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Detection of protein and carbohydrate in the extracellular matrix released by *Cochliobolus heterostrophus* during germination

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The extracellular matrix (ECM) of *Cochliobolus heterostrophus* (anamorph: *Bipolaris maydis*) was made visible by gold/silver and FITC-lectin staining at different stages of germ tube development. A proteinaceous material was released from conidia as germ tubes began to emerge and continued to be released from the germ tube tip throughout elongation. A material that did not stain for protein was observed to surround germ tubes upon their elongation. At later stages of maturation, germ tubes were surrounded by a sheath of proteinaceous material. After 15 h of incubation, staining with the FITC-labeled Concanavalin A revealed that a carbohydrate material surrounded and extended between hyphae. The ECM extract was separated into two fractions which were shown by SDS-PAGE and HPLC analyses to consist of proteins and carbohydrates. The results demonstrate that the composition and physical structure of the ECM change over time. Thus, the ECM is not a static material. Rather, the components of the ECM appear to be laid down at different stages of fungal morphogenesis, possibly related to germ tube emergence, elongation, and maturation.

Key Words——adhesion; *Bipolaris maydis*; *Cochliobolus heterostrophus*; germination; hyphal sheath.

The term extracellular matrix (ECM) is often used to refer to any material that is secreted or released from fungal spores either before, during, or after germination. However, because hyphal growth occurs from the tip of the hypha (Heath, 1990) the ECM can be expected to be associated with the entire mycelial thallus. Components of the ECM are sometimes considered to have a role in the fungal infection process (Nicholson and Kunoh, 1995). Initiation of the infection process for any fungal pathogen may involve recognition of the host surface, preparation of the infection court, adhesion, and infection structure differentiation. Because adhesion is an important phenomenon in the infection process, it is often thought that some components of the ECM function as adhesives (Nicholson, 1996; Nicholson and Epstein, 1991).

Evidence suggests that the ECM is a complex mixture of materials each of which has different functions (Nicholson, 1996; Nicholson and Kunoh, 1995). In *Colletotrichum graminicola* (Ces.) Wils., an ECM surrounds conidia within acervuli. This matrix is completely water soluble and includes high-molecular-weight glycoproteins (Ramados et al., 1985), low-molecular-weight materials such as the self-inhibitor mycosporine-alanine (Leite and Nicholson, 1992), and various enzymes (Nicholson, 1992; Pascholati et al., 1993). Such water-soluble ECM materials are known in some cases to be essential to the infection process and to the survival of the organism (Nicholson, 1992; Nicholson and Kunoh, 1995). However, water-soluble matrices are very different from materials assumed to be involved in adhesion, a condition that requires the adhesive material to be insoluble and to displace water (Epstein and Nicholson, 1997).

In *Cochliobolus heterostrophus* (Drechs.) Drechs. (anamorph: *Bipolaris maydis* (Nisikado & Miyake) Shoemaker), it is known that the emerging germ tube and hyphae are surrounded by a sheath of material which is composed of two layers (Braun and Howard, 1994; Evans et al., 1982). Our interest in the ECM and its role in fungal survival, adhesion, and the infection process led us to use *C. heterostrophus* as a system to investigate ECM structure and composition. Our initial attempts to isolate and characterize the ECM were frustrated by the relative insolubility of the materials. Therefore, we elected to first render the ECM visible in order to discern its appearance at various stages of fungal morphogenesis, from conidium germination to formation of elongated, interwoven hyphae. In the present investigation, we used a gold/silver stain procedure as well as stains with FITC-labeled lectins to demonstrate that protein and carbohydrate materials are components of the ECM. We also report the presence of an ECM component that surrounds hyphae and extends from one hypha to another.

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Materials and Methods

Fungus and culture procedures *Cochliobolus heterostrophus* was cultured on potato dextrose agar under constant fluorescent light (60 μE $m^{-2}s^{-1}$) at 21°C and transferred at 2-wk intervals. A modified Fries medium (Pringle and Scheffer, 1963) was added to the surface of 2-wk-old culture plates, and the conidia were suspended by scraping the surface of the culture with a sterile rubber policeman. The final conidial suspension, adjusted to 2×10^5 conidia/ml with Fries medium, was filtered through cheesecloth, and the suspension was used directly for each experiment.

Visualization of the extracellular matrix Conidia suspended in modified Fries medium were deposited onto the surface of precleaned, multiwell glass slides (Carlson Scientific, Peotone, IL) as required for individual experiments. Conidia were incubated at 23°C in a time study that ran for a period up to 15 h. Conidia were observed microscopically at 1-h intervals to evaluate changes in the ECM at different stages of morphogenesis.

To visualize the extracellular matrix that had been released from the germinating conidia, two staining procedures were used. In the first procedure, conidial germlings were stained with colloidal gold with silver enhancement (gold/silver) to detect the release of proteinaceous materials. A second visualization procedure was done by fluorescence microscopy with various fluorescein isothiocyanate (FITC)-labeled lectins for the detection of binding of the lectin to carbohydrate moieties.

