inside and outside the host for transmission. Hence, the lipid subclass of Cer-NDS plays a crucial role for Entamoeba cell differentiation and morphogenesis by alternating the membrane properties.

IMPORTANCE Entamoeba is a protozoan parasite that thrives in its niche by alternating its two forms between a proliferative trophozoite and dormant cyst. Cysts are the only form able to transmit to a new host and are differentiated from trophozoites in a process termed “encystation.” During Entamoeba encystation, cell metabolites, components, and morphology drastically change, which occur sequentially in an orchestrated manner. Lipids are plausibly among these metabolites. However, the involved lipid species and their metabolic pathways remain largely unknown. Here, we identified dihydroceramides (Cer-NDSs) containing very long N-acyl chains (C26 to C30) as a key metabolite for Entamoeba encystation by our state-of-the-art untargeted lipidomics. We also showed that these Cer-NDSs are critical to generate the membrane impermeability, a prerequisite for this parasite to show dormancy as a cyst that repels substances and prevents water loss. Hence, ceramide metabolism is essential for Entamoeba to maintain the parasitic lifestyle.

KEYWORDS Entamoeba, amoebiasis, ceramide, encystation, infectious disease, lipidomics
Entamoeba histolytica, a protozoan parasite belonging to the clade Amoebozoa, causes amoebiasis, for which the development of new therapeutic means is urgently needed due to ill‐prepared clinical options (1–3). As a parasitic strategy, *E. histolytica* alternates its form between a proliferative trophozoite and a dormant cyst (4, 5). The cyst is the only form able to transmit to a new host and is differentiated from trophozoites via stage transition, which is termed “encystation” (6). Encystation is a fundamental cell differentiation process, and the change in cell morphology is obvious; motile ameboid cells become rounded nonmotile cells (Fig. 1A). Substantial changes also occur concurrently in cell components. For example, a single nucleus becomes four nuclei, and ribosomes become aggregated and form chromatoid bodies (7–11). Cells become coated with a cyst wall, and cell membrane permeability decreases greatly, resulting in mature cysts being rigid and resistant to environmental assault, such as desiccation (12–14). These structural and physiological changes are closely linked to fluctuations of various metabolites from diverse biochemical pathways, which play crucial roles in *Entamoeba* encystation (15). Chitins, a major component of the cyst wall, are specifically synthesized during encystation (16–18). Lipids, whose composition affects the physical properties of membranes, such as fluidity and rigidity (19), are plausibly responsible for the decrease in membrane permeability of the *Entamoeba* cyst. However, the lipid species involved have not been identified, and their metabolic pathways remain largely unknown. In this study, to identify the lipid species fluctuating during *Entamoeba* encystation, we performed state‐of‐the‐art liquid chromatography‐mass spectrometry (LC‐MS)‐based untargeted lipidomics (20) and found ceramides containing nonhydroxy fatty acid and dihydrosphingosine (Cer‐NDSs) to be among the most induced lipid species.

Ceramides play versatile roles in homeostasis (21, 22). They are pivotal intermediates in the synthesis of a variety of sphingolipids that are essential membrane components, such as sphingomyelin (SM) and ganglioside. Ceramides and their derivatives also function as signaling molecules in cell proliferation, differentiation, and death. Typically, the ceramides are produced by de novo synthesis and salvage pathways. The de novo pathway consists of four sequential biochemical reactions (Fig. 1B) (21, 22): (i) condensation of serine and palmitoyl‐coenzyme A (CoA), the rate‐limiting step; (ii) reduction of the resulting 3‐keto‐dihydrosphingosine; (iii) acylation of hydroxyl species using acyl‐CoA; and (iv) desaturation of the dihydro product, Cer‐NDS. The salvage pathway includes SM hydrolysis by phospholipase C and sphingolipid degradation and recycling to provide intermediates for the de novo pathway (19, 23, 24). In *Entamoeba*, the presence of sphingolipids and the importance of sphingolipid metabolism in trophozoite proliferation, encystation, and excystation were previously described (25–30). Of note, based on AmoebaDB (http://amoebadb.org/amoeba/), *Entamoeba* possesses an atypical de novo pathway for ceramide synthesis in contrast to typical free‐living organisms, such as humans and yeast; the gene encoding the fourth enzyme in the de novo pathway, dihydroceramide desaturase, is absent from the *Entamoeba* genome (see Fig. 1B). In this study, we conducted comprehensive nontargeted lipidomics and successfully identified Cer‐NDS containing very long N‐acyl chains (C_{26} to C_{30}) (see Fig. 1C for the structure) as one of the most induced lipid species during *Entamoeba* encystation.

