Structure of the Cell Wall of *Pythium debaryanum*

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The structure of hyphal walls of *Pythium debaryanum* was investigated by electron microscopy of shadowed replicas and thin sections, before and after digestion by snail gut enzymes or by 1 N HCl at 100 C for 1 hr, and by X-ray diffraction. We found that the wall had two phases, one composed of microfibrils of unknown composition and a second consisting of an amorphous matrix, part of which stained like protein with potassium permanganate and part of which was removed by snail-gut enzymes. In the microfibrillar phase, there were two layers; an outer, thicker layer of randomly disposed microfibrils and an inner, thin layer of microfibrils oriented parallel to the hyphal axis. As in *Neurospora crassa*, the amorphous phase included a branching system of pores, 40–80 A in diameter. Unlike *N. crassa*, the cytoplasm of *Pythium* showed Golgi bodies frequently, and many lomasomes were observed between the cytoplasmic membrane and the wall. The relations between these organelles and the mechanism of wall formation in *Pythium* are not understood.

In a recent study of the structure and composition of the cell wall of *Neurospora crassa* (8), we confirmed earlier findings (13) that one of the two phases of this wall consists of randomly disposed microfibrils of chitin. We also found that the second phase of this cell wall was composed of an amorphous matrix containing both β, 1–3 glucan(s) and protein(s). A portion of the protein fraction was associated with a system of branching pores, 40 to 70 A in diameter, which opened into the medium and also approached the cytoplasmic membrane. If such pores are a general feature of fungal walls, they may be important in the transport of large molecules (14). Therefore, we wanted to determine if the cell wall structure (including the pores) of a cellulose-containing fungus is similar to that of *N. crassa*. *Pythium debaryanum* Hesse was selected for study because evidence indicated that the wall of this organism is composed entirely of cellulose (4).

**Materials and Methods**

Cultures of *P. debaryanum* Hesse (ATCC no. 9998) were grown in 125-ml Erlenmeyer flasks containing 30 ml of mycological broth (Difco), supplemented with 0.5% yeast extract and 0.5% malt extract, for 4 to 5 days at 20 to 25 C. The mycelium was removed and washed with distilled water over a stainless-steel sieve until free of the nutrient medium. Fungal hyphae were either fixed immediately for examination in the electron microscope or were treated further to isolate the walls from the cytoplasm. In either case, the methods were the same as those described previously for *N. crassa* (8). Procedures for electron microscopy or X-ray diffraction were exactly as described before (8), except for the preparation of fibers composed of hyphae. In this instance, small pieces of thoroughly washed mycelium were slowly stretched in one direction until a portion of the mass formed a thread in which the hyphae were fully extended and parallel to each other. The threads formed in this way were then allowed to dry at room temperature to form a coherent bundle of parallel filaments suitable for mounting in the X-ray diffraction apparatus.

**Results**

Intact hyphal walls. After permanganate fixation alone, the appearance of the cell wall of *P. debaryanum* was similar to that of *N. crassa* (Fig. 1). A zone, 0.1 to 0.3 μm wide, having a frayed exterior edge was observed outside the plasmalemma. Within this zone, there was little structure, with the exception of some fibers which had the same appearance as the fibers first observed in the wall of *N. crassa* B110 by Shatkin and Tatum (13). As with *N. crassa* (8), prior fixation with glutaraldehyde and then KMnO4 made these fibers more clearly visible (Fig. 2). In some places, the fibers were resolved into segments of two parallel or curved lines with a space between them. The segments varied in
Fig. 1. Transverse section of the wall of an intact hypha of *Pythium debaryanum* fixed by KMnO₄. Note the indefinite boundary of the outer edge of the wall and the indications of fibers in the wall (arrows). Unless stated otherwise, all magnification markers represent 0.1 μ.

Fig. 2. Transverse section of the wall of an intact hypha after fixation by glutaraldehyde and then KMnO₄. Note the segments of two parallel or curved lines with a space between them (pores), the occasional doughnut shaped objects which may be cross sections of pores (circles), and the examples of branching of the pores (squares). To display wall structure more clearly, the cytoplasm was overexposed.

Fig. 3. Transverse section of the wall of an intact hypha, showing four layers of different electron density within the wall. Note the thin inner layer of higher electron density.
length from 0.02 to 0.1 μ, and the outside edges of the lines were 40 to 80 A apart (Fig. 2). As in *N. crassa*, these lines appeared to be segments of a three-dimensional system distributed throughout the wall, since branching of the space between the lines occurred occasionally (Fig. 2). In addition, we found circular or elliptical structures which may be cross-sections of portions of this system. The remainder of the wall between the lines was uniformly granular, but, in some places, there were layers of different electron density across the intact hyphal wall (Fig. 3).

