Lipid Composition of Multilamellar Bodies Secreted by *Dictyostelium discoideum* Reveals Their Amoebal Origin

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When they are fed with bacteria, *Dictyostelium discoideum* amoebae produce and secrete multilamellar bodies (MLBs), which are composed of membranous material. It has been proposed that MLBs are a waste disposal system that allows *D. discoideum* to eliminate undigested bacterial remains. However, the real function of MLBs remains unknown. Determination of the biochemical composition of MLBs, especially lipids, represents a way to gain information about the role of these structures. To allow these analyses, a protocol involving various centrifugation procedures has been developed to purify secreted MLBs from amoeba-bacterium cocultures. The purity of the MLB preparation was confirmed by transmission electron microscopy and by immunofluorescence using H36, an antibody that binds to MLBs. The lipid and fatty acid compositions of pure MLBs were then analyzed by high-performance thin-layer chromatography (HPTLC) and gas chromatography (GC), respectively, and compared to those of amoebae as well as bacteria used as a food source. While the bacteria were devoid of phosphatidylcholine (PC) and phosphatidylinositol (PI), these two polar lipid species were major classes of lipids in MLBs and amoebae. Similarly, the fatty acid composition of MLBs and amoebae was characterized by the presence of polyunsaturated fatty acids, while cyclic fatty acids were found only in bacteria. These results strongly suggest that the lipids constituting the MLBs originate from the amoebal metabolism rather than from undigested bacterial membranes. This opens the possibility that MLBs, instead of being a waste disposal system, have unsuspected roles in *D. discoideum* physiology.

Multilamellar bodies (MLBs) are specialized lipid storage or secretory organelles of lysosomal origin. In cells, these are membrane-enclosed structures with a core composed of multiple concentric membrane layers. MLBs vary in diameter from 0.1 to over 2 μm (1). In mammals, MLBs are produced by many cell types. In many cases, MLBs are secreted and play various physiological roles, such as representing a particular source of specialized lipid components required for tissue protection and function (1).

MLBs are not produced only by higher eukaryotic cells, as they have also been observed in protozoa and, more particularly, in *Dictyostelium discoideum* (2–10). *D. discoideum* is a soil amoeba and a bacterial predator that internalizes its prey through phagocytosis. It lives as a unicellular organism when food is available. However, in the absence of food, its behavior changes, and it undergoes multicellular development, leading to cell aggregation and the production of fruiting bodies within approximately 24 h after starvation (11).

MLBs are produced in abundance when *D. discoideum* cells are grown in the presence of bacteria but are virtually absent when the cells are grown axenically in nutrient liquid medium (8, 9). MLBs ranging from about 0.5 to 2 μm are produced in the lysosomal compartments of the endocytic pathway of *D. discoideum* cells when internalized bacteria are digested (7, 9, 10). MLBs have also been found in starved cells at various stages of multicellular development that were initially grown with bacteria (*Klebsiella aerogenes* or *Escherichia coli*), but their abundance decreased as multicellular development progressed (8).

*D. discoideum* cells secrete their MLBs, and this phenomenon has been repeatedly seen at different steps of the multicellular process (2, 4–8). Despite the fact that bacterial lawns best mimic the natural environment of *D. discoideum*, the secretion of MLBs by vegetative cells grown on a bacterial lawn has been reported only once to our knowledge, with few details (10). This is probably due to the fact that the secretion of MLBs observed in most studies occurred under conditions where cell aggregation was induced by starvation or where vegetative cells could not be distinguished from starved cells (2, 4–8).

Some authors have proposed that MLBs are undigested bacterial material and consequently that MLBs secreted by *D. discoideum* are a kind of waste rejected by the cells (3, 6, 8, 10). On the other hand, secreted MLBs also contain some amoebal proteins, such as a cysteine proteinase, discoidin I, and other glycosylated proteins still not identified (2, 4, 5). Among these, with discoidin I being a marker of early development in *D. discoideum* (12), other authors suggested that MLB secretion may play a physiological role in the multicellular development and life cycle of *D. discoideum* (2).

Most of the data regarding MLBs resulted from microscopy studies and immunocytochemistry experiments carried out on fixed cells (2–10). Consequently, the biochemical composition of MLBs is lacking, especially the lipid one. It is difficult to elucidate the real physiological function and the origin of MLBs without such biochemical clues. Hence, determination of the lipid composition of MLBs would particularly help to clarify their bacterial or amoebal
origin because both amoebae and bacteria possess a distinct lipid profile (13–19). Thus, by comparing the lipid compositions of purified MLBs, amoebae, and bacteria, it would be possible to determine the origin of the lipids found in MLBs. If bacterial lipids are not taken up by \textit{D. discoideum} cells during the digestion process, one can expect that they would accumulate as waste in the MLBs. In this case, MLBs and bacteria would have roughly the same lipid profile. In contrast, if the lipid compositions of MLBs and amoebae are similar, this would suggest that the MLB membranes are mainly built by the metabolism of amoebae.