The gold/silver stain procedure was a modification of the technique described by Jones et al. (1995) for staining extracellular fungal matrices. For microscopic observations, glass slides with conidia were washed three times for 10 min each with Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5), and then rinsed briefly three times in distilled water. Conidia on the slides were stained by submersion in 25–30 ml of a colloidal gold protein stain solution (Bio-Rad, Hercules, CA) for 1 h. The slides were then rinsed in distilled water, immersed for 5 min in 0.2 M citrate buffer (pH 3.7), then they were transferred to a silver enhancement solution (hydroquinone/silver lactate) according to the manufacturer's specifications (Bio-Rad). The slides were placed immediately in complete darkness, and silver enhancement was allowed to proceed for 4 min. (It should be noted that a longer period of silver enhancement resulted in a loss of structural detail due to overstaining). A fixing solution (Bio-Rad) was then added for 5 min to stop the enhancement reaction. The slides were washed again in distilled water, allowed to dry, and then observed and photographed by light microscopy. To optimize the detection of the stained proteinaceous material, specimens were mounted in water and a coverslip was applied. Specimens were first observed without a coverslip and it was verified that applying the coverslip did not move or alter the appearance of the stained proteinaceous material.

Cytochemical analysis with FITC-conjugated lectins was carried out by the method of Mercure et al. (1995). Conidia and conidial germlings were stained by the following procedure. Glass slides with associated fungal material were washed twice with 1 ml of 0.05 M glycine in phosphate-buffered saline (PBS, 50 mM potassium phosphate buffer, pH 6.8, 150 mM sodium chloride) to block nonspecific binding of the lectins (Lehnen and Powell, 1989). Specimens were then stained for 30 min in the dark with either FITC-ConA (*Canavalia ensiformis* concanavalin A agglutinin, sugar specificity α-D-mannose and α-D-glucose), FITC-WGA (*Triticum vulgaris* wheat germ agglutinin, sugar specificity *N*-acetylglucosamine), FITC-LCA (*Lens culinaris* agglutinin, sugar specificity α-D-mannose, α-D-glucose, *N*-acetylglucosamine), or FITC-BPA (*Bauhinia purpurea* agglutinin, sugar specificity *N*-acetylgalactosamine) at a concentration of 200 μg of FITC-lectin per ml of PBS. Slides were removed from the lectin solution, rinsed with PBS, and coverslips were applied with PBS as a mounting medium. Specimens were observed and photographed by epifluorescence microscopy (beam splitter DM500, exciter filter BP490 peak excitation 490 [range 400–490], barrier filter 0515) with an Olympus Vanox microscope. Experimental controls to ensure that lectins bound only to their respective haptens were carried out in which each FITC-lectin (400 μg/ml) was mixed with an equal volume of a 400 mM solution of the known competitive hapten carbohydrate. The absence of fluorescence indicated that there was no nonspecific binding to other haptens.

Isolation and characterization of the extracellular matrix Glass Petri dishes were cleaned by washing with Alconox laboratory detergent (VWR Scientific Products, West Chester, PA) and rinsed three times with double glass distilled water. Petri plates (200) on which conidia of *C. heterostrophus* (10 ml per plate of a suspension of 2×10^5 conidia/ml) had been deposited were incubated for either 3 or 15 h at room temperature. The plates were first washed briefly with distilled water to remove ungerminated, unadhered conidia. Approximately 1 ml of a 0.1% sodium dodecyl sulfate (SDS) solution was pipetted into each plate and the plates were brushed with a soft, camel hair paint brush to remove fungal germlings and their associated extracellular matrices. A soft brush was used to ensure that conidial germlings would not be broken, a phenomenon that would result in the release of intracellular materials including proteins and organelles. Observations by light microscopy confirmed that conidial germlings were not disrupted. The entire procedure was performed at room temperature, and each plate was brushed immediately after addition of the SDS solution to remove fungal germlings. The germling/matrices mixture was then centrifuged (3,000 rpm, 5 min) to separate the ECM from the fungal germlings. The supernatant representing the ECM fraction was concentrated 20-fold in a dialysis membrane (1,000 MW exclusion limit) with polyethylene glycol (PEG, 20,000 MW) at room temperature. The final concentrate, approximately 10 ml, was then reduced to 1 ml in a Speed Vac Concentrator (Savant, Hicksville, NY). The preparation is referred to as the ECM extract.

Gel electrophoresis of the ECM extract Electrophoresis of the ECM extract was conducted with SDS polyacrylamide gels (SDS-PAGE). Separation of denatured proteins was accomplished with a 12% or 7.5% separating gel (7×8 cm×1.0 mm) and a 4% stacking gel run at 100 V (12% gel) or 60 V (7.5% gel) until the tracking dye reached the bottom of the gel. The sample buffer, a modified Laemmli buffer (Laemmli, 1970), contained 0.0625 M Tris-HCl buffer (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.05% bromphenol blue as a tracking dye. The samples were incubated at 100°C for 3 min in an equal volume of sample buffer, and 40 μl was loaded into each lane. Gels were stained for proteins by the silver stain method (Bio-Rad). Protein molecular weight standards were also from Bio-Rad.

