Wall Structure and Germination of Spores in *Cunninghamella echinulata*

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(Received 21 February 1975; revised 21 April 1975)

SUMMARY

The spore of *Cunninghamella echinulata* is contained inside a monosporic sporangium. The spore wall consists of two layers: an outer thin electron-dense layer and an inner thick electron-transparent layer. The inner layer appears fibrillar. During spore germination a localized area of new wall material is formed beneath the existing spore wall at the site where germ-tube formation will occur. This new wall pushes out through the spore and sporangial walls and forms the wall of the germ tube.

The germination of spores of fungi as a group is discussed. At present no taxonomic significance can be attached to the mode of germination.

INTRODUCTION

Cunninghamella echinulata (Thaxt.) Thaxt. produces its spores inside monosporic sporangia that are borne on clavate or subglobose ampullae. The spore and sporangial walls are fused, resulting in a propagule that is dispersed and germinates as a unit. Until recently these monosporic sporangia were considered to be conidia (Hawker, Thomas & Beckett, 1970; Dykstra, 1974). Khan & Talbot (1975) studied their development and showed that sporangial and spore walls are of different origin and comparable with the sporangial and sporangiospore walls of multisporic sporangia. It is only because the two are fused that an illusion is created of a single wall with many layers.

The mechanism of vegetative wall formation at the time of spore germination also has a bearing on the subject of the types of propagules in fungi. Bartnicki-Garcia (1968) has suggested three main types of vegetative wall formation, each characteristic of certain groups of fungi and certain types of spores. His type I included germination of conidia and spores of higher fungi where an already existing spore wall, or one of its inner layers, extends to become the wall of the germ tube. Type II, relating to zoospores, does not concern us here. In his type III, representing the germination of sporangiospores of Mucorales, a new vegetative wall is formed under the existing spore wall.

There are three conflicting reports about the origin of the wall of the germ tube in *Cunninghamella*: a new vegetative wall is formed (*C. elegans* Lendner; Hawker, 1966); the innermost lining of the existing inner layer of the spore wall is changed chemically and extends outwards to become the wall of the germ tube (*Cunninghamella* sp.; Hawker *et al.* 1970); the inner layer of the spore wall clothes the emerging germ tube, without a new wall being formed or any chemical change occurring (*C. vesiculosa* Misra; Dykstra, 1974).

Thus on the basis of germination type the propagules of *Cunninghamella* could be

sporangiospores, or conidia, or something between the two. This study was undertaken to resolve this problem.

METHODS

Cunninghamella echinulata was grown at 25 °C on neutral Czapek-Dox + yeast agar containing (g/l H₂O): NaNO₃, 0.33; KH₂PO₄, 0.166; MgSO₄·7H₂O, 0.083; KCl, 0.83; FeSO₄·7H₂O, 0.0016; sucrose, 5; agar, 16; Difco yeast extract, 0.083. The spores were harvested in clumps and put on the medium to germinate at 25 °C. Some spores were fixed after about 2 h, before any germ tubes could be detected under a binocular microscope. Others were fixed after 3 h, when most of the spores had formed germ tubes. The spores incubated for 2 h were fixed only in potassium permanganate, while the others were fixed in this or glutaraldehyde, i.e. unbuffered 1 % (w/v) potassium permanganate at room temperature for 45 min, or 2.5 % (w/v) glutaraldehyde in 0.2 M-sodium cacodylate buffer, pH 7.25, at 25 °C for 3 h were used. Post-fixation after aldehyde treatment was carried out for 12 h in 1 % (w/v) buffered osmium tetroxide at 4 °C. The material was dehydrated through a graded series of acetone and embedded in Spurr's (1969) standard medium.

Sections were cut with an LKB ultratome, stained with 2 % (v/v) aqueous uranyl acetate followed by lead citrate, and examined with either a Philips EM 100 C or a Siemens Elmiskop 1A electron microscope.

Abbreviations used in Figures. ER, endoplasmic reticulum; ES, electron-dense structures; G, Golgi-like body; GS, globular structure; GT, germ tube; GW, germ-tube wall; IL, interrupted layer; ISL, inner layer of the spore wall; L, lipid droplet; M, mitochondrion; MB, microbody; MT, microtubule; MVB, multivesicular body; N, nucleus; OSL, outer layer of the spore wall; P, deposits of polysaccharide-like material; R, ribosomes; S, spine; SW, sporangial wall; V, vesicles; VA, vacuole; VL, vegetative layer; VS, vesicle containing dark material.

