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ARTICLE

Various dictyostelids from the environment can produce multilamellar bodies

Alicia F. Durocher, Cynthia Gagné-Thivierge, and Steve J. Charette

Abstract: Multilamellar bodies (MLBs), structures composed of concentric membrane layers, are known to be produced by different protozoa, including species of ciliates, free-living amoebae, and *Dictyostelium discoideum* social amoebae. Initially believed to be metabolic waste, potential roles like cell communication and food storage have been suggested for *D. discoideum* MLBs, which could be useful for the multicellular development of social amoebae and as a food source. However, among dictyostelids, this phenomenon has only been observed with *D. discoideum*, and mainly with laboratory strains grown in axenic conditions. It was thought that other social amoebae may also produce MLBs. Four environmental social amoeba isolates were characterized. All strains belong to the *Dictyostelium* genus, including some likely to be *Dictyostelium giganteum*. They have distinctive phenotypes comprising their growth rate on *Klebsiella aerogenes* lawns and the morphology of their fruiting bodies. They all produce MLBs like those produced by a *D. discoideum* laboratory strain when grown on *K. aerogenes* lawns, as revealed by analysis using the H36 antibody in epifluorescence microscopy as well as by transmission electron microscopy. Consequently, this study shows that MLBs are produced by various dictyostelid species, which further supports a role for MLBs in the lifestyle of amoebae.

Key words: social amoeba, multilamellar body, *Dictyostelium*, environmental isolate.

Résumé : Les corps multilamellaires (CML), des structures composées de couches membranaires concentriques, sont connus pour être produits par différents protozoaires, y compris des espèces de ciliés, des amibes libres et l'amibe sociale *Dictyostelium discoideum*. On a d'abord cru qu'il s'agissait de déchets métaboliques, mais on a ensuite suggéré que les CML de *D. discoideum* joueraient des rôles potentiels dans la communication cellulaire et le stockage de nourriture, ce qui pourrait être utile au développement multicellulaire des amibes sociales ainsi que comme source de nourriture. Cependant, parmi les dictyostélides, ce phénomène n'a été observé que chez *D. discoideum*, et principalement avec des souches de laboratoire cultivées en conditions axéniques. On a pensé que d'autres amibes sociales pourraient aussi produire des CML. Quatre isolats environnementaux d'amibes sociales ont été caractérisés. Toutes les souches appartenaient au genre *Dictyostelium*, y compris certaines susceptibles d'être *Dictyostelium giganteum*. Elles possèdent des phénotypes distinctifs comprenant leur taux de croissance sur tapis de *Klebsiella aerogenes* et la morphologie de leurs corps fructifères. Elles produisent toutes des CML similaires à ceux produits par la souche de laboratoire de *D. discoideum* lorsqu'elles sont cultivées sur tapis de *K. aerogenes*, comme le révèlent les analyses en microscopie à épifluorescence avec l'anticorps H36 et en microscopie électronique. Ainsi, cette étude montre que les CML sont produits par diverses espèces de dictyostélides, ce qui confirme le rôle des CML dans le mode de vie des amibes.

Mots-clés : amibe sociale, corps multilamellaire, *Dictyostelium*, isolat environnemental.

Introduction

Multilamellar bodies (MLBs) are composed of concentric lipid membrane layers. They are involved in lipid storage and secretion in many eukaryotic cells (Schmitz and Müller 1991). MLBs can be produced during the phagocytosis process and can be secreted by *Dictyostelium discoideum* social amoebae upon feeding on digestible bacteria (Paquet et al. 2013).

In starvation conditions, such as when preys become inaccessible, *D. discoideum* enters a multicellular phase beginning with aggregation of the cells into a migrating slug, leading to the creation of spore-containing fruiting

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A.F. Durocher, C. Gagné-Thivierge, and S.J. Charette. Institut de Biologie Intégrative et des Systèmes, Pavillon Charles-Eugène-Marchand, Université Laval, 1030 avenue de la Médicine, Québec, QC G1V 0A6, Canada; Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec, Université Laval, 2725, chemin Sainte-Foy, Québec, QC G1V 4G5, Canada; Département de biochimie, de microbiologie et de bio-informatique, Faculté des sciences et de génie, Université Laval, 1045, avenue de la Médicine, Québec, QC G1V 0A6, Canada.

Corresponding author: Steve J. Charette (email: steve.charette@bcm.ulaval.ca).

bodies to withstand long-term starvation (Kessin 2001). MLB production is mostly observed during the unicellular vegetative state, although MLBs can also be produced by aggregating cells (Hohl 1965). Different MLB morphologies are possible depending on, among others, the density of membrane layers or the type of bacteria phagocytosed by *D. discoideum* cells (Hohl 1965; Paquet et al. 2013; Denoncourt et al. 2014).

While MLBs were originally considered to be metabolic waste, it was noted that the formation of MLBs represents a cost for the cell that seems too large for simple waste evacuation. Biochemical analyses of both the lipids and proteins found in these structures as well as functional analyses of *D. discoideum* MLBs suggested additional roles for MLBs, including extracellular nutrient storage and a potential intermediary in communication between cells (Paquet et al. 2013; Denoncourt et al. 2016, 2017). Lipid analysis in particular showed that MLB lipids were mostly of amoeboid origin, suggesting that although bacteria are necessary for MLB production, the process could be controlled by amoebae.