RESULTS

Identification of lipid species and their fluctuating levels (increase or decrease) during encystation. To comprehensively investigate the lipid species that fluctuate during encystation, we used in vitro culture of *Entamoeba invadens*. Generally, studies of *Entamoeba* encystation have adopted the in vitro culture of *E. invadens*, a reptilian parasite, and not that of *E. histolytica* as a model system (see Fig. 1A). This is because the strains of *E. histolytica* available in the laboratory do not encyst after adaptation to culture conditions. The *E. invadens* life cycle is the same as that of *E. histolytica*, and the symptoms caused by *E. invadens* infection are similar to those of *E. histolytica* (4, 5). Lipids were extracted from encysting *E. invadens* cells at designated time points after
the induction of encystation and then were analyzed by untargeted lipidomics. During encystation, a series of lipid species, including ceramide, ceramide phosphatidylinositol (PI-Cer) (see Fig. 1D for the structure), lysophosphatidylserine (LPS), and lysophosphatidylinositol (LPI) was significantly increased with time (Fig. 2A and see Fig. S1 in the supplemental material; see Fig. 1A for the morphological and ultrastructural changes). In contrast, levels of SM (see Fig. 1D for the structure) and lysophosphatidylcholine (LPC) species were significantly decreased. No significant fluctuations in phospholipid or other sphingolipid species (phosphatidylcholine [PC], phosphatidylethanolamine [PE], PI, phosphatidylserine [PS], and ceramide phosphoethanolamine [PE-Cer]) (see Fig. 1D for the structure) were observed throughout encystation. Ceramide molecules were
FIG 2 Comprehensive analysis of lipid species during Entamoeba encystation by untargeted lipidomics. (A) Fluctuation of major lipid classes during encystation. Signal intensity levels are shown as fold change relative to the level at time zero. Time course profiles of each lipid species are presented in (Continued on next page)
detected throughout the *Entamoeba* life cycle (trophozoite and cyst stages) and mainly comprised Cer-NDSs (Fig. 2B). In mammals, the ceramide lipid class is essential to prevent water loss from the skin (31). *Entamoeba* cysts are also resistant to desiccation (5, 32). We, therefore, focused on Cer-NDS species for further analyses.

In *Entamoeba* trophozoites (*E. invadens* cells before encystation induction), Cer 18:0/20:24/1, Cer 18:0/20:24/0, Cer 19:0/20:24/1, Cer 18:0/20:16/0, and Cer 17:0/20:24/1 were dominantly present (0 h in Fig. S1A), and the amount of these species increased by ≥3-fold during the course of encystation (Fig. 2C and E and Fig. S1A). In contrast, the amounts of very-long-chain Cer-NDS species, such as Cer 18:0/20:30/1, Cer 16:0/20/30:2, and Cer 18:0/20/28:1, were increased 10- to 80-fold between 16 and 24 h after encystation induction (Fig. 2C and E). At 72 h, the abundance of very-long-chain Cer-NDS species became evident (Fig. 2D). Among those ceramides consistently detected in three independent experiments (see Table S1), 10 species of very-long-chain Cer-NDS (≥26 acyl chain) were significantly elevated (Fig. 2E and Table S1).

**Revealing a de novo ceramide synthesis pathway in *Entamoeba***. Very-long-chain Cer-NDSs were not detected in bovine serum, which is the major lipid source in *Entamoeba* encystation-inducing culture medium (33); therefore, it was unlikely that very-long-chain Cer-NDSs were derived from the external milieu. Of interest, all necessary genes for the de novo ceramide synthesis are harbored by both the *E. histolytica* and *E. invadens* genomes except for one gene encoding dihydroceramide desaturase (Fig. 1B) (AmoebaDB, http://amoebadb.org/amoeba/); there are two types of genes encoding serine palmitoyl transferase (SPT), one gene for 3-dehydrosphinganine reductase (KDH), and five (*E. histolytica*) or six (*E. invadens*) genes for ceramide synthase (CerS) (27).

To show the capability of *Entamoeba* to synthesize ceramides de novo, proliferating trophozoites and encysting cells were metabolically labeled with L-[U-14C]serine, a substrate for the first enzyme (SPT) in the de novo pathway (see Fig. 1B). 14C-labeled bands corresponding to ceramides were detected in both trophozoites and encysting cells (Fig. 3A). During encystation, an accumulation of radiolabeled ceramide with time was observed. A dramatic increase of radiolabeled ceramide was observed between 16 and 32 h (Fig. 3B). Alkaline treatment did not change the intensity of the detected bands, ruling out the lipids being glycerolipids (see Fig. S2). These results clearly indicated that *Entamoeba* synthesized ceramides by de novo biosynthesis. Notably, the time course for the accumulation of 14C-labeled ceramide correlated well with the increased amount of very-long-chain Cer-NDSs between 16 and 24 h after encystation induction and reached a plateau after 24 h (Fig. 2C and Fig. S1A). Consistently, during the initiation phase of encystation, expression of a series of ceramide biosynthetic enzymes was coordinately induced in *Entamoeba* (Fig. 3C). These results indicated that the induction of very-long-chain Cer-NDSs during *Entamoeba* encystation appeared to be mediated by de novo biosynthesis.

**Identification of the ceramide synthase gene responsible for producing Cer-NDSs in *Entamoeba***. Variation in the acyl chain length of Cer-NDSs observed during *Entamoeba* encystation is likely to be generated by different CerS isoforms, as observed in other organisms (21, 22). To identify the CerS responsible for very-long-chain Cer-NDS biosynthesis in *Entamoeba*, we exploited an approach combining genomics and lipidomics. The genetic approach included gene knockdown mediated by transcriptional gene silencing via antisense small RNA (34, 35) and gene overexpression. **Fig 2 Legend (Continued)**