It is mandatory to count on an adequate purification protocol of secreted MLBs that removes all contaminants (amoebae, bacteria, and other material likely secreted by amoebae) to determine their lipid composition. We therefore improved the previous purification method proposed by Barondes and colleagues (2) to obtain highly purified MLB preparations. Using this new purification procedure, we performed analyses of the lipids that constitute the secreted MLBs as well as those of amoebae and bacteria used as a food source. Our results show that MLBs and \textit{D. discoideum} cells have mostly the same lipid composition, while being different from the one of bacteria. These results strongly suggest that MLBs originated from the amoeba’s metabolism.

**MATERIALS AND METHODS**

**Amoebae, bacteria, and antibodies.** \textit{D. discoideum} DH1-10 cells (20) were grown at 21°C in HL5 medium (21) supplemented with 15 μg/ml of tetracycline. Cells were subcultured twice a week in fresh medium to prevent confluence of the cell culture. Alternatively, \textit{D. discoideum} cells were grown in cocultures with bacteria as described below.

\textit{K. aerogenes} bacteria (laboratory strain), kindly provided by P. Cosson (22), were grown on LB agar at 37°C typically for 2 days before they were used for bacterium-amoeba cocultures.

The H161, H72, and H36 mouse monoclonal antibodies have been described previously (21, 23, 24) and were generously furnished by P. Cosson.

**Bacterium-amoeba cocultures and purification of MLBs.** \textit{D. discoideum} cells were grown with \textit{K. aerogenes}. Amoebae and bacteria were mixed in a proportion of 1:1,000 and

![FIG 1](https://example.com/figure1.png)

**FIG 1** \textit{D. discoideum} cells grown on \textit{K. aerogenes} produce and secrete MLBs. (A) Image of a phagocytic plaque. In the center (ce) of the plaque, amoebae have consumed virtually all of the bacteria and have begun multicellular development. At the periphery (pe) of the plaque, amoebae are still in the vegetative form and are crawling and feeding on bacteria. (B) TEM image of \textit{D. discoideum} cells grown in liquid medium (HL5). (C to F) TEM images of material from the periphery of a phagocytic plaque of \textit{D. discoideum} cells grown on \textit{K. aerogenes}. In panel C, amoebae (*), bacteria (pinheads), and secreted MLBs (arrows) are highlighted. An MLB in a lysosomal compartment is shown in panel D, while secreted MLBs are shown in panels E and F. Scale bars: A, 2 mm; B and C, 2 μm; D and E, 0.2 μm; F, 0.5 μm.
plated on HL5 medium containing 2% agar, and the coculture was incubated at 21°C. After 5 or 6 days, the bacterial lawn was almost entirely consumed by *D. discoideum* cells. At this moment, the coculture on the surface of the medium was harvested with a sterile scraper and resuspended either in 10 ml of phosphate-buffered saline (PBS) (1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 154 mM NaCl) or in 10 ml of starvation buffer (2 mM Na₂HPO₄, 14.7 mM KH₂PO₄, 100 mM sorbitol, 100 μM CaCl₂, 1% HL5 medium) (25).

When used, Barondes’ method of purification of secreted MLBs (2) was performed on amoeba-bacterium cocultures resuspended in PBS. The amoeba-bacterium suspensions were centrifuged at 1,800 g for 5 min, and the pellets were resuspended in PBS. The centrifugation was repeated to pellet cells, fruiting bodies, debris, and contaminating bacteria. The supernatants were then centrifuged at 10,000 × g for 20 min to pellet MLBs. The final ultracentrifugation step at 100,000 × g for 1 h was omitted. The purified material was then processed for TEM and immunofluorescence.

Alternatively to Barondes’ purification method, we developed a new purification protocol (see the Results section). According to the new procedure, the amoeba-bacterium cocultures were harvested and resuspended in starvation buffer before being centrifuged at 450 × g for 5 min to pellet amoebae. The supernatant was placed in the presence of 5 × 10⁷ *D. discoideum* cells freshly grown in liquid HL5 medium. This new coculture was shaken at 200 rpm overnight at 21°C. This step was necessary to eliminate the remaining bacteria by using the increased phagocytic capacity of the freshly grown amoeba added to the solution compared to that of the one removed by centrifugation. After 24 h, the coculture was centrifuged at 450 × g for 5 min. After this stage, the supernatant contained MLBs and particles (<0.5 μm) of various appearances. To concentrate MLBs and separate them from the particulate material, 1 ml of supernatant was placed on top of a glass tube containing a 6-ml sodium bromide density gradient (1 ml of six different densities ranging from 1.0 to 1.5 g/ml). The tube was centrifuged at 3,220 × g for 45 min at room temperature. A yellowish aggregate between white layers was visible. This aggregate corresponded to the fraction of pure MLBs (see the Results section) and was collected with a Pasteur pipette and transferred in a new 1.5 ml tube. The purified MLBs were washed two times in PBS, and they were pelleted by centrifugation (17,000 × g) for 10 min between each wash. The purified MLBs were kept at 4°C.