MLB production has been observed in a few protozoa so far, including free-living amoeba such as *Acanthamoeba* and ciliates including *Tetrahymena* (Berk et al. 2008; Chekabab et al. 2012). In the case of the dictyostelid group, MLBs have only been observed with strains of the *D. discoideum* species (Gezelius 1959, 1961; Mercer and Shaffer 1960; Hohl 1965; Barondes et al. 1985; Cooper et al. 1986; Fukuzawa and Ochiai 1993; Emslie et al. 1998; Marchetti et al. 2004). Considering that some of these strains were severely modified at the genetic level to permit axenic growth, it cannot be excluded that MLB production by dictyostelids could be a laboratory artifact without the observation of the same phenomenon with amoebae retrieved from the environment and from species other than *D. discoideum*. Observing MLB production with different environmental strains would strengthen current theories about their additional roles.

It was hypothesized that environmental dictyostelids can produce MLBs as well as *D. discoideum* laboratory strains can, as the possible roles suggested for these structures would be useful to wild strains of social amoebae (Denoncourt et al. 2016, 2017): intercellular communication is essential to coordinating multicellular development, and a role as food storage could be beneficial in starvation periods. This study aimed to characterize environmental social amoebae and compare them with *D. discoideum* DH1-10, a common laboratory strain (Cornillon et al. 2000). All environmental isolates, from various dictyostelid species, were found to produce MLBs similarly to the laboratory strain.

Materials and methods

Isolation of social amoebae

Samples of soil from temperate deciduous forest ground were taken from different places in Québec, Quebec, Canada. Soil samples were serially diluted in water (1/10, 1/100, and 1/1000). A 150 μL volume of each dilution was mixed with a 150 μL volume of *Klebsiella aerogenes* bacteria grown overnight and plated on a 10-cm-diameter Petri dish containing SM medium (10 g/L bacteriological peptone, 1 g/L yeast extract, 2.2 g/L KH_2PO_4, 1 g/L K_2HPO_4, 1 g/L $MgSO_4$, 20 g/L agar, 1% glucose), followed by incubation at room temperature. *Klebsiella aerogenes* bacteria were generously gifted by Pierre Cosson (Benghezal et al. 2006). After 4–5 days of incubation, a part of the biomass from the border of phagocytic plaques displaying fruiting bodies was sampled using a micropipette tip and inoculated on the center of an SM medium Petri dish containing a fresh bacterial lawn. Twelve isolates were obtained using this method.

Amoeba strains

Four strains (JLJV2, E1, E2, and 3.2-2) displaying various phenotypes of fruiting bodies and speed of growth on bacterial lawns were selected from the 12 initially isolated, upon confirmation that they could be serially cultivated on bacterial lawns and kept frozen at –70 °C.

18S rRNA identification

PCR amplification of the 18S rRNA gene was conducted with primers Euk-4/18-F (5′-CTGGTTGATYCTGCCAGT-3′) (Hendriks et al. 1989) and EukR (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin et al. 1988). For JLJV2 and 3.2-2, Accustart II polymerase (Quantabio, USA) was used for PCR amplification, whereas E1 and E2 were amplified with GoTaq polymerase (Promega, USA). Hybridization was conducted at 55 or 60 °C. PCR products were sequenced using Sanger technology (ABI3730xl DNA analyzer). Sequences obtained were analyzed with BLAST to allow identification of the amoeba strains, along with comparison of the percent identity between isolates (Altschul et al. 1990).

Characteristics of environmental amoebae growth on bacterial lawns

For each amoeba strain, 40 cells suspended in HL5 medium were plated on a 15-cm-diameter Petri dish of SM agar along with 300 μL of a *K. aerogenes* suspension at an optical density at 595 nm of 2. Two plates were prepared per isolate and were incubated in the dark at room temperature. The growth rate of 10 phagocytic plaques per plate of each isolate was measured over the course of a week at least once a day, or more often as necessary, judging from the rate of growth of the plaques. Appearance of fruiting bodies was also examined at the end of the incubation with a Motic SMZ-168 series stereomicroscope equipped with a Moticam Pro 252A camera (Motic, Canada). Additional pictures were taken with an iPhone 6S through the stereomicroscope eyepiece.

H36 immunofluorescence

Cells were harvested from the periphery of a phagocytic plaque, suspended in HL5 medium, and the cell

Table 1. Sequence identities in 18S rDNA of the isolates and closest relative.

Isolate name	JLJV2	E1	E2	3.2-2	Closest relative (% identity)
JLJV2		93.6%	93.6%	94.8%	*Dictyostelium discoideum* (99.94%)
E1			97.4%	98.5%	*Dictyostelium giganteum* (99.09%)
E2				98.2%	*Dictyostelium giganteum* (98.03%)
3.2-2					*Dictyostelium giganteum* (98.93%)