Fig. S1 in the supplemental material. (B) Comparison of the total intensity of Cer-NS and Cer-NDS detected in encysting cells at the indicated times. The colors used for indicating the time are as in panel A. (C) Changes in the ceramide species profile during *Entamoeba* encystation. LC-MS/MS signal intensity levels are shown as fold change relative to the level at time zero. The colors used for indicating the time are as in panel A. (D) Dynamics of the increased levels of a broad range of ceramides. Stacked bar graph of ceramide species, which were detected in encysting cells at 0, 24, and 72 h after encystation induction and classified based on their acyl chains, are shown with different colors. (E) List of 15 most abundant ceramide species in cysts (72 h after induction), all of which were reproducibly detected in three independent experiments (Table S1). Red letters indicate ceramide species whose levels were >10-fold higher than those in trophozoites (0 h after induction). Representative data (Sample 1 in Table S1) are shown from three independent experiments.
We used *E. histolytica* instead of *E. invadens* as the host because the genetic systems for *E. invadens* have not been widely adopted. In *E. histolytica* trophozoites, CerNSD species were similarly detected as in *E. invadens* trophozoites (see Fig. S3A). A gene knockdown experiment was performed using five *E. histolytica* gene silencing

![Graphs showing ceramide synthesis and gene expression during encystation](http://msphere.asm.org/)
(gs) transformants, EhCerS2gs to EhCerS6gs, in each of which a single gene among the five EhCerSs was knocked down. Note that *E. histolytica* does not have a counterpart of *E. invadens* CerS1 (EiCerS1) (see Fig. 1B). After verifying the level of gene knockdown in each transformant by quantitative reverse transcription-PCR (qRT-PCR) (Fig. S3B), the lipidomic profiles of Cer-NDS species in all EhCerSgs (except for EhCerS3gs) and mock transformants were individually determined (Fig. 4A and Fig. S3C to E). One transformant, EhCerS3gs, showed a severe growth defect, which hampered long-term subculture. Among the transformants tested, only EhCerS4gs showed a significant reduction in Cer-NDS levels; the most significant reduction was observed in Cer 18:0/20:24:1 and the amounts of Cer 17:0/20/24:1 and Cer 19:0/20/24:1 were also reduced (Fig. 4A). In EhCerS4gs, both EhCerS4 and EhCerS5 transcripts were significantly downregulated (0.25% ± 0.03% and 4.2% ± 0.3%, respectively, relative to the mock transformant 100% control) (Fig. S3B). However, a contribution of EhCerS5 was ruled out because the amounts of Cer-NDS species were not changed in EhCerS5gs, in which only the EhCerS5 transcript was reduced (4.4% ± 0.6%) (Fig. S3B and D). These results indicated that EhCerS4 was responsible for synthesizing Cer-NDSs containing a C24:1 acyl chain. None of the remaining three transformants (EhCer2gs, -5gs, and -6gs) showed obvious changes in their Cer-NDS species profile, probably because of genetic redundancy (Fig. S3C to E).

Overexpression experiment of each EhCerSs was also performed; each EhCerS gene (see Fig. 1B) was separately overexpressed as a hemagglutinin (HA)-tagged protein to yield *E. histolytica* transformants, namely, EhCerS2-HA to EhCerS6-HA (Fig. 4B to F). In EhCerS2-HA, EhCerS5-HA, and EhCerS6-HA, only the targeted EhCerS was selectively upregulated (see Fig. S4A). In EhCerS2-HA, levels of Cer 18:0/20:28:2, Cer 18:0/20:30:1, and Cer 18:0/20:30:2 were selectively increased (Fig. 4B). In EhCerS5-HA, levels of Cer 17:0/20:26:0, Cer 18:0/20:26:0, Cer 18:0/20:26:1, Cer 18:0/20:28:0, Cer 19:0/20:28:0, Cer 17:0/20:28:1, Cer 18:0/20:28:2, Cer 18:0/20:30:1, and Cer 18:0/20:30:2 were selectively increased (Fig. 4E). In EhCerS6-HA, levels of Cer 18:0/20:20:0, Cer 18:0/20:26:0, Cer 17:0/20:28:1, Cer 18:0/20:28:1, Cer 19:0/20:28:1, Cer 18:0/20:28:2, Cer 18:0/20:30:1, and Cer 18:0/20:30:2 were selectively increased (Fig. 4F). These results indicate that variation of acyl chain length in Cer-NDSs was generated by ectopic overexpression of CerS isozymes. EhCerS2 produces C28:2, C30:1, and C30:2-Cer-NDSs, EhCerS5 produces C26:0, C26:1, C28:0, C28:1, C28:2, C30:1, and C30:2-Cer-NDSs, and EhCerS6 produces C20:0, C26:0, C28:2, C28:3, and C30:2-Cer-NDSs. These results were consistent with the encysting *E. invadens* cells; the transcription levels of EiCerS2, -5, and -6, were significantly upregulated (Fig. 3C), while the amount of Cer-NDS species containing C26:0, C28:0, C28:1, C28:2, and C30:2 were substantially increased (Fig. 2C). Overlap in the Cer-NDSs produced by EhCerS2, -5, and -6 reinforces our premise that genetic redundancy among these three CerS results in these single gene knockdown strains having no mutant phenotype. Of note, EhCerS6-HA, in which Cer-NDS levels were dramatically increased (Fig. 4F), displayed a growth defect (Fig. S4B). This may have resulted from the toxicity of a very high level of Cer-NDSs that accumulated in trophozoites. EhCerS4-HA showed significant increase of Cer 19:0/20:24:1 and Cer 19:1/20:24:1 compared to that in the control (Fig. 4D). Therefore, EhCerS4 appeared to be responsible for synthesizing Cer-NDS with a C24:1 acyl chain, which does not overlap the Cer-NDS species synthesized by functionally redundant EhCerS2, -5, and -6. EhCerS3-HA did not show obvious changes in Cer-NDS levels (Fig. 4C). These results indicated that the variation of Cer-NDS species in *Entamoeba* was generated by the different CerS isozymes (Table 1).