**TEM.** *D. discoideum* cells, extracellular materials from the periphery of the phagocytic plaques (Fig. 1A), bacteria, or purified material was fixed for 3 h in 0.1 M sodium cacodylate buffer (pH 7.3) containing 2% glutaraldehyde and 0.3% osmium tetroxide. Samples were washed with sodium cacodylate buffer three times and then dehydrated for 5 min in 30% ethanol, 5 min in 50% ethanol, 5 min in 70% ethanol, 10 min in 95% ethanol, and 1 h in 100% ethanol. The samples were then embedded in Epon resin and incubated overnight at 37°C, followed by 3 days at 60°C. Very thin slices (60 to 80 nm) were cut and stained for 8 min with 0.1% lead citrate and then for 5 min with 3% uranyl acetate. They were then examined using a transmission electron microscope (JEOL 1230) at 80 kV.

**Immunofluorescence.** Samples from purified material were allowed to adhere to glass coverslips for 90 min and then were fixed for 30 min in 4% paraformaldehyde. The samples were rinsed with PBS containing 40 mM NH₄Cl, permeabilized for 2 min in methanol at −20°C, and then immersed in PBS containing 0.2% bovine serum albumin (PBS-BSA) at room temperature for at least 5 min to block nonspecific binding sites. The glass coverslips were then incubated for 45 min at room temperature with the appropriate primary mouse antibodies diluted in PBS-BSA. A second incubation with Alexa 488-coupled anti-mouse IgG secondary antibody (Invitrogen, Burlington, Ontario, Canada) and DAPI (4’-6-diamidino-2-phenylindole) diluted in PBS-BSA was done for 30 min at room temperature. Between each step, at least three washes with PBS-BSA were done. The coverslips were then mounted on glass slides with Prolong Gold (Invitrogen, Burlington, Ontario, Canada). In colabeling experiments, the H72 and H161 antibodies were fluorescently prestained with Alexa 488 using Zenon technology (Invitrogen, Burlington, Ontario, Canada), and H36 antibody was detected using an Alexa 594-coupled anti-mouse IgG secondary antibody (Invitrogen, Burlington, Ontario, Canada). The capacity of the Zenon-coupled antibodies to detect their epitope was verified using *D. discoideum* cells (data not shown). Images of purified material were acquired using an Axio Observer Z1 microscope equipped with an Axiozoom camera (Carl Zeiss, Toronto, Ontario, Canada).

**Lipid extraction.** The lipids of purified MLBs were extracted using a modification of the Bligh and Dyer protocol (26). A suspension of purified MLBs (1 ml) was mixed with 1 ml of chloroform–methanol (2/1 [vol/vol]) in a screw-cap tube, and the mixture was heated at 80°C for 1 h. Then, after addition of 1 ml NaCl (2.5% [wt/vol]) to the extract, the medium was mixed for 1 min and centrifuged for 5 min at 80% of the maximum speed of the Labtop centrifuge (Hettich EBA 35), which corresponds to approximately 1,000 g. The lower phase (the organic one) was filtered onto a Pasteur pipette filled with a cotton pad and collected in a new tube. The remaining aqueous phase was extracted a second time with 1 ml of chloroform. After centrifugation, the organic phase was treated as described previously and pooled with the first one. The total chloroformic extract was evaporated and dried by a N₂ stream. The resulting dry lipid extract was dissolved in 0.1 ml of chloroform–methanol (1/1 [vol/vol]).

**Lipid separation.** The lipid extract was layered onto a high-performance thin-layer chromatography (HPTLC) plate, which was developed with the solvent system methyl acetate–CHCl₃–isopropanol–MeOH–0.25% KCl (5/5/5/2/1.8 [vol/vol/vol/vol/vol]) in order to separate the polar lipids (27). After migration, the plate was dried, and the lipids were revealed by a prilumin solution and visualized using an ImageQuant 300 apparatus at 254 nm. The lipids were identified by comparison to different lipid standards (sphingomyelin [SPH], phosphatidylethanolamine [PE], phosphatidylcholine [PC], cerebrosides [Cer], and phosphatidylglycerol [PG]) from Sigma (St. Louis, MO) layered on the same plate. The different lipids dyed by prilumin were quantified using Image J and/or by measuring their contents in fatty acids as described below.

**Fatty acid analysis.** Each spot corresponding to a different lipid was scraped, deposited into a screw-cap tube, and submitted to a transesterification treatment in the presence of 1 ml of MeOH·H₂SO₄ containing 5 μg of heptadecanoic acid (C₁₇:0) at 110°C for 1 h. After the tubes had cooled, 1 ml of 2.5% NaCl and 0.3 ml hexane were added, and the mixture was mixed to extract the fatty acid methyl esters (FAMES). The tubes were centrifuged as described above, and 0.1 ml of the hexane layer was transferred to vials for gas chromatography (GC) analysis. The FAMES (1 μl) were injected in an Agilent 7890 gas chromatograph equipped with a Carbowax column (15 m by 0.53 mm, 1.2 μm) (Alltech Associates, Deerfield, IL) or a CP-Sil column (100 m by 0.25 mm, 0.2 μm) and flame ionization detection. The temperature gradient was 160°C for 1 min, increased to 190°C at 20°C min⁻¹, increased to 210°C at 5°C min⁻¹, and then remained at 210°C for 5 min. FAMES were identified by comparing their retention times with those of commercial standards (Sigma, St. Louis, MO): C₁₆:0 (palmitic acid), C₁₈:0 (stearic acid), C₁₈:1 (oleic acid), C₁₈:2 (linoleic acid), C₁₈:3 (linolenic acid), C₂₀:0 (arachidic acid), and C₂₂:0 (behenic acid). FAMES were quantified by using an integration ChemStation to calculate the peak surfaces and then comparing them with the C₁₇:0 response.