density was adjusted to 500 000 cells/mL. A total of 500 000 cells of each strain were placed on a sterile coverslip and left to adhere for 2–3 h. Once the cells had adhered to the coverslip, H36 immunofluorescence was conducted as previously described by Paquet et al. (2013). The H36 antibody was detected with an Alexa 568-coupled anti-mouse IgG secondary antibody (Invitrogen, Burlington, Ontario, Canada). Samples were also stained with DAPI (4′,6-diamidino-2-phenylindole) to facilitate observations and discrimination between cells and cellular debris. Samples were observed with a Zeiss Axio Observer Z1 microscope equipped with an Axiocam camera (Carl Zeiss, Canada), in differential interference contrast and epifluorescence microscopy at 630× magnification. H36 immunofluorescence was reproduced at least three times for each amoeboid isolate. Average cell size for all isolates was also determined based on the measure of 30 cells per isolates from immunofluorescence images using Fiji/ImageJ (Schindelin et al. 2012, 2015). To verify H36 staining of MLBs, the cell suspension was centrifuged at 200*g* for 5 min, to concentrate most of the amoeboid cells in the pellet and recover mostly MLBs in the supernatant fraction. The supernatant was collected and placed on the coverslip and processed for immunofluorescence in the same way as the samples in which no amoeboid cells were removed.

Western blot of H36

Cells were harvested from the border of phagocytic plaques using micropipette tips. The biomass was resuspended in 4 mL of HL5 in a 15 mL Falcon tube and centrifuged at 200*g* for 5 min. The supernatant containing bacteria was discarded. Amoebae were washed an additional time using the same volume of HL5 and centrifugation force to remove the remaining bacteria. The amoebae were resuspended in 4 mL of HL5 and an aliquot was used to count the amoebae with a hemocytometer. The cells were centrifuged again and resuspended to a concentration of 300 000 cells/15 μL in HL5 mixed with one-half volume of 3× TEX loading buffer (0.22 mol/L Tris (pH 6.8), 23.5% glycerol, 9% SDS, and traces of Bromophenol blue). A control lysate was also prepared at 300 000 cells/15 μL with axenic *D. discoideum* DH1-10 cells grown in HL5.

The solubilized proteins were separated on 12% SDS–PAGE in reducing conditions (5% (*v*/*v*) 2-mercaptoethanol added to the samples loaded on the gel). Proteins in the gels were electrotransferred to a nitrocellulose membrane and then stained with Ponceau S to confirm the quantity of proteins on the membrane. The latter was then immersed in 50 mL of TBS (10 mmol/L Tris (pH 7.4) and 150 mmol/L NaCl) for 5 min and afterwards incubated with TBSM (TBS with 7% skim milk) for 2 h at room temperature to block nonspecific binding. The membrane was washed five times for 5 min with TBST (TBS with 0.1% Tween 20) and was incubated for 90 min at room temperature with H36 (ascite diluted 1:10 000 in TBST). The membrane was then washed three times for 5 min in TBST and was incubated for 1 h at room temperature with goat anti-mouse IgG IRDye 680RD (Li-cor, USA) according to the manufacturer's instructions. The membrane was washed six times for 5 min in TBST. The protein bands were acquired with the Odyssey Fc Imaging System (Li-cor, USA).

Transmission electron microscopy (TEM)

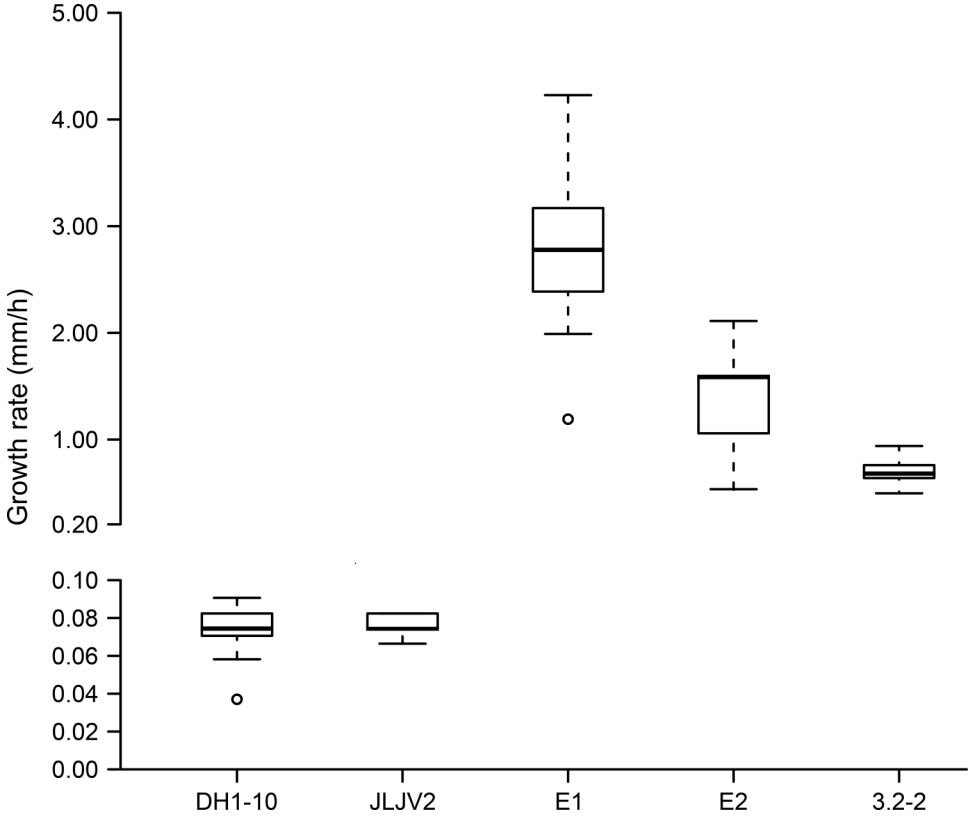
Samples from amoeboid cultures on SM medium supplemented with a *K. aerogenes* lawn were collected from the periphery of phagocytosis plaques and resuspended in 1× glutaraldehyde, and then processed as previously described (Paquet et al. 2013). Observations were made using a transmission electron microscope (either a JEOL 1230 for strains E1 and E2 or a FEI Tecnai Spirit G2 for strains JLJV2 and 3.2-2) at 80 kV. TEM micrographs were used to measure average MLB size (n = 38).