RESULTS AND DISCUSSION

The sporangia of *C. echinulata* are spherical (Fig. 1*c*) and covered with spines (Figs. 1*a*, 2*a*, 5). Because of earlier reports of swelling of sporangia during germination (Hawker, 1966; Hawker *et al.* 1970; Dykstra, 1974) camera lucida drawings were made soon after putting the sporangia on agar, and later of the same spores after germ-tube emergence. No increase in size was noticed except for a small bulge (Fig. 1*d*) at the point of germ-tube emergence. Thus there was no swelling of the sporangium during germination in *C. echinulata*.

The quiescent spore was filled with lipid droplets, mitochondria, several nuclei and some electron-dense globular bodies in the centre (Fig. 1*a*). A few membranous structures that may have been pieces of endoplasmic reticulum were also present; poor fixation and staining made comment on these, and ribosomes, difficult.

The wall of the propagule consisted of four distinct zones (Fig. 2*a*): an outermost sporangial wall, next an electron-dense interrupted layer, followed by a thin electron-dense outer layer of the spore wall and a thick electron-transparent inner layer of the spore wall. The sporangial wall and the interrupted layer appeared fibrillar, while both layers of spore wall showed no substructure, only a granular pattern. However, at the point of emergence of the germ tube (Fig. 3*b*, *c*) the microfibrillar nature of the inner layer of the spore wall became evident. It appeared to be composed of tightly interwoven randomly orientated fibrils. The fibrils in the sporangial wall and the interrupted layer seemed to be arranged sub-parallel to the periphery of the spore (Fig. 2*c*, *d*).

Fig. 1. Quiescent and germinated sporangia. (a) Quiescent sporangium showing lipid droplets (L), nuclei (N), dark globular structures (GS), mitochondria (M), and endoplasmic reticulum-like profiles (ER). Glutaraldehyde-osmium tetroxide fixation. (b) Germinated sporangium. Note the complete disappearance of dark globular structures and the appearance of vacuoles (VA). Ring-shaped mitochondrial profiles (arrows) are common. Endoplasmic reticulum (ER) is closely associated with nuclei (N). Potassium permanganate fixation. (c) Quiescent sporangium. (d) Germinated sporangium. Note the bulge (arrow head) at the point of germ-tube emergence (GT).

Fig. 2. Wall structure of germinating and germinated sporangia. (a) Transverse section of germinating sporangium showing four distinct zones in the wall. Glutaraldehyde–osmium tetroxide fixation. (b) Transverse section of vegetative wall after the removal of the spore and sporangial wall but before it becomes the wall of the germ tube. The vesicles are fusing with plasmalemma. The fibrillar substructure of the wall is distinct. Glutaraldehyde–osmium tetroxide fixation. (c) Outer layer of the spore wall and the interrupted layer. The former shows granularity, the latter fibrillar substructure (arrow heads). Glutaraldehyde–osmium tetroxide fixation. (d) Sporangial wall, in transverse section, showing fibrillar substructure (arrow heads). Glutaraldehyde–osmium tetroxide fixation.

Fig. 3. Stages in the formation of a germ tube. (a) The vegetative layer (VL) is deposited locally (between arrows) in the form of a cap. No change in the thickness of inner layer of spore wall (ISL) is evident. The sporangial wall (SW) and spore wall have already started to break from outside probably because of pressure from inside. Potassium permanganate fixation. (b) The pressure from inside results in the loosening of the fibrils of inner layer of spore wall. The fibrils form a network (arrow heads). Glutaraldehyde-osmium tetroxide fixation. (c) The germ tube is protruding after breaking the spore and sporangial wall. The sporangial wall has separated from the outer layer of the spore wall (OSL). The fibrils of the inner layer of the spore wall are covering the emerging germ tube (arrows). Glutaraldehyde-osmium tetroxide fixation. (d) The germ tube (GT) has emerged. The wall of the germ tube is continuous with the vegetative layer (VL) that does not surround the entire spore protoplast. The arrow head marks the starting point of the vegetative layer. Glutaraldehyde-osmium tetroxide fixation.