**Ceramide metabolism in *Entamoeba***. To understand ceramide metabolism in *Entamoeba*, we investigated the effect of myriocin, a known inhibitor for the first enzyme (SPT) in the *de novo* pathway for ceramide biosynthesis (see Fig. 1B). Myriocin dose-dependently inhibited cyst formation in *in vitro* cultures of *E. invadens*, which was consistent with the previous report (27, 28). The 50% inhibitory concentration (IC₅₀) was calculated as 68.6 ± 12.5 nM (n = 3) (Fig. 5A). Also, the physiological changes...
Knockdown (A) and overexpression (B to F) of CerS genes change the ceramide profile in *E. histolytica*. (A) Percentages of each ceramide species relative to the total amount in EhCerS4gs and the control strain. Red arrows indicate the ceramide species showing (Continued on next page)
during the course of encystation were monitored by flow cytometry (37). Evans blue (EB) was used as an indicator of membrane permeability, and calcofluor (CF) was used as an indicator of the level of chitin, a major component of the cyst wall (38). As shown in the control in Fig. 5A, it appeared that the \( \text{CF}^- \text{EB}^+ \) population (proliferating trophozoites) was gradually changed to a \( \text{CF}^+ \text{EB}^- \) population (mature cysts) through \( \text{CF}^- \text{EB}^- \) and \( \text{CF}^+ \text{EB}^+ \) populations. At 12 h after induction, myriocin treatment did not affect the phenotype, but at 16 h, cell differentiation was paused, resulting in the accumulation of an irregular \( \text{CF}^+ \text{EB}^\text{strong} \) population (abnormal cells) at 20 h. These results indicated that myriocin impaired the encystation process at 16 to 20 h postinduction. Importantly, this time frame correlated well with the lipidomic changes of very-long-chain Cer-NDSs dramatically increased between 16 and 24 h after induction of encystation (Fig. 2C). These results indicated that inhibition of very-long-chain Cer-NDS biosynthesis by myriocin halted cyst formation.

Next, we determined the consequence of *Entamoeba* encysting cells treated with myriocin. After 24 h, when the effect of myriocin was first apparent on cyst formation, both control and myriocin-treated live cells were stained by CF and EB (Fig. 5B). Flow cytometry analysis showed that the level of CF fluorescence in myriocin-treated cells was comparable to that of untreated cells (Fig. 5A). A change in the CF signal reflects the synthesis and degradation of chitin polymers. Therefore, these results indicate that chitins are synthesized and placed in the cyst wall at similar levels in both myriocin-treated and untreated cells. However, a distinct physiological change was observed in myriocin-treated cells. The fluorescence signal of EB, an indicator of membrane permeability, abnormally accumulated inside the cells, indicating that myriocin treatment increased the membrane permeability of encysting cells (Fig. 5B).

To further determine the structural changes induced by myriocin treatment, we performed transmission electron microscopy analysis of encysting cells in either the presence or absence of myriocin (Fig. 5C). Cells were prepared by rapid freezing and freeze-substitution to preserve the membrane structure (39). The myriocin-treated cells were withered, and accumulations of abnormal vacuoles were observed throughout the cytoplasm. Notably, the cell membranes of myriocin-treated cells were more compressed and disconnected. Furthermore, the cyst wall regions of treated cells were swollen, and cell components randomly filled the spaces between the regions of disrupted membranes and the cyst walls. It is worth mentioning that obvious changes in lipidome of encysting cells (24 h postinduction) by myriocin treatment was observed.

**TABLE 1** Variety of dihydroceramide species generated in *Entamoeba histolytica* by ceramide synthase isozymes (EhCerS2 to -6)

<table>
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<tr>
<th>Product</th>
<th>EhCerS2</th>
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<th>EhCerS4</th>
<th>EhCerS5</th>
<th>EhCerS6</th>
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<tr>
<td>Dihydroceramide species</td>
<td>nd(^a)</td>
<td>( \text{C}_{24:1} \text{Cer} )</td>
<td>( \text{C}_{26:0} \text{Cer} )</td>
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<td>( \text{C}_{26:1} \text{Cer} )</td>
<td>( \text{C}_{26:0} \text{Cer} )</td>
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<td>( \text{C}_{28:2} \text{Cer} )</td>
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\(^a\text{nd, not determined.}\)

FIG 4 Legend (Continued)

the most significant differences. Changes in the levels of ceramide species in EhCerS2-HA (B), EhCerS3-HA (C), EhCerS4-HA (D), EhCerS5-HA (E), and EhCerS6-HA (F) strains. Signal intensity levels are shown as fold change to that of the control strain. Red bars indicate the ceramide species increased by >1.5-fold (B to D), 3-fold (E), and 5-fold (F). Representative data are shown from two independent experiments.
FIG 5 Effects of myriocin on *Entamoeba* cyst formation. (A) Encystation assay. Immediately after induction, *E. invadens* was cultivated in encystation medium in the presence of various concentrations of myriocin (111 to 1,000 nM). Flow cytometry results obtained at the indicated times after induction are shown. The number inside each panel indicates the percentage of the boxed cell population. The red numbered panels indicate the conditions under which myriocin had an effect on encysting cells. EB, Evans blue; CF, calcofluor. (B, left) Fluorescence microscopy images of a cell treated with 1 μM myriocin (Continued on next page)
in Cer-NDSs containing very long \( N \)-acyl chains (≥26 carbon) and their metabolites, PI-Cers. The level of LPS was also affected by myriocin treatment, but to a small extent compared to that for Cer-NDSs (see Fig. S5; Table S2). These results indicate that the off-target effect of myriocin on lipid metabolism in *Entamoeba* encysting cells was quite limited under this experimental condition. These results indicated that the stage-specific induction of Cer-NDSs with very long \( N \)-acyl chains (C26 to C30) were indispensable to develop membrane impermeability.