**RESULTS**

**Vegetative *D. discoideum* cells secrete MLBs.** First, we wanted to determine whether MLB secretion by *D. discoideum* is associated only with cell starvation and multicellular development or whether it also occurs in vegetative cells. To achieve that, *D. discoideum* cells collected from the periphery of phagocytic plaques (Fig. 1A), where they are in their vegetative state (28), as well as other cells grown axenically in liquid medium, were analyzed by TEM. Axenic cells displayed endosomal compartments that were mostly devoid of electron-dense material (Fig. 1B). No MLBs were...
seen inside or outside cells grown in liquid medium. On the other hand, the material collected from the periphery of phagocytic plaques included amoebae, bacteria, extracellular MLBs ranging in size from 0.5 to 2 m, and unidentified smaller particles of various morphologies (Fig. 1C). In addition, amoebae grown on a lawn of K. aerogenes contained endosomes that were mainly filled with bacteria being digested or with MLBs (Fig. 1C and D). The MLBs, both intracellular and extracellular, were composed of concentric membrane lamellae with a more or less spherical morphology (Fig. 1D, E, and F). Given that a large number of secreted MLBs were visible in the periphery of phagocytic plaques and that the amoebae were still in the vegetative state, MLB secretion does not appear to be linked to multicellular development per se and is likely a continuous process associated with amoebae grown in the presence of bacteria.

To confirm that MLBs are secreted by vegetative D. discoideum cells, amoebae were also grown in liquid HL5 medium in the presence of K. aerogenes to prevent their starvation and multicellular development. As shown in Fig. 2, MLBs were visible inside the cells in endocytic compartments as well as outside the cells. These results demonstrated that vegetative D. discoideum cells are able to secrete MLBs when grown in the presence of bacteria and confirmed the possibility of using vegetative cells instead of cells engaged in the multicellular process to further study MLB secretion. In addition, this opened the possibility to produce large quantities of MLBs.

The H36 antigen is present on secreted MLBs. As the biochemical composition of MLBs is still unknown, tools to evaluate the purity of MLB preparations, such as protein markers or antibodies, are lacking. We have used three antibodies (H36, H72, and H161) that recognize membrane-associated proteins found in the endocytic pathway or at the plasma membrane of D. discoideum cells (21, 23, 24) to determine their MLB-binding capacity. The antigen of the H161 antibody is p80, a transmembrane protein likely involved in ion transport (24). The H36 and H72 antibodies bind to proteins with molecular masses of 47 and 25 kDa, respectively (21, 24). The identities of these two proteins are unknown.

Using the protocol published by Barondes et al. (2), a large quantity of MLBs has been purified from amoebae grown on K. aerogenes (Fig. 3A and B). The purified MLBs reacted with H36 but not H72 and H161 under immunofluorescence (Fig. 3C to E). K. aerogenes cells were not reacting with the H36 antibody (data not shown). Even if a clear association of the H36 antibody with secreted MLBs could be seen, a small fraction of the MLBs (DAPI-negative structures) did not react with the antibody or had sparse or incomplete staining (pinheads in Fig. 3C).

To confirm the specific staining of MLBs with H36, a colabeling of MLBs with H36 and either H72 or H161 was also performed (Fig. 3F and G). Here again, only H36 antibody specifically stained secreted MLBs. These results suggested that the antigen detected by the H36 antibody is associated with secreted MLBs, and this antibody appeared to be a good tool to check the purity of MLB preparations.

A new purification protocol for secreted MLBs. Barondes’ purification protocol is not optimal, since about 20 to 60% of the purified biomass was bacteria (DAPI-positive structures in Fig. 3) (data not shown). Moreover, this procedure did not permit the separation of MLBs from other small (<0.5 m) and abundant material seen in the images in Fig. 3A and B. We therefore decided to improve Barondes’ method for the purification of secreted MLBs. This improved purification procedure is shown in Fig. 4A. All steps added to the protocol were designed to get MLBs virtually free of other biological contaminants as this was a prerequisite for the subsequent biochemical analysis of MLBs.

In order to decrease bacterial contamination, a new step consisting of a second coculture with freshly grown D. discoideum cells (Fig. 4A, step 2) was included. This step allowed the elimination of more than 95% of the remaining bacteria. A sodium bromide density gradient (Fig. 4A, step 4) was then used to separate MLBs from contaminating smaller particles of various morphologies. This step was inspired by a protocol for Bacillus subtilis spore purification (29). It was noticed in our preliminary assays that MLBs had the propensity to aggregate and that this was favored by the use of phosphate-buffered saline (PBS) for the purification procedure compared to HL5 medium (data not shown). This characteristic of the MLBs to aggregate in PBS was usefully combined with the purification of the crude MLB fraction by density centrifugation.