Results

The amoebae used in this study were isolated from forest soil in Québec, Quebec, Canada. A precise 18S rRNA identification was only possible for isolate JLJV2, which was identified as *Dictyostelium discoideum* (Table 1). For the other isolates, a partial identification was attained. All strains were found to be *Dictyostelium* species, similar to *D. giganteum*, *D. bruneum*, *D. gargantuum*, and *D. purpureum*, but closest to *D. giganteum*. Percent identity was high among all isolates, with JLJV2 being the most distinct from the other strains. However, it should be noted that only a 797 bp forward sequence could be obtained for isolate E2. Thus, only forward sequences were compared to obtain the percent identity between the isolates. For identification of the isolates, forward and reverse sequences were used with JLJV2, E1, and 3.2-2.

Strains E1, E2, and 3.2-2 had a much higher growth rate than *D. discoideum* DH1-10, used in this study for comparison, as measured by phagocytic plaque growth rate on *K. aerogenes*. JLJV2 and DH1-10 grew at a similar rate (Fig. 1).

Fig. 1. Growth of the amoeboid isolates on *Klebsiella aerogenes*. The graph presents the growth rate of phagocytic plaques (mm/h) for the various isolates and DH1-10 on *K. aerogenes*. The mean for the growth of 20 phagocytic plaques per condition is shown with the standard deviation. The rectangle spans the 25th and 75th percentiles, the horizontal line inside the rectangle is the median, and the whiskers indicate the minimum and maximum values. Outliers (outside the percentile range) are indicated by open circles.



Growth rates were statistically different between all strains except DH1-10 and JLJV2, as shown by a p value under 0.05 following analysis with the Mann–Whitney U test or Student's t test.

Typical multicellular development of the amoeboid isolates is presented in Fig. 2. Strains E2 and 3.2-2 produced similar abundant fruiting bodies. These structures were quite large and tall compared with those produced by DH1-10. Strain JLJV2 produced short fruiting bodies, similar to the ones of DH1-10 but with a lower abundance. Strain E1 had the most distinctive multicellular development, as this strain was defective for the culmination step of fruiting body development (Fig. 2C). Only slugs, mounds, and occasional finger formations could be observed for this isolate.

JLJV2 and DH1-10 cells were of a similar size, while E1 and 3.2-2 were larger and E2 was the strain with the smallest cells (Fig. 3). Cell sizes were significantly different from one another, as indicated by a p value under 0.05 following analysis of variance (ANOVA) using either the Mann–Whitney U test or Student's t test, except for these pairs: DH1-10 and JLJV2, DH1-10 and 3.2-2, and E1 and 3.2-2.

In *D. discoideum* grown axenically in HL5, the proteins recognized by the H36 antibody are mostly present in membranes but are excluded from phagocytic cups and phagosomes (Mercanti et al. 2006; Denoncourt et al. 2016). MLB staining has also been reported with this antibody (Paquet et al. 2013; Denoncourt et al. 2016). Figure 4 shows fluorescence images of H36 staining, which was positive for cells of all wild isolates and DH1-10. Staining is concentrated at the plasma membrane for all strains, as is typically observed with axenic DH1-10 (Mercanti et al. 2006), with some cytoplasmic signal observed in some strains. Inserts for JLJV2, E1, E2, and DH1-10 show characteristic staining of MLBs, with staining for JLJV2, E2, and DH1-10 forming a ring around the MLB (Paquet et al. 2013). H36 staining of MLBs was not observed with isolate 3.2-2.

The H36 antibody recognizes *N*-acetylglucosamine-1-phosphate, a post-translational modification that can be found on many proteins (Denoncourt et al. 2016). A Western blot was performed to determine the profile of bands detected by H36 with the amoeboid isolates (Fig. 5). The first interesting observation is that the band pattern obtained for DH1-10 is different between cells cultured axenically in HL5 and cells grown in the presence of bacteria. An intense band at around 30 kDa and other additional bands are visible for cells grown in the presence of bacteria compared with axenic cells. JLJV2 has a band profile equivalent to that of DH1-10. For their part, E1, E2, and 3.2-2 have a different band pattern compared

Fig. 2. Multicellular development of the amoeboid isolates. Typical images of fruiting bodies and phagocytosis plaques produced by each strain are presented. Top row shows stereomicroscope images taken with a Motic camera (scale bar: 1 mm; magnification: × 100) while the bottom row shows images taken through the eyepiece of the stereomicroscope (scale bar: 2 mm; magnification: A and B, × 100; C to E, × 64).