**DISCUSSION**

*Entamoeba* encystation is a crucial process for maintaining the life cycle of this parasitic species. Encystation is a fundamental cell differentiation and morphogenesis process that involves a variety of pathways, which function as an orchestrated network (5). Here, we performed an untargeted lipidomic analysis of encysting cells over time. This approach enabled us to reveal that the levels of Cer-NDSs were significantly induced during encystation. We also unraveled unique features of an *Entamoeba* metabolic pathway and its physiology. First, *Entamoeba* possesses an atypical de novo ceramide synthesis pathway that produces Cer-NDSs possessing a broad range of acyl chains (C16, C20, C22, C23, C24, C26, C28, C30:0, C24:1, C28:1, C28:2, C28:3, C30:1, and C30:2) as terminal metabolites. Second, during encystation, the amounts of very-long-chain Cer-NDSs with an acyl chain length of ≥26 were increased, coinciding with transcriptional upregulation of the three genes encoding CerS2, -5, and -6. Those enzymes were functionally redundant and responsible for producing those ceramides. Third, inhibition of de novo ceramide synthesis caused damage to the plasma membrane and increased membrane permeability to a nonphysiological level, resulting in the formation of aberrant cysts.

Differentiation into dormant cysts from proliferative trophozoites is necessary for *Entamoeba* to be resistant to environmental assaults inside as well as outside the host and to be transmitted to a new host. This dormant process involves rounding and strengthening of the cells, which requires alternations to properties of the plasma membrane, such as changes to the composition and topology of the lipid bilayer. Halting Cer-NDS production by adding myriocin to *in vitro* encystation induction cultures resulted in proliferating trophozoites becoming rounded as normal but that differentiated into aberrant cysts. Therefore, newly synthesized Cer-NDSs in encysting cells are not involved in the cell rounding process, but they do have crucial roles after the encysting cells became rounded. The underlying molecular mechanisms need to be elucidated; however, in myriocin-treated encysting cells, a partly disconnected plasma membrane and abnormal accumulation in the cytoplasm of EB, a membrane impermeable dye, were simultaneously observed. Furthermore, we can estimate the content of very-long-chain Cer-NDSs with the acyl chain length of ≥26 in a single encysting cell to be significantly elevated because cell numbers should not theoretically increase, as encystation is a differentiation process, but they empirically decreased a bit. Cell volume also became around half during encystation. These findings indicate that very-long-chain Cer-NDSs have critical roles in maintaining the plasma membrane impermeability and that *Entamoeba* regulates intracellular ceramide metabolism to provide very-long-chain Cer-NDSs to enable transmission to a new host. Cer-NSs were also present in *Entamoeba* despite the absence of dihydroceramide desaturase gene in *Entamoeba* genome (AmoebaDB). Therefore, these ceramides containing nonhydroxy fatty acid and sphingosines (Cer-NSs) are plausibly acquired from the culture medium. Interestingly, a species difference of *Entamoeba* in the ratio of Cer-NS to ceramide was

**FIG 5 Legend (Continued)**

myriocin or a control cell observed at 24 h after encystation induction. Bars, 10 μm. Representative images are shown from two independent experiments. (Right) Electron microscopy images of 1 μM myriocin-treated (top) or control (bottom) cells observed at 24 h after encystation induction (left, whole cells; right, magnified images of boxed areas). Arrow indicates the disconnected plasma membrane. Representative images from more than 20 sections are shown. CB, chromatoid body; CW, cyst wall; MV, multivesicular body; PM, plasma membrane.
seen (Fig. 2B and see Fig. S3A in the supplemental material). This may reflect that the *E. histolytica* capability for de novo ceramide synthesis is less than that of *E. invadens*.

Furthermore, we showed evidence that Cer-NDS species may also be essential for *Entamoeba* to multiply as trophozoites. Both *E. histolytica* and *E. invadens* trophozoite proliferations were impaired by myriocin with IC\textsubscript{50} values of 46.7 ± 11.5 nM (n = 3) and 1.90 ± 0.10 μM (n = 3), respectively. The growth impairment by myriocin was complemented by the gene knockdown analyses targeting the enzymes in the *E. histolytica* de novo ceramide biosynthesis pathway (see Fig. 1B). Both knockdown strains, EhSPT1gs and EhSPT2gs (see Fig. S6A), showed severe growth defects, which hampered long-term subculture. Another knockdown strain, EhKDHrgs, also showed a growth defect (Fig. S6A and B). Another knockdown strain, EhCerS3gs, in which the downregulation of EhCerS3 was confirmed in an early subculture (Fig. S3B), showed severe growth defects, similar to those of EhSPT1gs and EhSPT2gs. Alternating its forms between a proliferating trophozoite and dormant cyst is a parasitic strategy for surviving in different niches. Hence, ceramide metabolism plays crucial roles in the *Entamoeba* life cycle.