Carbohydrate analyses of the ECM extract ECM extract that had been prepared by the brushing procedure and reduced to a volume of 1 ml was separated into two fractions upon centrifugation (10,000 rpm, 2 min), an upper layer of less dense material, and a more dense material that pelleted. The less dense material was stained with FITC-labeled ConA and WGA and observed by bright field and fluorescence microscopy. Both the low and high density materials were analyzed for carbohydrate content by HPLC as described by Yadav et al. (1994).

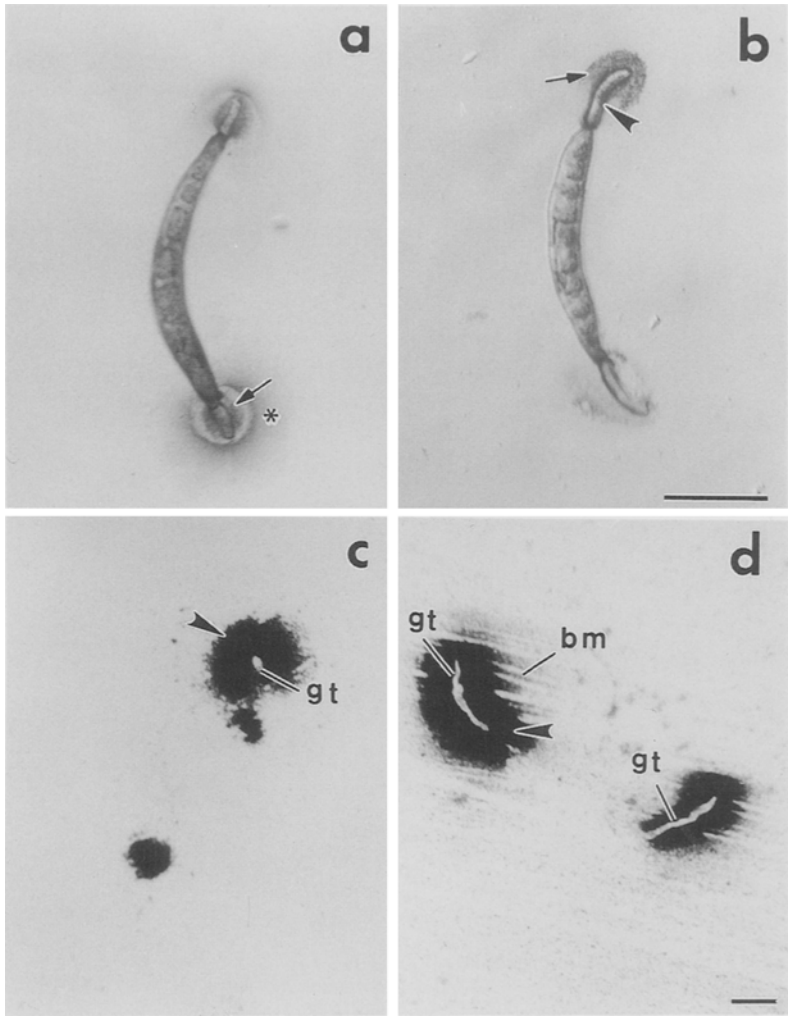

Fig. 1. Gold/silver staining for protein in the extracellular matrix (ECM) released from conidia of *Cochliobolus heterostrophus* upon germination.
a. After incubation for 1 h on a glass substratum a proteinaceous material surrounded the emerging germ tube. Two darkly stained zones were observed. The first zone (arrow) was immediately adjacent to the emerging germ tube. This was separated from the second zone (asterix) by a zone that was either only slightly stained or was not stained. b. After incubation for 2 h, a germ tube (arrowhead) had emerged from a distal cell of the conidium and was surrounded by a densely stained proteinaceous material (arrow). Bar for a and b=50 μm. When the staining procedure was performed after conidial germlings were removed by brushing after incubation for 1 h (c) or 2 h (d), the proteinaceous ECM material remained attached to the surface of the substratum. Marks made by the brush (bm) are evident in d. Sites where germ tubes had been present are indicated by gt (c, d). Bar for c and d=10 μm.

Results

Detection of the ECM during various stages of germination One hour after inoculation onto the glass substratum, gold/silver staining demonstrated the release of a proteinaceous material at the tips of conidia where germ tubes were emerging (Fig. 1a). As shown in Fig. 1a, distinct zones of stain were observed, two darker zones separated by a zone that was either only slightly stained or was not stained. The first stained zone was immediately adjacent to the emerging germ tube. After 2 h, these zones seemed to disappear and were replaced by a single zone of stain that surrounded the elongating germ tube (Fig. 1b). (It should be noted that, although the