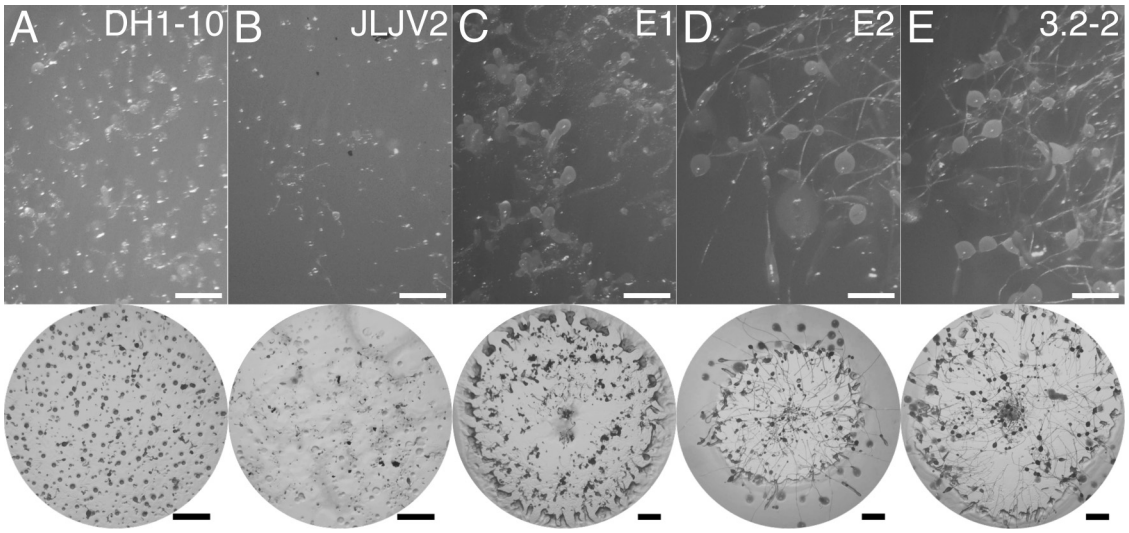
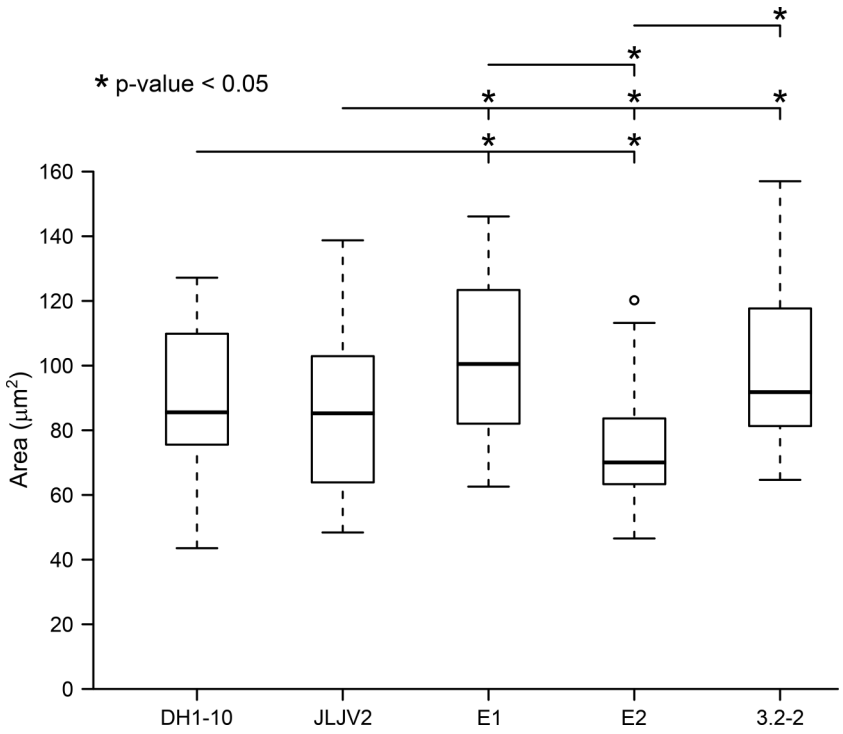