Figure 4B shows that four different fractions of stacked material were detected in the sodium bromide density gradient. All of these fractions were analyzed by immunofluorescence using H36 and DAPI staining to detect the layer containing pure MLBs. Fraction 1, the first one from the top of the gradient, contained material of small size (<0.5 m) (Fig. 4C). Between fraction 1 and fraction 2, there was a streak of material impossible to collect with precision. Fraction 2 appeared as a small aggregate stuck on the
tube wall. This aggregate corresponded to MLBs (H36-positive structures) free of contaminants and was recovered and further analyzed (see the next section). Layers 3 and 4 had a similar composition. MLBs were present in good proportion in these fractions, but bacteria and small particles (<0.5 μm) were also visible. The purification procedure was performed many times (>10), and there was always the same profile after the sodium bromide density gradient, demonstrating the robustness of the purification protocol.

**Purity evaluation of the MLB fraction.** TEM images indicated that the aggregate (fraction 2) was enriched in pure MLBs, with sizes ranging from about 0.5 to 2 μm (Fig. 5A). At higher magnification (Fig. 5B), MLBs appeared as roughly spherical structures

![FIG 3](image1.png) **FIG 3** H36 labeling of partially purified secreted MLBs. Secreted MLBs were purified using the protocol proposed by Barondes et al. (2). The purified material was processed for TEM (A and B) or deposited on a glass slide and processed for DAPI staining and immunofluorescence using the H36 (C), H72 (D), or H161 (E) antibody or for colabeling with H36 and H72-Zenon (F) or with H36 and H161-Zenon (G). (A and B) TEM images. Bacteria (pinheads), secreted MLBs (arrows), and small, unidentified particles (*) are highlighted in panel B. (C) Epifluorescence microscopic images. DIC, differential interference contrast. Some MLBs (DAPI-negative structures) with H36 labeling are indicated with arrows in panel C. Some MLBs with sparse, irregular, or no H36 labeling are indicated with pinheads. Some MLBs are also indicated with arrows in panels D and E. Scale bars: A, 2 μm; B, 0.5 μm; C to G, 5 μm.

![FIG 4](image2.png) **FIG 4** Protocol for purification of MLBs secreted by *D. discoideum*. (A) Schematic representation of the purification protocol of secreted MLBs. When the bacterial lawn was almost entirely eaten by amoebae, the biomass found on the petri dish was harvested and resuspended in starvation buffer (step 1). Amoebae were removed by centrifugation. In step 2, the supernatant was put back into a coculture with fresh amoebae for 24 h. This step allowed the elimination of residual bacteria. Amoebae were removed again by centrifugation (step 3). In step 4, part of the supernatant was deposited on the top of a sodium bromide density gradient and then centrifuged to concentrate MLBs in a small aggregate separated from other contaminants. Finally, MLBs were recovered from the gradient and washed twice in PBS (step 5). (B and C) Images of a sodium bromide density gradient after centrifugation. MLBs were found in many area of the gradient but were in a pure state only in the aggregate (layer 2). The aggregate from the gradient in panel B is highlighted (white circle) in the magnified image shown in panel C.
presenting a tight multilayered profile, which is typical of secreted MLBs (2, 4–8, 10). Moreover, purified MLBs had nothing in common at the morphological level with \textit{K. aerogenes} used as a food source (compare Fig. 5A with C). Contaminant bacteria or amoe- bal elements other than MLBs were virtually not detected in this fraction, indicating that the steps added to the purification protocol strongly increased the purity of the MLBs.

In addition to TEM observations, which allowed only a partial analysis of the sample (a few slices of material analyzed from a block of about 1 mm$^3$), the purity of MLBs present in the aggregate recovered from the sodium bromide gradient was also extensively observed by epifluorescence microscopy to determine the presence of bacteria in MLB preparations using DAPI (Fig. 5D and E). Virtually no bacterium was visible. H36 was used to visualize MLBs on the same samples. In some cases, large aggregates of MLBs were visible (Fig. 5E).

The purity of three MLB preparations was quantified by monitoring H36 and DAPI staining (Table 1). In each case, more than 1,800 structures were observed to determine the proportion of bacteria in the samples. It appeared that less than 3 bacteria per 100 MLBs were detectable. Based on these results, the purification protocol developed here decreased by at least 10-fold the presence of contaminants in the MLB preparation compared to the previous method. This high purity of MLB preparation opened the door to biochemical characterization of their lipid content.

**FIG 5** Purity of MLB preparations confirmed by TEM and immunofluorescence. After the passage throughout the new purification method, the purity of MLBs from layer 2 was confirmed by TEM (A to C) and immunofluorescence (D and E). (A) Field of purified MLBs. (B) Single MLB. The purified MLBs have the typical multilayered morphology already described. (C) \textit{K. aerogenes} in pure culture. (D and E) Two series of images from immunofluorescence analyses of two different fields are shown. By differential interference contrast (DIC), a lot of small, round structures corresponding to MLBs were visible (D) as well as small (D) or large (E) MLB aggregates. The H36 antibody stains these structures. The two fields shown here are DAPI negative, suggesting the absence of DNA (i.e., bacteria) after the purification procedure was performed. Scale bars for D and E, 4 µm.