Our lipidomic analysis detected PE-Cers, PI-Cers, and SMs, the precursors of which are ceramides (Fig. 2A and Fig. S1B to D), which is consistent with the previous studies (29, 30). Furthermore, a drastic increase of some very-long-chain PE-Cer species, such as PE-Cer 18:0/20:6 and PE-Cer 18:0/28:1, was observed during *E. invadens* encystation (Fig. S1B), although the total amount of PE-Cers in cells did not change (Fig. 2A). Because changes in the level of PE-Cer-NDs and Cer-NDs levels were well correlated during the course of cyst formation (Fig. 2C and S1A and B), PE-Cer-NDs appeared to be synthesized de novo via Cer-NDs. Note that previous studies determined the effects of *E. histolytica* and *E. invadens* CerS2 gene knockdown or overexpression on trophozoite proliferation, encystation, and excystation (25, 26). The observed phenotypes, at least for *E. histolytica* trophozoite proliferation, were inconsistent with our present results from the *E. histolytica* genetic study (Fig. S4B). We attribute this inconsistency to the functional redundancy among EhCerS2, -5, and -6. This genetic redundancy may also affect the encystation and excystation, because *E. invadens* possesses all of these counterparts (AmoebaDB) (26) (Fig. 1B). However, the possibility that CerS2 specifically functions in these processes cannot be ruled out; therefore, alternative approaches, such as pharmacological blockage of specific CerS, are required for elucidating the roles of Cer-NDs species, products of CerS, during *Entamoeba* encystation and excystation. Taken together, *Entamoeba* provides the necessary diversity of sphingolipids, such as Cer, PE-Cer, PI-Cer, and SM. However, the precise physiology of these sphingolipids in *Entamoeba*, including identification and characterization of sphingolipid synthase(s) and the uptake mechanism of SM from the host, needs to be unraveled.

As well as ceramides, sphingolipid and glycerophospholipid diversity are generated by variations in acyl chains, i.e., the number of carbon atoms and the level of unsaturation (Fig. S1E to K). The acyl chain variations in these lipids are principally introduced by a ubiquitous enzyme, acyl-CoA synthetase, which uses various fatty acids as a substrate. Organisms typically utilize fatty acids per se, which are either scavenged from the external milieu or synthesized by a de novo pathway. After elongation and desaturation by fatty acid elongases and desaturases, respectively, these provide fatty acids. Unlike typical organisms, such as human and yeast, *Entamoeba* relies totally on the external milieu as the fatty acid source because genes for neither type I nor II fatty acid synthases, responsible for de novo synthesis, are present in the genome (34, 40, 41). Furthermore, fatty acid desaturases are not encoded. In contrast, all enzymes necessary for fatty acid elongation, which proceeds via a four-step biochemical cycle (42, 43), are encoded in *Entamoeba* genomes (AmoebaDB) (34, 40) (see Fig. S7A). Consistently, during encystation, significant upregulation of *E. invadens* genes that encode enzymes involved in fatty acid elongation was observed (Fig. S7B). Notably, knockdown of the gene encoding the second enzyme of the pathway in *E. histolytica* produced a severe growth defect. Therefore, *Entamoeba* fatty acid elongation, along with other lipid
metabolism, such as sphingolipid and sulfolipid metabolism, is crucial for maintenance of the life cycle (this study, 34).

In conclusion, we have shown the overall scheme of *Entamoeba* sphingolipid metabolism and its unique features. These findings substantiate the importance of lipid metabolism in *Entamoeba* encystation and indicate a new role for ceramides in organism homeostasis. This contributes not only to the advances in understanding *Entamoeba* physiology but also to the field of sphingolipid and membrane biology.

**MATERIALS AND METHODS**

**Parasite cultures.** *E. histolytica* (G3 and HM-1:IMSS cl6) were routinely maintained as previously described (44). *E. invadens* (IP-1) was routinely maintained in a glass tube filled with 6 ml Bi-S-33 (proliferation medium). To induce encystation, 2.5 × 10^5 *E. invadens* trophozoites were seeded in a Nunc cell culture flask with a solid cap (catalog number [no.] 163371; Thermo Fisher Scientific, Waltham, MA, USA) filled with 56 ml Bi-S-33 medium and cultivated at 26°C for 5 days. Trophozoites were harvested from the required numbers of flasks and transferred to encystation medium (37) at a final concentration of 6 × 10^5/ml.

**LC-MS/MS-based lipidomics.** *E. invadens* cyst formation was induced as previously described in either the absence or presence of 1 μM myricin (37). One micromolar myricin was freshly diluted from 5 mM stock, which was prepared by dissolving myricin powder (Cayman, MI, USA) in dimethyl sulfoxide (DMSO) and stored at −30°C. Sample containing 0.02% DMSO was used as a control of myricin treatment. Briefly, trophozoites suspended in encystation medium (6 × 10^5 cells/ml) were seeded in 24-well culture plates (2 ml per well) and sealed as described (45) using Parafilm (Bemis Company, Inc., Oshkosh, WI, USA). Then, plates were incubated at 26°C for the period indicated in the text and figures. Cell pellets were collected from two wells of a 24-well plate were collected in a single 15-ml tube using 10 ml phosphate-buffered saline (PBS) and then centrifuged at 770 × g for 5 min at 4°C. The cell pellet was washed with 6 ml PBS and resuspended in 4 ml PBS. One milliliter of the cell suspension was then dispensed into each of four 1.5-ml tubes, and cells were pelleted by centrifugation. Cell pellets in tubes were kept at −80°C until use.