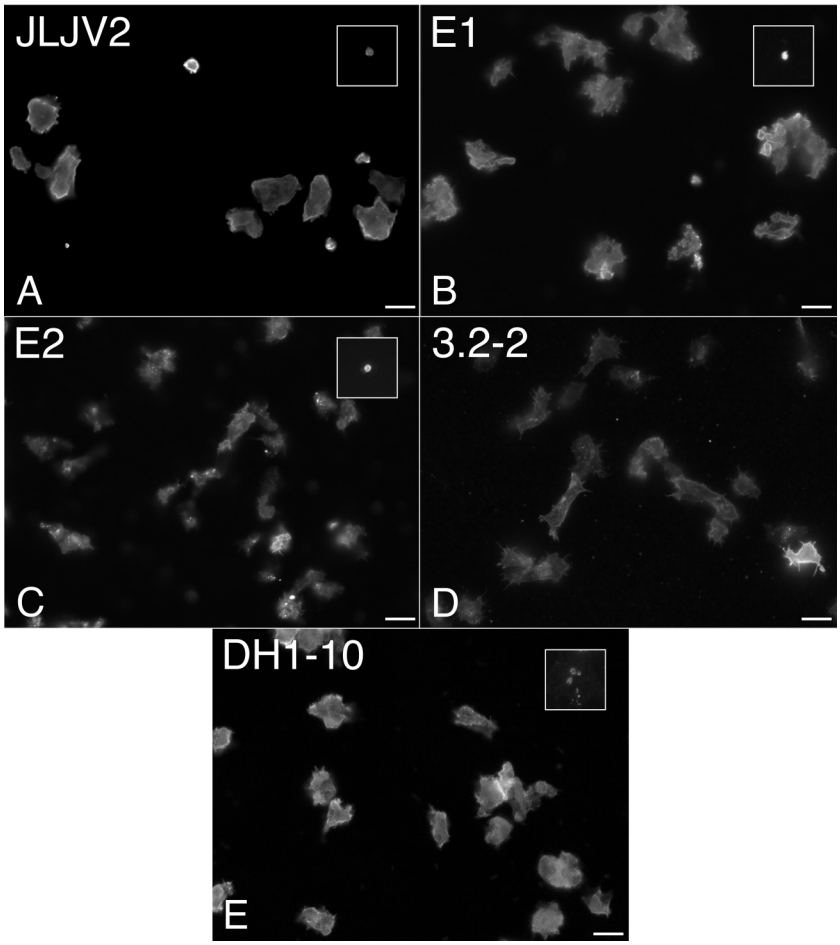
Fig. 3. Average cell size (μm²) of the amoeboid isolates. Asterisks (*) indicate $p < 0.05$ between two values. The rectangle spans the 25th and 75th percentiles, the horizontal line inside the rectangle is the median, and the whiskers indicate the minimum and maximum values. Outliers (outside the percentile range) are indicated by open circles.



with DH1-10. In addition, even if their band pattern is similar, E1, E2, and 3.2-2 show some differences with a few bands common to two out of three isolates, again suggesting that they are distinct strains.

Figure 6 presents TEM images of MLBs produced by each amoeboid isolate, both in formation inside the cells and secreted in the medium. The MLBs produced by JLJV2, E1, E2, and 3.2-2 all had a similar appearance whether inside or outside the cells. Some MLBs produced by JLJV2 and E1 contained tighter membrane layers. This morphology was observed both inside and outside of the cell. All strains also produced looser MLBs, including some that were not entirely round. Some MLBs presented "U-shaped" layers of membrane, such as in Figs. 6F and 6H. MLBs may also include amorphous material as previously described (Denoncourt et al. 2016). The size of MLBs

Fig. 4. H36 staining is positive for all of the amoeboid isolates. JLJV2 (A), E1 (B), E2 (C), and 3.2-2 (D) cells show a staining pattern similar to that observed with DH1-10 (E) upon immunofluorescent staining with the H36 antibody. Scale bars equivalent to 10 μm. The H36 antibody labels several structures inside the cells, so it is not possible to be sure which intracellular elements marked by the antibody correspond to multilamellar bodies (MLBs) (Denoncourt et al. 2017). All pictures taken at × 630 magnification. Insets show H36 staining on MLBs produced by the associated isolate, except for 3.2-2 where no MLB could be conclusively targeted with H36 staining.



was on average 1.1 ± 0.3 by 1.3 ± 0.3 μm, a size close to that of a bacterium, which is in accordance with MLB sizes that have been reported previously, ranging from 0.1 to about 2 μm (Schmitz and Müller 1991).

Discussion

The production of MLBs by all isolates studied suggests that these structures could be produced by dictyostelids in general. These results support theories proposing a supplementary role for MLBs beyond being a metabolic by-product by demonstrating that MLB production is not an artefact resulting from, among other reasons, the axenization of the laboratory strains (Denoncourt et al. 2016, 2017).

Identification of the species based on 18S rRNA gene sequencing was not entirely conclusive, which was expected as there is a high level of homogeneity within social amoebae. This is particularly the case for the species *D. giganteum* and *D. purpureum*, which seem to exist in multiclonal groups with different genotypes (Sathe et al. 2010). Percent identity between the isolates is also high, as expected from the identification obtained. JLJV2 is the only strain conclusively identified as *D. discoideum*, and its sequence was consequently not as similar to the other isolates. However, because the four isolates displayed very divergent phenotypes, especially for the growth rates and fruiting body characteristics, as shown in Figs. 1–3, it is plausible that this study has analyzed the production of MLB from four distinct dictyostelid species. Strain E1 was particularly interesting because of its culmination defect, as seen in Fig. 2C. Culmination of fruiting bodies is controlled by many genes (Kessin 2001; Loomis 2015), and since a full genome sequencing of E1 was not conducted, the exact reasons for this defect are unknown. However, it was interesting to compare this likely mutant strain with culminating strains, and it should be noted that while being defective for culmination, strain E1 still produces MLBs similar to other strains. Therefore, in future studies on the role of MLBs,