**Isolated hyphal walls.** Layers of different electron density were more prominent in thin sections of isolated, sonic-treated, fully extracted cell walls (8) which were fixed in KMnO4 (Fig. 4). These sections also showed the segments of two parallel lines with a space between them. The layers varied in width, electron density, or both; the cause of this variation is unknown. During sonic treatment, the wall ruptured longitudinally and sometimes the layers separated. The edges of the separated layers then curled inwardly (Fig. 5). In some places, the partial separation of a distinct inner layer from the remainder of the wall was observed (Fig. 6).

Treatment of the isolated, fully extracted cell walls with snail-gut enzyme (8) before fixation with KMnO4 swelled the tissue, separated the layers, and reduced the electron density of the granular matrix of the fragments (Fig. 7). If the action of this enzyme on *P. debaryanum* is similar to its action on *N. crassa*, then the amorphous portion of the wall probably contains one or more β-glucans. In contrast to the granular matrix, the electron density of the segments of lines was not reduced by the above treatment (Fig. 7). The failure of the enzyme to affect the segments is consistent with the appreciable protein content of the boundaries of the pores, as in *N. crassa*.

Replicas of isolated, sonic-treated and extracted walls showed a uniform outer surface with regularly dispersed granules (Fig. 8). Replicas of the outer surface of walls digested by snail-gut enzyme revealed a network of randomly dispersed microfibrils (Fig. 9). When the walls were extracted by use of 1 N HCl at 100 C for 1 hr, we discovered similar microfibrils arranged in the same way, but the use of hot 17% NaOH for 1 hr had little effect. In contrast, microfibrils on the inner side of the walls were oriented parallel to each other and to the cell axis (Fig. 10 and 11). These microfibrils formed only a thin layer (1 to 2 threads in depth) within the wall, and, under prolonged sonic vibration, the fragments separated as long bundles (Fig. 12).

By use of methacrylate as an embedding medium, we demonstrated that there are two distinct layers in the wall containing microfibrils of different thicknesses and orientations. Methacrylate was removed from transverse sections of the hyphal walls which had been digested with snail-gut enzyme by ethylene dichloride, and the residue was shadowed with palladium-gold (Fig. 13). A thick mat, consisting of portions of randomly oriented microfibrils surrounded by amorphous material, was on the outside of the wall, and a single layer of short segments of approximately parallel microfibrils was on the inner side. Cell walls from 5-week-old cultures did not increase their deposition of inner layers as compared to 5-day-old cultures. In confirmation of these observations, removal of the embedding medium from tangential sections showed only long randomly disposed microfibrils (Fig. 14).

**X-ray diffraction.** The X-ray diffraction patterns of isolated, sonic-treated fully extracted walls and of the material which had been digested three times (with 1 N HCl for 1 hr at 100 C) are shown in Fig. 15; patterns from Atlantic squid chitin and bacterial cellulose are also shown. The patterns from both samples were different from standard cellulose I or from chitin. Therefore, we were unable to confirm Frey’s report (4) that cellulose is present in the walls of *P. debaryanum*. The patterns reflect characteristic spacings of 2.6, 3.9, and 4.8 Å. Because the sharpness of the patterns increased as a portion of the amorphous matrix of the wall was removed by digestion with 1 N HCl (Fig. 15), the patterns were probably due to the microfibrillar component which remained (Fig. 9).

Since the inner layers of the microfibrils were so strongly oriented parallel to the axis of the hypha, we thought that it would be possible to demonstrate this orientation by an X-ray diffraction diagram of a bundle of hyphae. However, two attempts to accomplish this indicated that the orientation of the thin inner layer was not sufficient to be detected above the background imposed by the thicker, randomly disposed outer network.

**Cell wall formation and deposition.** With few exceptions, growth in fungi is apical, and new wall material is deposited at the tips (2). If this is true for *P. debaryanum*, then one may be able to detect evidence for deposition of microfibrils in the thicker, outer portion of the wall before deposition of the oriented inner layer. Accordingly, hyphal tips cut from growing colonies on agar plates were isolated. The tips were extracted with 1 N HCl at 100 C for 1 hr, and replicas were
FIG. 4. Transverse section of an isolated, sonic-treated fully extracted cell wall showing pronounced layering within the wall after fixation with KMnO₄. Note the segments of lines with spaces between them (arrows).

FIG. 5. Transverse section of a separated layer of the wall illustrating the tendency of such layers to curl up like a roll of papyrus.

FIG. 6. Transverse section of an isolated, sonic-treated fully extracted cell wall fixed in KMnO₄. Note the granular structure and the indications of pores (black arrows). Also note that the inner layer of the wall is almost separated from the remainder (white arrow).
FIG. 7. Transverse section of a separated layer from a hyphal wall after digestion of the hypha with snail-gut enzyme and fixation in KMnO$_4$. Note the swelling of the pores, the dispersed appearance of their boundaries (arrows), and the reduced electron density of the matrix.

FIG. 8. Replica of the outer surface of an isolated, sonic-treated fully extracted hyphal wall.