For *E. histolytica* transformants, stably subculturing cells (1.5 × 10^5) in the presence of 20 μg/ml G418 disulfate (Nacalai Tesque, Kyoto, Japan) were collected in a 5-ml tube by centrifugation at 440 × g for 5 min at 4°C. The cell pellet of each transformant was washed with 4 ml PBS and resuspended in 1.5 ml PBS. Five hundred microliters of the cell suspension was dispensed into each of three 1.5-ml tubes, and cells were pelleted by centrifugation at 770 × g for 5 min at 4°C. Cell pellets were then kept at −80°C until use.

Lipids were extracted from cells using single-phase extraction as previously described (46) with minor modifications. The cell pellet prepared as described above was mixed with 0.5 ml methanol, sonicated for 2 min, and incubated for 1 h at ambient temperature. After 0.2 ml of the obtained suspension was mixed with 0.1 ml CHCl3, in a new glass tube, the sample was incubated for 1 h at ambient temperature. Then, 20 μl water was added to the sample, and the mixture was incubated for 15 min at ambient temperature. After the extract was centrifuged at 2,000 × g for 10 min at ambient temperature, the supernatants were collected and dried. The obtained lipids were resuspended in 50 μl methanol (MeOH)-CHCl3-H2O solution (2:1:0.2 [vol/vol/vol]), and were then kept at 4°C until use.

LC-MS/MS analysis was carried out using a quadrupole time of flight mass spectrometer, TripleTOF 6600 (SCIEX, Framingham, MA, USA) coupled with an ACQUITY ultraperformance liquid chromatography (UPLC) system (Waters, Milford, MA, USA). All analyses were performed using data-dependent MS/MS acquisition (DDA) at the high-resolution mode in MS1 and at the high sensitivity mode in MS2. The UPLC peptide ethylene-bridged hybrid (BEH) C18 (50 by 2.1 mm; 1.7 μm) column was maintained at 45°C at a flow rate of 0.3 ml/min. The LC separation was performed with a gradient elution of mobile phase A (methanol-acetonitrile-water: 1:1:3 [vol/vol/vol] containing 5 mM ammonium acetate [Wako Chemicals, Osaka, Japan]) and mobile phase B (isopropanol containing 5 mM ammonium acetate and 10 mM EDTA). The LC gradient and mass spectrometer settings were the same as previously described (46). The data analysis was performed as previously described (20). The obtained data were, as a result, normalized by adjusting the cell numbers processed; for encoding cells, the cell numbers were those treated for encystation induction, whereas for transformants, those treated for lipid extraction were used.

**Metabolic labeling of *E. invadens* and lipid analysis.** *E. invadens* trophozoites suspended in proliferation medium (1.5 × 10^5/ml) or encystation medium (6 × 10^5 cells/ml) were seeded in 96-well culture plates (240 μl per well). After adding U-13C-labeled L-serine (173.6 mCi/mmol) (Moravek, Brea, CA, USA) to each well (final radioactivity, 3 μCi/ml), the plates were sealed and incubated at 26°C for the period indicated as described above. For each time indicated, cell cultures from four wells of a 96-well plate were collected in a single 6-ml glass tube, and cells were pelleted by centrifugation at 1,500 × g for 5 min at 4°C. The cell pellet in each tube was washed twice with PBS. Then lipids were extracted by successive addition of 3.8 ml chloroform-methanol-0.15 N HCl (5:104 [vol/vol/vol]), 1 ml chloroform, and 1 ml 1% KCl (wt/vol deionized water) with thorough mixing at each addition. Phases were separated by centrifugation at 770 × g for 5 min at ambient temperature, and the organic phase was recovered and dried. The lipids extracted from 2.88 × 10^6 cells were resolved by thin-layer chromatography (TLC) on Silica Gel 60 high-performance TLC plates (Merck, Darmstadt, Germany) with chloroform-methanol-15 N
NH3 (60:35:8 [vol/vol/vol]). Each spot on the TLC plates was quantified using a Fuji imaging analyzer and Multi Gauge 2.2 software (FLA-7000, Fujifilm, Tokyo, Japan).

**Alkaline treatment of lipids.** The lipids obtained from 2.88 × 10^5 cells, as described above, were suspended in 600 μl 0.1 M KOH in chloroform-methanol (2:1 [vol/vol]) and incubated for 2 h at 37°C. After incubation, the lipid solution was sequentially mixed with 21 μl 4 M formic acid, 200 μl chloroform, and 400 μl deionized water. Then, the phases were separated by centrifugation at 770 × g for 5 min at ambient temperature, and the organic phase was recovered, dried, and dissolved in 50 μl chloroform-methanol (1:1 [vol/vol]) (47). The obtained lipids were resolved by TLC on Silica Gel 60 high-performance TLC plates (Merck, Darmstadt, Germany) with chloroform-methanol-15 N NH3 (60:35:8 [vol/vol/vol]). Each spot on the TLC plates was analyzed as described above.

**Real-time qRT-PCR.** Real-time qRT-PCR was performed as previously described (48) with minor modifications. Total RNA from Entamoeba cells was extracted with RNAiso Plus (TaKaRa Bio Inc., Kyoto, Japan), and cDNA was synthesized using the ReverTra Ace qPCR RT master mix with genomic DNA (gDNA) remover (Toyobo Co. Ltd., Osaka, Japan). Real-time PCR was performed using StepOnePlus (Thermo Fisher Scientific, Waltham, MA, USA), Thunderbird SYBR qPCR mix (Toyobo), and appropriate primer sets (see Table S3 in the supplemental material). For encysting *E. invadens* cell analysis, the cell pellets from one aliquot prepared for lipidomics were suspended in 1 ml RNAiso Plus (TaKaRa), and the resulting samples were similarly processed and analyzed.