**Lipid composition of purified MLBs.** HPTLC analysis of the lipids extracted from purified MLBs revealed that these structures were composed of neutral lipids (NL) and phospholipids, which represent 23 and 60%, respectively, of the total lipid fraction (Fig. 6). Phospholipids were constituted by five species: phosphatidylethanolamine (PE) and phosphatidylycerol (PC), which were the major species, as well as phosphatidylglycerol (PG), phosphatidyl-
TABLE 1 Purity of the MLB fraction monitored by immunofluorescencea

| Purification step | MLBsb | Bacteriaa | MLB purity (%):d |
|------------------|-------|-----------|------------------|
| 1                | 1,827 | 18        | 99.01            |
| 2                | 2,008 | 49        | 97.56            |
| 3                | 3,432 | 105       | 96.9             |

Avg ± SD 97.82 ± 1.08

a Observations were made on random fields.
b Structures (0.5 to 2 µm) visible by differential interference contrast and presenting DAPI-negative and H36-positive signals.
c Structures (~1 µm) visible by differential interference contrast and presenting DAPI-positive and H36-negative signals.
d [no. of MLBs/(no. of MLBs + no. of bacteria)] × 100.

Serine (PS), and phosphatidylinositol (PI), which were present in smaller proportions. Two other lipid classes, L1 and L2, which had the same retention factor (Rf) values as cerebrosides and sulfolipid standards, respectively, were also detected. As a control, analysis of the lipid composition of both K. aerogenes bacteria and D. discoideum cells was carried out using the same protocol. As shown in Fig. 6, K. aerogenes only contained PE and PG plus PS as phospholipids, in agreement with PE being the most abundant phospholipid in many Gram-negative and Gram-positive bacterial species (15, 30). In contrast to the MLB situation, PC, PI, and lipid L1 were not detected in K. aerogenes. The lipid profile of D. discoideum cells contained all of the lipids detected in the MLBs as well as cardiolipid and lysophosphatidylcholine (LysoPC) (Fig. 6). If lipids L1 and L2 were less abundant in amoeba than in MLBs, the proportions of NL and all phospholipids were in the same range and in agreement with those from previously published analyses (17, 19). Altogether, these results showed that the lipid composition of MLBs was most similar to that of amoeba and rather different from that of the bacteria used as the food source. These data suggested that the bacterial lipids were not integrated as a whole in MLBs but that lipids synthesized by D. discoideum were used to assemble these structures.

The total lipid extracts of MLBs, amoebae, and K. aerogenes were then submitted to a methanolic transesterification for the fatty acid methyl esters analysis by GC. In MLBs, the C16:0, C18:1, and C18:2 fatty acids were the most abundant fatty acids and represented about 75% of the total content (Fig. 7). In K. aerogenes, C16:0 was the major fatty acid under the growth conditions used and represented more than 60% of the total (Fig. 7). The C17 cyc and C19 cyc fatty acids, which contain a cyclopropane cycle and are typical of several bacteria (15), were also detected in K. aerogenes (Fig. 7). Finally, the fatty acid profile of D. discoideum contained large amounts of C18:1 and C18:2 fatty acids, as in the case of MLBs and was devoid of cyclopropane-type fatty acids (Fig. 7). In contrast to bacteria, which contained very low levels of unsaturated C18 fatty acids, both amoebae and MLBs were highly enriched in C18:1 and C18:2 fatty acids. Despite the fact that MLBs contained a relatively high level of C16:0, their fatty acid composition shared greater similarity with the one of amoebae than with that of bacteria. Overall, these results suggested that fatty acids constituting the MLB lipids originated from amoebae rather than from bacteria and that a specific subset of fatty acid originating from cell metabolism accumulated in MLBs.

DISCUSSION

In the present study, we revisited the biogenesis and secretion of MLBs to obtain new clues about their origin. We first confirmed the observation by Mercer and Shaffer that MLB exocytosis also occurs when the cells are still in their vegetative form and not solely when they are starved or aggregating (10). Then, we demonstrated that H36 antibody is a useful tool that allows the detection of secreted MLBs. These observations opened the door to the design of an improved method to purify secreted MLBs. The purification of secreted MLBs has been done to help understand their origin by comparative analysis of lipids contained in MLBs with those of amoeba and bacteria, the latter being used as a food source for D. discoideum cells.