During germination there was complete disappearance of electron-dense globular bodies and subsequent appearance of vacuoles (Fig. 1*b*). Buckley, Sjaholm & Sommer (1966) reported a similar phenomenon at the time of germination of *Botrytis cinerea* conidia. They suggested that vacuoles developed by evacuation of membrane-bounded electron-dense bodies. In *C. echinulata*, too, the stored food in globular bodies may have been used up, resulting in the formation of the vacuoles. There was an increase in the number of mitochondrial profiles which were of different shapes (Figs. 1*b*, 4).

The number of lipid bodies decreased during germination, but complete disappearance as was reported by Hawker *et al.* (1970) for the germination of *Cunninghamella* spores did not occur (Figs. 1*b*, 5). The lipids may be used and contribute to the vacuolar system.

Deposits of polysaccharide-like material, an important constituent of developing spores (Khan & Talbot, 1975) and absent from dormant spores, reappeared during germination (Fig. 5).

A considerable amount of endoplasmic reticulum was present in germinating spores (i.e. after incubation but before germ-tube formation) as well as in germinated spores (i.e. after germ-tube formation). It was closely associated with (Fig. 1*b*), and sometimes connected to, the nuclei. Golgi-like bodies were common and consisted of either single or up to three cisternae. Certain unit-membrane-bounded electron-dense structures were common in germinating spores and germ tubes (Figs. 1*b*, 5). Microbodies were also common (Figs. 2*a*, 4).

Multivesicular bodies and vesicles were present in germinating as well as germinated spores. In both, ribosomes were common (Figs. 2*a*, 5), either free in the cytoplasm or attached to the cisternae of rough endoplasmic reticulum. Microtubules were seen occasionally (Fig. 3*d*).

In spores fixed with potassium permanganate, a light-staining zone was visible between the inner layer of the spore wall and the plasma membrane (Fig. 4). A similar zone was seen by Hawker *et al.* (1970), but they took it to be a continuation of the germ-tube wall. During germination, wall material was deposited locally underneath the spore wall at the place where a germ tube would emerge. The light-staining zone could be seen even in this area (Fig. 4). Because of this deposition it is assumed that pressure was exerted on all the wall layers outside this area. There was no thinning of the inner layer of the spore wall: the thickness remained the same in areas both with or without this new localized wall (Figs. 3*a*, 4). However, fibrils began to loosen on the outer side of this area (Fig. 3*b*) and the wall of the propagule started to break from the outside (Fig. 3*a*). The sporangial wall separated from the spore wall because of the pressure from within, and finally both walls broke. The new localized vegetative layer later formed the wall of the germ tube, which came out through the break in all the wall layers (Figs. 3, 5).

Germ tubes sometimes came out very close to one another and sometimes were widely separated. When several were formed there were localized strips of new wall material that could give an illusion of a complete wall layer in some sections.

Endoplasmic reticulum, Golgi-like bodies and vesicles were common in the area of incipient germ tubes (Fig. 3*a*, 4). The vesicles were small and appeared to fuse with the plasma membrane (Figs. 2*b*, 4). Though the vesicles were neither as large nor as abundant as those in the tips of growing hyphae (Grove & Bracker, 1970) they still appeared to be involved in the formation and growth of the germ tube as suggested by Bracker (1971) for *Gilbertella persicaria*.

The new vegetative wall layer was electron-dense and appeared to consist of microfibrils arranged sub-parallel to the spore periphery (Fig. 2*b*). It was not a chemically changed

Fig. 4. Near-median section through two incipient germ tubes. The cap-like deposits of wall material (between unlabelled small black arrows) forming the vegetative layer (VL) are restricted to the tips of germ tubes. Vesicles (V), Golgi-like bodies (G) and endoplasmic-reticulum cisternae are common in the spore protoplast in the vicinity of the germ tube. The vesicles appear to fuse with the plasmalemma (white arrows). No change in the thickness of the inner layer of the germ tube (ISL) is evident. Note a zone of greater electron transparency (large arrow head) between the plasmalemma and the wall layers. The sporangial wall and the interrupted layer have already broken away. Potassium permanganate fixation.

Fig. 5. Near-median section through one germ tube. The lipid droplets (L) are still present. The wall of the germ tube is continuous with the vegetative layer (VL) and distinct from the inner layer of the spore wall (ISW). Glutaraldehyde-osmium tetroxide fixation.

part of the inner layer, as no decrease in the thickness of the latter was noticed on its formation. Thus this layer appeared to be different from the inner layer of the spore wall, both in electron density and architecture.