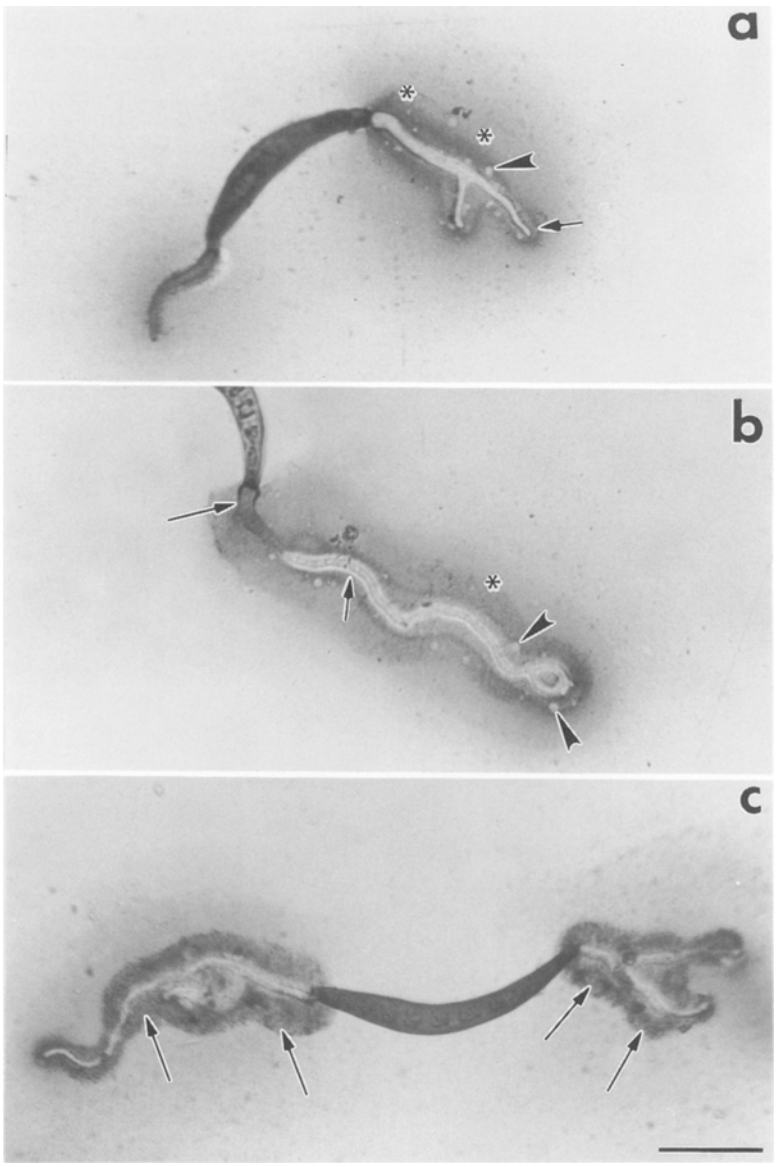

Fig. 2. Gold/silver staining for protein in the extracellular matrix (ECM) released from conidia of *Cochliobolus heterostrophus* at various times during germ tube elongation.
a. Conidium incubated for 3 h and stained for the presence of extracellular protein. The germ tube was surrounded by a sheath of material that stained positively for protein (asterix). A zone of more intense staining was observed at the tip of the germ tube (arrow). A material that did not stain, or that stained only faintly for protein appeared as spots that were interspersed along the length of the germ tube (arrowhead). b. Conidia incubated for 5 h and stained for protein. A sheath of material that stained positively for protein surrounded the germ tube (asterix). The oldest portion of the germ tube was stained heavily for protein (large arrow). A material that did not stain for protein, or that stained only faintly, appeared as spots along a portion of the length of the germ tube (arrowheads). An apparently unstained zone was present along the length of the germ tube (short arrow). c. A conidium incubated longer than 5 h and stained for protein. A sheath of material that stained for protein surrounded the germ tubes (arrows). Bar = 50 μm.

proteinaceous material around the lower germ tube in Fig. 1b was not stained as intensely as that around the upper germ tube, complimentary experiments showed that it would have been stained had silver enhancment been carried out for a longer time). Conidia were easily removed from the substratum by the brushing procedure; however, staining of the brushed substratum revealed that, although the fungus had been removed, material that stained for protein remained on the glass surface at both the 1 and 2 h time intervals (Figs. 1c, d). In this experiment, the silver enhancement was performed for a longer time to allow for maximal visualization of the proteinaceous material.