**Gene knockdown in *E. histolytica*.** Construction of pSAP2-g-multi-based plasmids for gene silencing using appropriate primers (Table S3) was performed essentially as described previously (34). Plasmid transfection into *E. histolytica* (G3) trophozoites using Lipofectamine LTX and establishment of stable transformants were performed as described (34). The knockdown levels of the targeted genes in the established transformants were evaluated by real-time qRT-PCR using suitable primer sets (Table S3) as described above.

**Overexpression in *E. histolytica*.** Overexpression of HA-tagged proteins was achieved using the pEEx-m-HA vector, which was derived from pEEx-HA (36). The EcoRI-BglII fragment of pEEx-HA was replaced with a newly PCR-amplified 5′ conserved sequence (CS) region appended with EcoRI and Nheli-HindIII-BgIII sites at the either end. PCR amplimers harboring open reading frames (ORFs) of target genes were obtained using suitable primers sets (Table S3), digested with Nheli and BgIII, and inserted into the corresponding sites of pEEx-m-HA. The resulting correct plasmids were then introduced into *E. histolytica* (HM-1:IMSS c6) trophozoites, and stable transformants were established as described above. The levels of overexpression of the targeted genes in the established transformants were evaluated by real-time qRT-PCR using suitable primer sets (Table S3) as described above.

**Flow cytometry and fluorescence microscopy.** *E. invadens* trophozoites treated for encystation were suspended in encystation medium (37) sustaining either various concentrations of myriocin or the solvent control, DMSO. Note that the DMSO content in all wells was 1% (vol/vol). Then, the cell suspensions were seeded in 96-well culture plates, sealed, and incubated at 26°C for the designated period, as described above. The medium containing myriocin was prepared by adding each myriocin stock solution at 1/100 before trophozoite inoculation. For a solvent control, in place of the myriocin stock solution, DMSO was added at 1/100 before trophozoite inoculation. For a solvent control, in place of the myriocin stock solution, DMSO was added at 1/100 before trophozoite inoculation. For a solvent control, in place of the myriocin stock solution, DMSO was added at 1/100 before trophozoite inoculation.

**Flow cytometry.** For flow cytometry using Evans blue (EB) and calcein (CF), cells in the above-described cultures were treated and processed, and the obtained data were analyzed as described previously (37). The IC_{50} of myriocin for the cyst formation at 72 h after inducing encystation was also determined by this flow cytometry method.

**Transmission electron microscopy.** Transmission electron microscopy analysis, based on a rapid freezing and freeze-fixation method, was outsourced to Tokai Electron Microscopy, Inc. (Nagoya, Japan). The cells treated for encystation were cultivated either in the presence of 1 μM myriocin or DMSO for 24 h in 6 wells of a 96-well plate as described above. Then, the cells were collected in a single 1.5-ml tube using 1 ml PBS and pelleted by centrifugation at 5,200 × g for 1 min at 4°C. Each cell pellet was sandwiched between copper disks and quickly frozen in liquid propane at −175°C. The resulting samples were then freeze-substituted with 2% glutaraldehyde and 1% tannic acid in ethanol containing 2% distilled water (vol/vol) at −80°C for 48 h. Subsequently, they were transferred to a −20°C freezer and kept at −20°C for 3 h, followed by warming to 4°C by 4 h. The samples were then dehydrated for 30 min three times in absolute ethanol at ambient temperature and left in absolute ethanol at ambient temperature overnight. On the following day, the samples were soaked for 30 min twice in propylene oxide (PO) and once in a mixture of PO and resin (Quetol-812; Nissin EM Co., Tokyo, Japan) (70:30 [vol/vol]) for 1 h. The tube cap was left open overnight.
to completely volatilize the PO. Then, the samples were freshly soaked in 100% resin and incubated at 60°C for 48 h to polymerize the resin. The samples embedded in the polymerized resins were sectioned at an ultrathin thickness of 70 nm using an ultramicrotome (Ultracut UCT; Leica, Vienna, Austria). These samples were stained with 2% uranyl acetate at ambient temperature for 15 min and washed with distilled water. Then, the samples were secondarily stained with lead stain solution (Sigma-Aldrich, St. Louis, MO, USA) at ambient temperature for 3 min. The resulting samples were then examined at 100 kV acceleration voltage under a transmission electron microscope (JEM-1400 Plus; JEOL, Tokyo, Japan). Images were acquired using a charge-coupled-device (CCD) camera (EM-14830RUBY2; JEOL).

Data availability. All raw mass spectrometry data are freely available on the RIKEN DROP Met website (http://prime.psc.riken.jp/menta.cgi/prime/drop_index), under index number DM0036.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
FIG S1, TIF file, 1.6 MB.
FIG S2, TIF file, 0.8 MB.
FIG S3, TIF file, 1.6 MB.
FIG S4, TIF file, 1.5 MB.
FIG S5, TIF file, 1.2 MB.
FIG S6, TIF file, 0.8 MB.
FIG S7, TIF file, 1.7 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, XLSX file, 0.2 MB.
TABLE S3, PDF file, 0.1 MB.

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