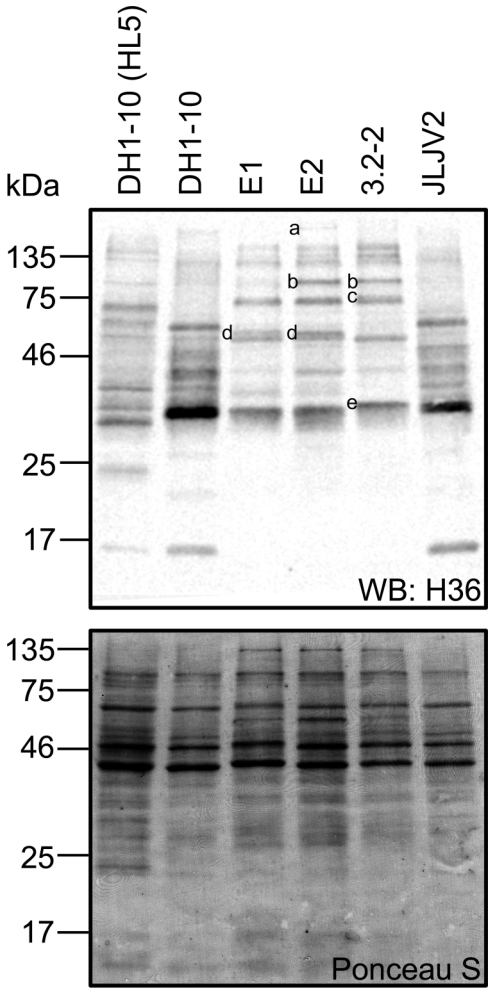


Fig. 5. The H36 antibody displays different protein band patterns in the amoeboid isolates. Proteins from cell extracts were separated on a 12% SDS–PAGE and transferred on a nitrocellulose membrane. Before performing the Western blot (WB) with the H36 antibody, the proteins on the membrane were stained with Ponceau S to confirm equivalent amount of proteins in each lane. On the image of the WB, letters correspond to bands specific to E1, E2, and 3.2-2 individually or in pairs.

it should be considered that if they are indeed involved in intercellular communication, they do not seem to be involved in culmination, or that at least a culmination defect does not hinder their production.

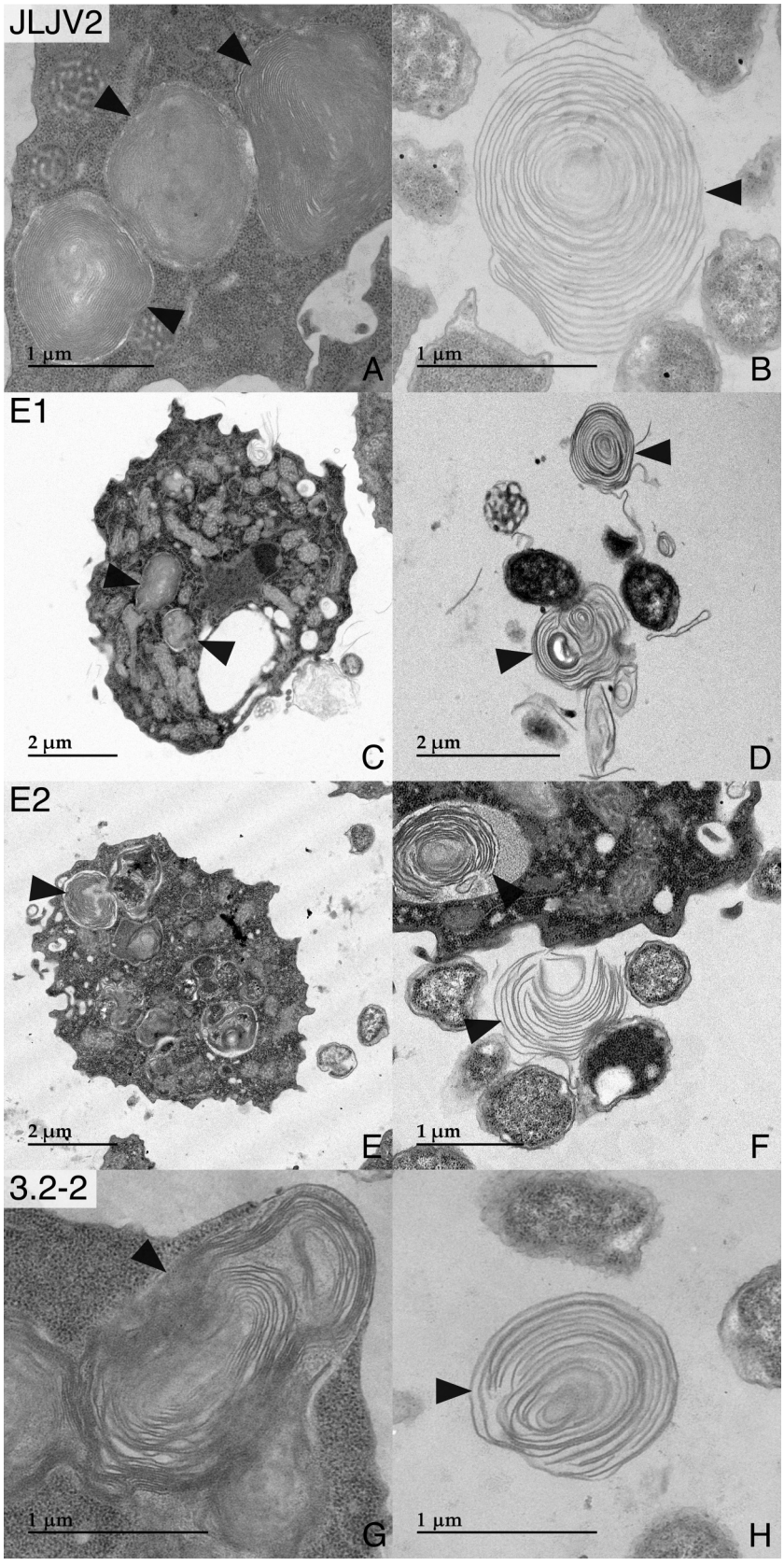
In a previous study, analysis of the lipid composition of the membranes constituting MLBs showed that they were of amoeboid origin, rather than being formed from bacterial remains, as was suggested earlier (Hohl 1965; Paquet et al. 2013). Subsequently, some proteins associated with MLBs, such as discoidin, a lectin involved in adhesion, or SctA, a constitutively secreted protein, suggest that these bodies could play a role in intercellular communication (Barondes et al. 1985; Denoncourt et al. 2016). PhoPQ, a hypothetical protein similar to AprA, has been detected in MLBs. AprA, which regulates proliferation in *D. discoideum*, can act as a chemorepellent for vegetative cells, but the effect is not observed on starved cells (Brock and Gomer 2005; Phillips and Gomer 2012). Since starvation triggers multicellular development in social amoebae, the presence of PhoPQ in MLBs is particularly interesting for a possible role in intercellular communication. A study has also shown that naïve *D. discoideum* cells exposed to MLBs produced by another *D. discoideum* population will ingest and degrade these MLBs, which could indicate a role as food storage (Denoncourt et al. 2017). Therefore, MLBs could be more than a by-product of bacteria digestion.