When germ tube elongation had proceeded for a longer time, the staining procedure revealed that the germ tube was surrounded by a proteinaceous sheath of material (Fig. 2). After 3 and 5 h of germination, the sheath stained intensely at the tips of germ tubes (Fig. 2a). In addition, there were circular spots that were either not stained or only lightly stained. These spots typically were found along the length of the germ tube and interspersed within the proteinaceous sheath material (Figs. 2a, b). Often the sheath stained more intensely around the oldest portion of the germ tube, which had

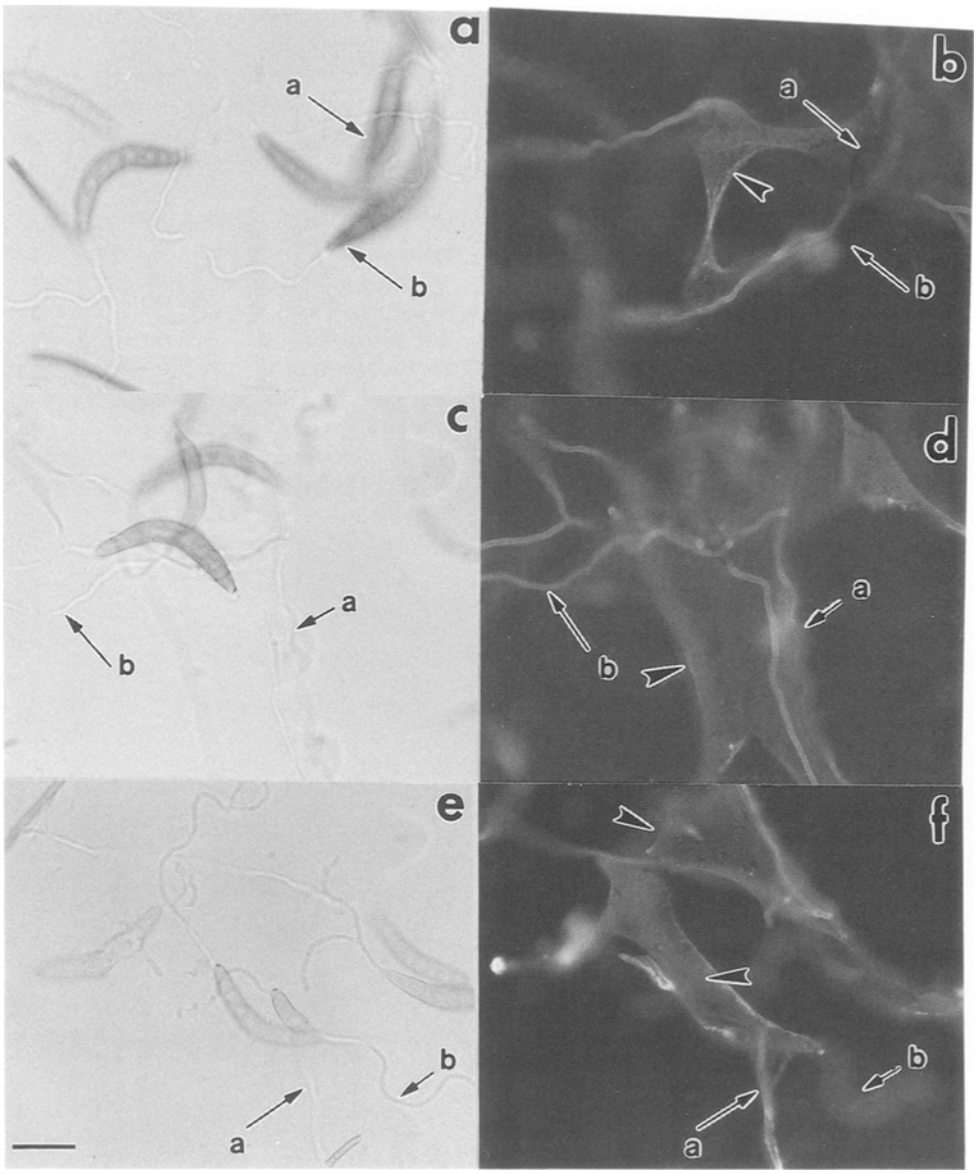

Fig. 3. Carbohydrate staining with FITC-ConA to reveal components of the ECM.
When stained with FITC-ConA, a material not observed by bright field microscopy was easily visualized by UV fluorescence microscopy (compare a with b, c with d, and e with f). In some places, the material appeared as a film (arrowheads) that extended between hyphae (b, d, f). The arrows a and b in each figure point to the same structures, either conidia or hyphal germ tubes. Bar = 50 μm.

emerged from the conidium first (Fig. 2b). After 5 h of mycelial development, it appeared that the sheath was composed of zones that stained differently, an inner zone immediately adjacent to the wall of the germ tube that stained very lightly (or not at all) and a broad outer zone that appeared to consist of layers that stained with different intensities (Fig. 2b). When germ tube elongation had proceeded for a longer time, e.g., >5 h, the staining procedure revealed an apparently uniform sheath of material that surrounded the entire germ tube (Fig. 2c).

Staining with FITC-labeled lectins Conidial germlings were stained with various FITC-labeled lectins to ascertain whether carbohydrate was associated with the sheath material. Components of the ECM stained with the FITC-labeled lectins ConA and WGA but did not stain with either the FITC-labeled BPA or LCA.

Staining with FITC-ConA Conidia were allowed to germinate for 15 h, stained with FITC-ConA, and observed microscopically. Extensive growth of the fungus occurred with germ tubes often entangled and growing in close proximity (Figs. 3a, c, e). Observation of specimens by fluorescence microscopy revealed that some of the ECM appeared as an amorphous sheet of material that surrounded and spread between hyphae (compare Figs. 3a with 3b, 3c with 3d, and 3e with 3f). This ECM component was only observed by staining with FITC-ConA.