The elimination of residual bacteria by incubating the MLB suspension with new amoebae displaying a high phagocytic activity and the use of a sodium bromide gradient to separate MLBs from other amoeba-secreted material are the two main breakthroughs of our method. The new improved protocol yielded highly pure preparations of MLBs, making it possible to analyze their lipid composition. However, the purity of the MLB preparations may be lower than suggested by the results in Table 1. Since free bacteria might not bind efficiently to glass coverslips, this could result in an overestimation of the efficiency of the MLB purification procedure. However, electron microscopy observa-

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FIG 6 Total lipid profile of purified MLBs, amoebae, and K. aerogenes. The lipids were extracted, separated by HPTLC, and quantified. The results are expressed as the mean values ± standard deviations of 3 independent analyses for the MLBs, 6 for amoebae, and 3 for K. aerogenes (Ka).
In addition, if the major phospholipid and fatty acids that were not present in $D. ~disscoideum$ cells are established from some $K. ~aerogenes$ cells, which again is not consistent with our analyses and suggest that MLBs get their full maturation in postlysosomes (lysosomal compartments older than 60 min) (18). Bis (monoacylglycerol)-phosphate (BMP) is a major lipid in younger lysosomes (30 min) but not in postlysosomes. Since PE, PC, and neutral lipids are the more abundant lipids (13) (Fig. 6). More precisely, lipids found in the endocytic pathway of axenic $D. ~disscoideum$ cells already have been characterized by magnetic fractionation (17, 18). Rodriguez-Paris and colleagues showed that PE and PC are the predominant classes of phospholipids in postlysosomes (lysosomal compartments older than 60 min) (18). Bis (monoacylglycerol)-phosphate (BMP) is a major lipid in younger lysosomes (30 min) but not in postlysosomes. Since PE, PC, and neutral lipids are the more abundant lipids in MLBs, the results previously obtained for the lipid composition of the endocytic pathway of $D. ~disscoideum$ cells are consistent with our analyses and suggest that MLBs get their full maturation in postlysosomes by addition of specific lipids and exclusion of others (i.e., BMP) from the membrane of the lysosomal compartments. Moreover, even if MLBs have a fatty acid profile quite similar to the one of amoebae, C18:1 and C20:0 are specific to MLBs, or other fatty acids like C16:0 are more present in these structures than in $D. ~disscoideum$ cells. These results suggest that a specific subset of fatty acids is transferred in lysosomal compartments and accumulates in MLBs during their production/maturation.

Purified MLBs can be stored for at least 6 months at 4°C with no obvious alterations (data not shown), suggesting that these structures are quite stable, which raises further questions about the physiological role(s) of these structures. In addition, because MLBs are produced uniquely when $D. ~disscoideum$ cells are in the presence of an abundant source of food (i.e., bacteria), it is possible to imagine that the production of MLBs may represent a kind of food storage.

In the future, it will be of interest to determine if MLBs in the suspension are engulfed during their incubation with the second batch of $D. ~disscoideum$ cells, the one allowing the elimination of residual bacteria (step 2 in Fig. 4A). Since free MLBs can be purified after this incubation with amoeba, one can conclude that MLBs either are not internalized or are secreted again by the cells after their internalization. This question of reinternalization of MLBs by $D. ~disscoideum$ cells is important. An answer to this question will contribute to understanding the role of secreted MLBs. For example, if reinternalization occurs, MLBs may serve as a kind of communication device between cells. If this is not the case, one
can imagine that a molecular signal exists on MLBs to prevent their phagocytosis. For the moment, no tool is available to answer this question of reinternalization. Some amoeba proteins are already known to be associated with MLBs. This is the case of discoidin I, but this protein might not be an appropriate marker, since it is present on MLBs only in specific situations (2, 5). Other glycosylated D. discoideum proteins, including a cysteine proteinase, are also known to be associated with the MLBs, but their identity remains unknown disqualifying them, for the moment, as potential MLB markers (4). Further analyses, including an analysis of the protein repertoire on secreted MLBs, will be the next step in deducing the function of these structures.

Knowing which amoeba proteins are present on MLBs will also be the first step in studying the biogenesis of these structures. Studying protein sorting during MLB biogenesis as well as the function of these structures will be the next step in studying the biogenesis of these structures.