These results demonstrate that other *Dictyostelium* species are able to produce MLBs, opening the door to new research questions. For example, it can be asked if MLBs produced by one species can be recognized, phagocytosed, and digested by another *Dictyostelium* species, and whether the MLBs of the first species may affect the second species. As a first step to answer these questions, a biochemical characterization of MLBs from these isolates would be mandatory to see if they are similar to those produced by *D. discoideum* (Barondes et al. 1985; Paquet et al. 2013; Denoncourt et al. 2016) and, for example, to see if cell signaling molecules are found in these MLBs. However, a limitation for studies of this kind is the fact that these environmental isolates are not axenic, compared with laboratory strains, and that axenic cultures can be useful for some experiments (Paquet et al. 2013; Denoncourt et al. 2017). Axenization was attempted for these strains using HL5 medium, which is routinely used for *D. discoideum* culture. It was partly conclusive for isolate JLJV2, which was the only isolate to grow in axenic medium. However, axenic JLJV2 cells appeared damaged when observed in TEM (data not shown).

Although we used environmental isolates, the production of MLBs was only observed in a laboratory setting. It was shown here, however, that H36 staining could be used to detect MLBs from JLJV2, E1, and E2. This finding could be used in further research to detect MLBs directly from environmental samples. However, since H36 is also detected in amoeboid cells, another marker could be used. This alternative marker would be especially useful because the H36 antibody detects a post-translational protein modification rather than a specific protein (Denoncourt et al. 2016), which likely explains the difference in the signal observed depending on the amoeboid strain, as shown by our results with 3.2-2. Although 3.2-2 is similar to E1 and E2 based on sequence identity, no H36 signal could be detected from MLBs. The post-translational modification recognized by H36 may not be present in 3.2-2 MLB proteins, as suggested by the difference seen in the band patterns recognized by H36 in Western blot for E1, E2, and 3.2-2, especially the d band specific to E1 and E2 (Fig. 4).

Another candidate for antibody detection of MLBs would be SctA, a protein found in pycnosomes, which could be embedded into MLBs. SctA could be an interesting

Fig. 6. Multilamellar bodies (MLBs) produced by amoeboid isolates were visible both inside the cells and in the medium. MLBs (arrowheads) present inside (A) and outside (B) JLJV2 cells, inside (C) and outside (D) E1 cells, inside (E) and outside (F) E2 cells, and inside (G) and outside (H) 3.2-2 cells. Magnification: A, × 13 500; B, × 18 500; C, × 2500; D, × 4000; E, × 2500; F, × 6000; G, × 4800; H, × 18 500.



target for detection of dictyostelid MLBs in the environment, as the protein is constitutively secreted and is not found in significant quantities inside cells (Sabra et al. 2016; Denoncourt et al. 2016). SctA staining of MLBs produces a less intense fluorescent signal than what is observed with H36, but the specificity to MLBs could allow an easier detection. In fact, detection of SctA combined with the use of H36 to label MLBs would be an interesting approach for analysis of environmental samples. SctA staining was not attempted here because the *sctA* gene has not been detected in the *D. giganteum* genome. However, it has been identified in other *Dictyostelium* species, such as *D. discoideum* and *D. purpureum*, as determined using BLAST (Altschul et al. 1990). Additional antibodies could be developed to facilitate detection of MLBs from complex samples. Identification of MLBs directly in environmental samples would be crucial to support theories of a supplementary role for MLBs.

Conclusion

Our results suggest that MLBs can be produced by various dictyostelid species from the forest soil environment, similarly to laboratory strain production of these structures. Such results are consistent with proposed roles for MLBs, such as intercellular communication or food storage. As new research constantly unveils new levels of complexity in the lifestyle of social amoebae, MLBs could be a crucial element to understanding the particularities of these microorganisms.

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