Staining with FITC-WGA Figure 4-a shows a conidium with elongated germ tubes at 15 h after inoculation. Staining with FITC-WGA revealed that the lectin bound to components that ensheath and surround the germ tubes close to where they emerge from the conidium (Figs. 4b, c). Some stained material appeared amorphous with little evidence of structure, whereas the lectin also bound to materials that give the impression of distinct spots. It is significant that the FITC-WGA labeling revealed that material that surrounded the entire germ tube and extended outward toward the tip of the germ tube. This pattern of staining did not occur when FITC-ConA was used.

Analysis of the ECM extract The ECM extract was separated into two components, one less dense than the other. At times earlier than 3 h, the less dense material was not detected. Microscopic observation of the less dense ECM component revealed amorphous sheets of material with no consistent structure (Figs. 5a, d). The sheets stained with both FITC-ConA and FITC-WGA, each giving the same pattern of fluorescence. Staining with both lectins often appeared as small areas of intensely bright spots (Figs. 5b, c), whereas other areas of the sheet-like materials stained in a manner that made them appear as diffused, fluorescent films (Figs. 5e, f).

This material was also analyzed for proteins by SDS-PAGE and for carbohydrate content by HPLC analyses (Yadav et al., 1994). SDS-PAGE of the amorphous, less dense fraction of the ECM extract revealed the presence of two distinct protein bands with molecular weights of 64 and 72 kDa (Fig. 6). In addition, there was an apparent band of high-molecular-weight material that just barely migrated into the gel. Carbohydrate analyses of the less dense fraction of the ECM extract revealed the presence of the neutral sugar residues rhamnose, arabinose, galactose, glucose, and mannose, plus an unidentified uronic acid (Fig. 7).

Electrophoresis of components derived from the high density material precipitated from the ECM revealed the presence of proteins. Material isolated at 3 h showed a diffuse band at about 200 kDa (Fig. 8, lane b). For the

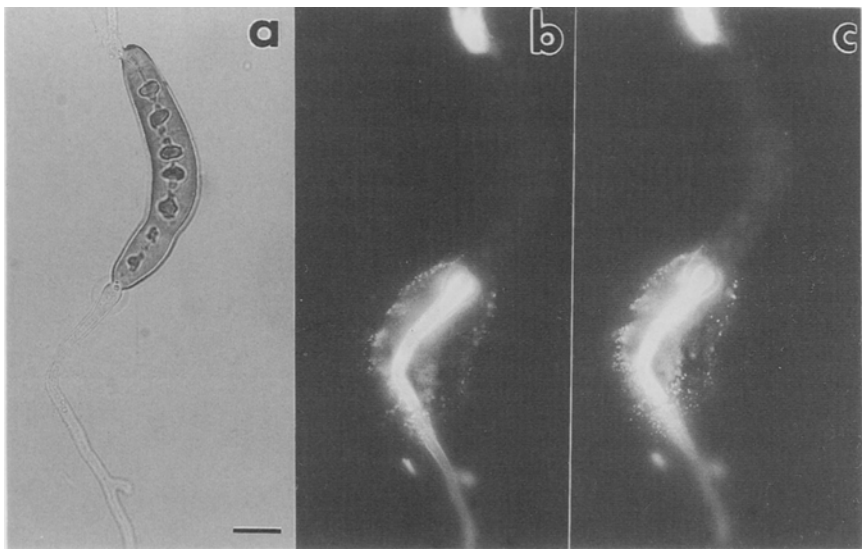

Fig. 4. Carbohydrate staining with FITC-WGA to reveal components of the ECM.
a. Visualization of a germinated conidium by bright field microscopy. b and c. Staining of the ECM materials on the same conidium as shown in a) when observed by UV fluorescence microscopy. Figures b and c show two different focal planes of the same germling and reveal that some of the material was amorphous and some appeared as distinct spots of fluorescence. Bar = 10 μm.