This study shows that the germ-tube wall is a new entity completely different from the existing wall layers of the spore. Hawker *et al.* (1970) noticed a 'cap over the apex of the germ tube initial consisting of a thin layer of relatively electron-dense material'. The wall of the germ tube was 'clearly an extension of the original germ tube cap'. This 'could be a new wall layer', but its appearance was 'more consistent with a change in the chemical nature of the wall'. The deposits of new wall material at the tip of the incipient germ tube in *C. echinulata* corresponded with this cap, but because there was no change in the thickness of the inner layer of the spore wall on the formation of the vegetative wall (i.e. the 'cap') and the two differed in electron density as well as architecture, the vegetative wall could not be a part, or a chemically changed part, of the inner layer of the spore wall.

Dykstra's (1974) work is based on the study of germinated spores, making it impossible to see the early stages of germ-tube formation and to interpret the final structures correctly. His fig. 7 shows that the inner layer of the spore wall and the wall of the germ tube are different. In Dykstra's study, as well as that of Hawker *et al.* (1970), only two layers were reported in the wall of the propagule, while here it has been shown to have four distinct zones.

There are two other reports of localized vegetative-wall formation: during the germination of cysts of *Phytophthora palmivora* (Hegnauer & Hohl, 1973) and during conidial germination in *B. cinerea* (Gull & Trinci, 1971). As with *Cunninghamella*, earlier workers on *P. palmivora* (Hemmes & Hohl, 1971) and on *B. cinerea* (Hawker & Hendy, 1963; Buckley *et al.* 1966) did not see the deposition of a new wall during germination; further examples may well be encountered as incipient germ tubes are studied in greater detail.

The germination of fungal spores

Many ultrastructural studies of fungal spore germination have been carried out on several types of spores. The emphasis has been on the origin of the wall of the germ tube, together with other structural changes. There have been some conflicting reports, as in the germination of cysts of *P. palmivora* and conidia of *B. cinerea*. There have also been reports showing that spores belonging to species of the same form-genus, or to genera placed in the same class or subdivision of fungi, may produce their germ-tube walls in different ways. For example, in *Aspergillus oryzae* (Tanaka, 1966) the vegetative wall originates from the inner layer of the thick spore wall; in *A. niger* (Hawker, 1966) and *A. nidulans* (Border & Trinci, 1970) a new vegetative wall layer is laid down. In *Penicillium megasporum* (Remsen, Hess & Sassen, 1967) and *Penicillium chrysogenum* (McCoy, Girard & Kornfeld, 1971) the innermost layer of the conidial wall elongates to form the germ-tube wall. *Fusarium culmorum* (Marchant, 1966) is another conidial fungus in which a new vegetative wall is formed. Turning to basidiospore germination, the germ-tube wall in *Psilocybe* sp. (Stocks & Hess, 1970) is formed *de novo*; in *Coprinus lagopus*, on the other hand, the existing spore wall layer extends out to form the germ-tube wall (Heintz & Niederpruem, 1971).

The differences reported in the origin of the germ-tube wall of fungi classified in the same major taxon may possibly be due to problems with penetration of the fixatives and to the use of potassium permanganate which has been shown to be inferior to Kellenberger's medium or aldehyde fixatives in showing the wall layers in germinating spores (Border &

Trinci, 1970; Gull & Trinci, 1971). I agree with Border & Trinci (1970) that a better fixative should be used before deciding whether a new wall layer is laid down. It is possible that all spores on germination produce a vegetative wall, but that this may be a complete layer in some spores and a localized one in others.

The brief survey above shows that the formation of a new wall layer surrounding the entire protoplast of the germinating spore is not restricted to Mucorales as was suggested earlier (Bartnicki-Garcia, 1968; Hawker *et al.* 1970) but also occurs with some conidia and basidiospores. Localized formation of the vegetative wall is also not restricted to a particular type of spore; it occurs with a cyst in *P. palmivora*, a conidium in *B. cinerea* and a sporangiospore in *C. echinulata*. Thus contrary to the suggestion of Dykstra (1974), no taxonomic significance can as yet be attached to the mode of germination. The evolutionary theory based on the mechanism of germ-tube wall formation (Hawker *et al.* 1970) should also be reconsidered.

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