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REFERENCES

1. Schmitz G, Muller G. 1991. Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. J. Lipid Res. 32:1539–1570.
2. Barondes SH, Haywood-Reid PL, Cooper DN. 1985. Discoidin I, an endogenous lectin, is externalized from Dictyostelium discoideum in multilamellar bodies. J. Cell Biol. 100:1825–1833.
3. Cooper DNW, Haywood-Reid PL, Springer WR, Barondes SH. 1986. Bacterial glycoconjugates are natural ligands for the carbohydrate binding-site of discoidin-I and influence its cellular compartmentalization. Dev. Biol. 114:416–425.
4. Emslie KR, Birch D, Champion AC, Williams KL. 1998. Localisation of glycoproteins containing type 3 O-linked glycosylation to multilamellar bodies in Dictyostelium discoideum. Eur. J. Protistol. 34:321–328.
5. Fukuzawa M, Ochiai H. 1993. Different subcellular localizations of discoidin I monomer and tetramer in Dictyostelium discoideum cells using conformation-specific monoclonal antibodies. Exp. Cell Res. 204:61–72.
6. Gezelius K. 1959. The ultrastructure of cells and cellulose membranes in Acrasiae. Exp. Cell Res. 18:425–453.
7. Gezelius K. 1961. Further studies in the ultrastructure of Acrasiae. Exp. Cell Res. 23:300–310.
8. Hohl HR. 1965. Nature and development of membrane systems in food vacuoles of cellular slime molds predatory upon bacteria. J. Bacteriol. 90:755–765.
9. Marchetti A, Mercanti V, Cornillon S, Alobaud L, Charette SJ, Cosson P. 2004. Formation of multivesicular endosomes in Dictyostelium. J. Cell Sci. 117:6053–6059.
10. Mercer EH, Shaffer BM. 1960. Electron microscopy of solitary and aggregated slime mould cells. J. Biophys. Biochem. Cytol. 7:353–356.
11. Kessin RH. 2001. Dictyostelium: evolution, cell biology, and the development of multicellularity. Cambridge University Press, Cambridge, United Kingdom.
12. Maeda Y. 2005. Regulation of growth and differentiation in Dictyostelium. Int. Rev. Cytol. 244:287–332.
13. Davidoff F, Korn ED. 1963. Fatty acid and phospholipid composition of the cellular slime mold, Dictyostelium discoideum. The occurrence of previously undescribed fatty acids. J. Biol. Chem. 238:3199–3209.
14. Dunnick JK, O’Leary WM. 1970. Correlation of bacteria lipid composition with antibiotic resistance. J. Bacteriol. 101:892–900.
15. Manter DK, Hunter WJ, Vivanco JM. 2011. Enterobacter soli sp. nov.: a lignin-degrading gamma-proteobacteria isolated from soil. Curr. Microbiol. 62:1044–1049.
16. Martinez-Morales F, Schobert M, Lopez-Lara JM, Geiger O. 2003. Pathways for phosphorylcholine biosynthesis in bacteria. Microbiology 149:3461–3471.
17. Nolta KV, Rodriguez-Paris JM, Steck TL. 1994. Analysis of successive endocytic compartments isolated from Dictyostelium discoideum by magnetic fractionation. Biochim. Biophys. Acta 1224:237–246.
18. Rodriguez-Paris JM, Nolta KV, Steck TL. 1993. Characterization of lysosomes isolated from Dictyostelium discoideum by magnetic fractionation. J. Biol. Chem. 268:9110–9116.
19. Weeks G, Herring FG. 1980. The lipid composition and membrane fluidity of Dictyostelium discoideum plasma membranes at various stages during differentiation. J. Lipid Res. 21:681–686.
20. Cornillon S, Pech E, Benghezal M, Ravelan K, Gaynor E, Letoueuf F, Bruckert F, Cosson P. 2000. Php1p is a nine-transmembrane protein superfamily member involved in Dictyostelium adhesion and phagocytosis. J. Biol. Chem. 275:34287–34292.
21. Mercanti V, Charette SJ, Bennett N, Ryckewaert JJ, Letourneur F, Cosson P. 2006. Selective membrane exclusion in phagocytic and macropinocytic cups. J. Cell Sci. 119:4079–4087.
22. Benghezal M, Fauvarque MO, Tournebize R, Froquet R, Marchetti A, Bergeret E, Lardy B, Klein G, Sansonetti P, Charette SJ, Cosson P. 2006. Specific host genes required for the killing of Klebsiella bacteria by phagocytes. Cell. Microbiol. 8:139–148.
23. Charette SJ, Mercanti V, Letoueuf F, Bennett N, Cosson P. 2006. A role for adaptor protein-3 complex in the organization of the endocytic pathway in Dictyostelium. Traffic 7:1528–1538.
24. Ravelan K, de Chassey B, Cornillon S, Benghezal M, Zulianello L, Gebbie L, Letoueuf F, Cosson P. 2001. Membrane sorting in the endocytic and phagocytic pathway of Dictyostelium discoideum. Eur. J. Cell Biol. 80:754–764.
25. Smith EW, Lima WC, Charette SJ, Cosson P. 2010. Effect of starvation on the endocytic pathway in Dictyostelium cells. EurKaryot. Cell 9:387–392.
26. Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–917.
27. Heape AM, Juguéin H, Boiron F, Cassagne C. 1985. Improved one-dimensional thin-layer chromatographic technique for polar lipids. J. Chromatogr. 322:391–395.
28. Artemenko Y, Swaney KF, Devreotes PN. 2011. Assessment of development and chemotaxis in Dictyostelium discoideum mutants. Methods Mol. Biol. 769:287–309.
29. Nicholson WL, Law JF. 1999. Method for purification of bacterial endospores from soils: UV resistance of natural Sonoran desert soil populations of Bacillus spp. with reference to B. subtilis strain 168. J. Microbiol. Methods 35:13–21.
30. Ratledge C, Wilkinson SG. 1988. Microbial lipids. Academic Press, London, United Kingdom.
31. Charette SJ, Cosson P. 2007. A LYST/beige homolog is involved in biogenesis of Dictyostelium secretory lysosomes. J. Cell Sci. 120:2338–2343.
32. Charette SJ, Cosson P. 2008. Altered composition and secretion of lysosome-derived compartments in Dictyostelium AP-3 mutant cells. Traffic 9:588–596.