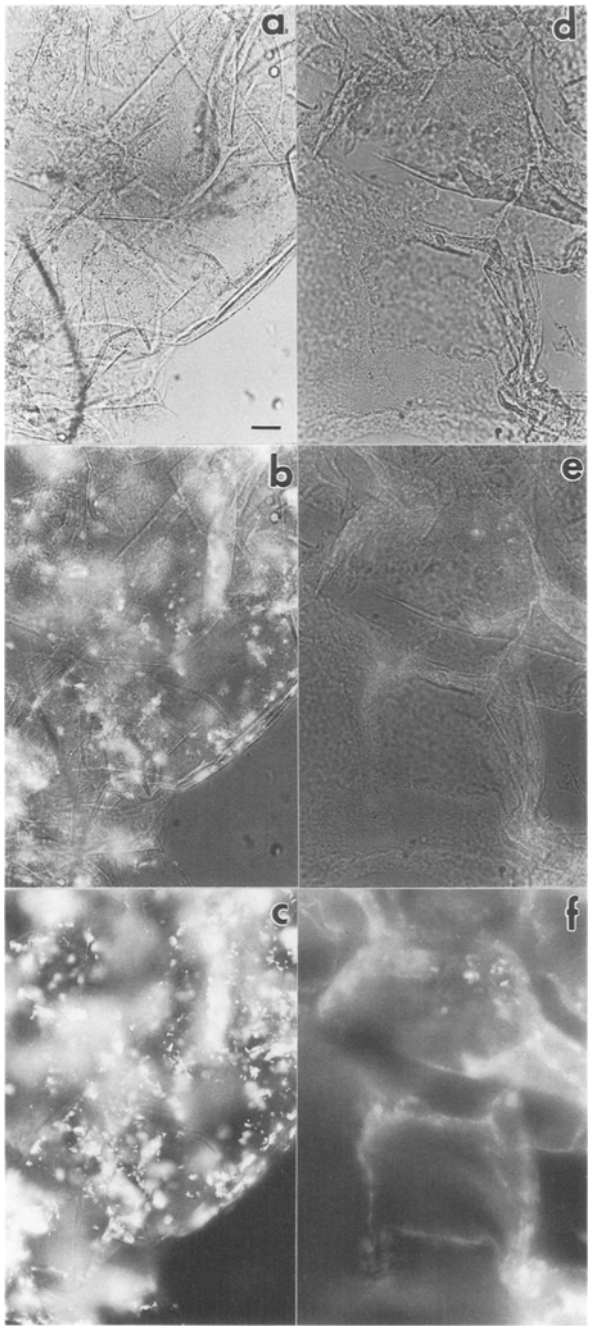

Fig. 5. Microscopic observation of the 15 h less dense fraction of the ECM extract.
The extract was stained with FITC-ConA (a, b, c) or with FITC-WGA (d, e, f). When viewed by brightfield microscopy the extract appeared as sheets of an amorphous material (a, d). When viewed by a combination of UV and brightfield light the material showed areas which stained as a sheet of diffused fluorescent material, sometimes with fluorescent spots (b, e). When viewed only with UV light, the material appeared as amorphous, fluorescent films (c, f). Bar = 50 μm.

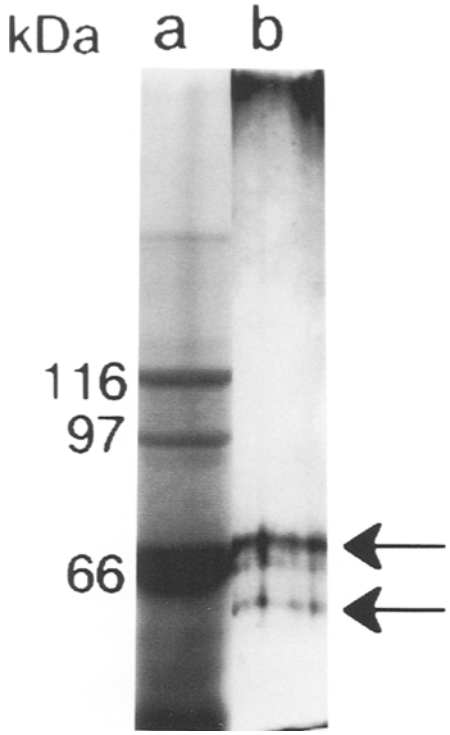

Fig. 6. SDS-PAGE in a 7.5% running gel of the less dense fraction isolated from the ECM extract.
Lane a, molecular weight markers, β-galactosidase (116 kDa), phosphorylase B (97 kDa) and bovine serum albumin (66 kDa). Lane b, arrows indicate the two protein bands with molecular weights of 64 and 72 kDa.

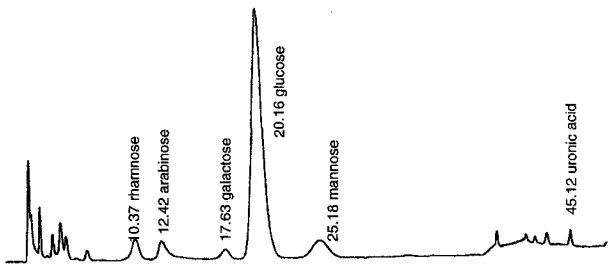

Fig. 7. HPLC analysis of the carbohydrate composition of the less dense material isolated from the ECM extract.
Analysis was by the method of Yadav et al. (1994). Values beside peaks represent retention times. Peak size is a measure of relative abundance of the compound.

ECM extract isolated at 15 h, numerous bands were detected, including a high-molecular-weight band similar to that observed in the extracts from 3 h (Fig. 8, lane c). When the 15 h, high density ECM fraction was analyzed for carbohydrate, the same sugars were found as the less dense ECM fraction, but apparently in lower quantity (data not shown).

Discussion

The existence of sheaths around germ tubes and hyphae

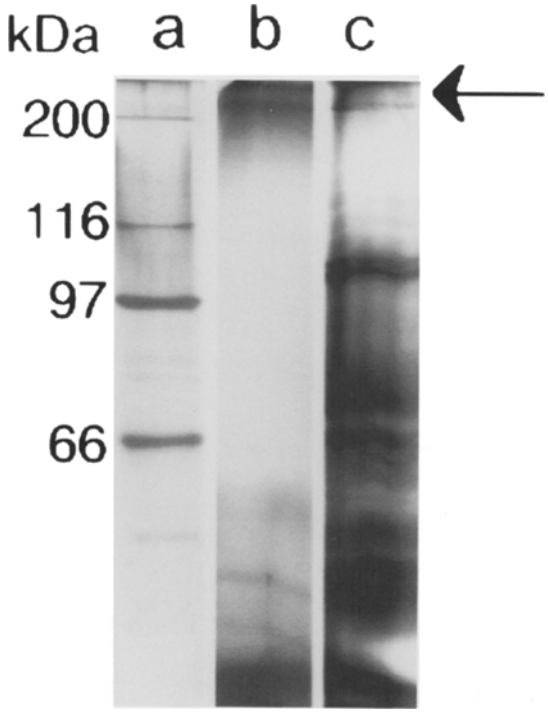

Fig. 8. SDS-PAGE in a 12% running gel of the high density fraction isolated from the ECM extract.
Lane a, molecular weight markers, myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa) and bovine serum albumin (66 kDa). Lane b, material isolated at 3 h. Lane c, material isolated at 15 h. Arrow indicates a high molecular weight band at about 200 kDa.

of a variety of fungi is well established (Hau and Rush, 1992; Nicholson and Epstein, 1991; Palmer et al., 1983a, b). The pathogen *C. heterostrophus* also produces an extensive sheath, which was shown by Evans et al. (1982) to be composed of two layers. Braun and Howard (1994) also presented evidence that the sheath, or extracellular matrix, of *C. heterostrophus* is composed of two layers.

The results of the present investigation suggest the possibility that the ECM is composed of more than two layers which include proteins and carbohydrates. Layers with different intensities of protein stain are evident in the ECM, and their appearance seems to depend on the stage of morphogenesis of the germ tube or hypha. The ECM materials are released at different times (germ tube emergence vs. germ tube elongation) and have different physical appearances (Fig. 1 vs. Fig. 2), suggesting that the ECM materials may have different functions.

Braun and Howard (1994) showed that adhesion of *C. heterostrophus* germlings was correlated with the release of an ECM from conidia just prior to germ tube emergence. We suggest that the proteinaceous ECM that is released with the onset of germination (Fig. 1) is the same material described by Braun and Howard (1994) and that it functions as an adhesive. Evidence for this is that, although germlings could be removed from the surface, the proteinaceous material remained attached to the substratum (Figs. 1c, d).

Evans et al. (1982) used scanning electron microscopy with *C. heterostrophus* and demonstrated what they referred to as a ragged sheath of material that surrounded hyphae but was observed only infrequently. Similar observations were made by Palmer et al. (1983a, b) on the fungi grouped as white and brown-rotters and involved in wood decay. They used reflectance light microscopy to demonstrate an extensive sheathlike material that surrounded and connected hyphae. In the present investigation, we have demonstrated that a component of the ECM was observed only after 15 h of hyphal growth and only after staining with FITC-ConA (Fig. 3). The material not only surrounded hyphae but also extended between hyphae and sometimes appeared to extend over great distances (Fig. 3). With the staining procedure used, the material appeared to be translucent. How this ECM component develops, contacts, and extends between hyphae is unknown. It is possible that its formation is the simple result of physical contact of ECM materials of one hypha with those of another. ConA binds to the material; thus, it must contain glucose and/or mannose residues. Whether this ECM component is the same as the sheath materials observed by Evans et al. (1982) and Palmer et al. (1983a, b) is unknown.

The material that was isolated and referred to as the ECM extract was divided into dense and less dense components by centrifugation. The less dense fraction was shown to be composed, at least in part, of proteins with molecular weights of 64 and 72 kDa, and of carbohydrate, mainly glucose. Whether this fraction is associated with the ECM material that bound with ConA and appeared to surround hyphae is unknown. Analysis of the dense ECM fraction for protein content by SDS-PAGE showed that the protein composition of the fraction changed depending on when the fraction was isolated. Thus, isolation after 3 h of incubation revealed that the primary component was a very high molecular weight protein (>200 kDa) (Fig. 8, lane b). In contrast, when conidia were allowed to germinate for 15 h, several lower molecular weight proteins in addition to the 200 kDa material were revealed by SDS-PAGE (Fig. 8, lane c).

The results of this investigation demonstrate that the ECM is not a static material. Rather, its composition and physical structure change over time. We suggest that the components of the ECM are laid down at different stages of fungal morphogenesis, possibly related to germ tube emergence, elongation, and maturation. Thus, to successfully isolate and chemically characterize the different components of the ECM, it will be necessary to carefully define the time when each component is produced and to develop techniques required for its isolation.

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