FIG. 9. Replica of the outer surface of an isolated, sonic-treated fully extracted hyphal wall after digestion by snail-gut enzyme. Note the randomly disposed micro-fibrils.
FIG. 10. Left side of photograph: replica of the inner surface of an isolated, sonic-treated fully extracted hyphal wall after digestion by 1 N HCl at 100 C for 1 hr. Note the extreme orientation of some of the microfibrils, as compared with the random disposition of microfibrils in the outer layer (right side). The cell wall fragment from which the replica was made was large enough to enable distinction between the outer and inner layers.

FIG. 11. Replica of another portion of the material used in Fig. 10 to illustrate the regular, parallel orientation of the microfibrils in the inner layer of the wall.

FIG. 12. Replica of a fragment of the inner layer of microfibrils, separated by sonic vibration. The dimension marker here represents 0.4 μ. 

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Fig. 13. Transverse section of a hyphal wall digested by snail-gut enzyme, after the removal of methacrylate from the section. Note the line of short parallel segments of microfibrils on the inner surface of the wall and the lack of any discernible orientation in the outer portion of the wall. The dimension marker represents 0.2 $\mu$m.

Fig. 14. Microfibrils from a tangential section of the outer layer of the wall of the same material as in Fig. 13. Note the lack of orientation of the microfibrils.
prepared as described previously (8). Replicas of untreated tips resembled those of isolated and extracted mature cell walls (Fig. 16). The outside of the acid-extracted tips showed a network of random microfibrils (Fig. 17) which was quite similar to that of mature hyphal walls. Torn tips which exposed the inner surface did not reveal any parallel microfibrils. Therefore, the inner layer of parallel microfibrils may not be formed at the tip but may be deposited later during the growth of the hypha.

Cell organelles. Thin sections of hyphae fixed in KMnO₄ exhibited the presence of Golgi bodies having numerous vesicles in the cytoplasm (Fig. 18). Occasionally, these vesicles touched the plasma membrane (Fig. 19a, b), possibly preparatory to crossing its surface, as in green plants (11). Sometimes the vesicles were observed between the cell wall and the plasma membrane (Fig. 20).

In addition to the frequent occurrence of Golgi bodies in the cytoplasm of P. debaryanum, numerous lamosomes were observed between the plasma membrane and cell wall (Fig. 21a, b). These vesicular structures may be groups of tubules, as illustrated by Fig. 21 c and d. Figure 22 indicates that these organelles may be produced in the cytoplasm, become enclosed in an envelope, and then migrate to the surface of the cells to discharge their contents.

**DISCUSSION**

We could not confirm the presence of cellulose in the cell wall of P. debaryanum. However, we found that, in general, the cell wall of this organism resembled that of N. crassa, since both possessed a two-phase system with similar morphology; i.e., microfibrils were embedded in an amorphous matrix which included both β-glucans and pores which were associated with protein. Although the presence of pores in the cell wall of P. debaryanum is interesting, it must not be overemphasized. We have no evidence that these pores are anything other than branching tubular channels (composed, at least in part, of protein) which may cross the cell wall and connect the space next to the plasmalemma with the external medium. In particular, there is no evidence that these pores represent a type of fine penetration of the cell wall by the cytoplasm. Finally, these structures are not small plasmodesmata. Bearing the above in mind, it is still interesting that these pores transport ferritin across the walls of N. crassa (8). If the pores are a general feature of fungal cell walls, such passages may be the means by which fungi are able to absorb large molecules from the external medium or to excrete other large molecules across their thick supporting shells (14). The study of the mechanisms and control of such transport presents a fascinating challenge to both biochemists and electron microscopists.

Nonetheless, despite the above similarities between Pythium and Neurospora, these fungi differ in the properties of the amorphous carbohydrates of their cell walls and in the composition and arrangement of their microfibrils. In Pythium, there are at least two distinct layers in the cell wall of mature hyphae. These layers differ in the arrangement of their microfibrils; the outer layer contains randomly dispersed microfibrils and the inner has a parallel longitudinal arrangement. In contrast, N. crassa possesses a cell wall which contains only a random arrangement of chitin microfibrils. Furthermore, the β-glucans of Pythium are less soluble in hot 17% NaOH than are the β-glucans of Neurospora. Finally, the microfibrils of N. crassa are composed of chitin (8, 13), whereas the microfibrils in the walls of P. debaryanum ATCC no. 9998 either gave no reflections or those which they gave were different from cellulose I or chitin (Fig. 15). Because Frey (4) did not publish the diagrams or the characteristic spacings he observed for P. debaryanum, it would not be profitable to speculate on the causes of the disparity between his general conclusion and our observations. However, the composition of the microfibrils and the...
FIG. 16. Replica of a surface of an untreated tip of a hypha. The dimension marker represents 0.3 μ.

FIG. 17. Replica of the surface of a tip digested by 1 N HCl at 100 C for 1 hr. Note the random network of microfibrils.
**FIG. 18.** Transverse section of a hypha fixed by KMnO₄. Golgi bodies (G) and associated vesicles (V) can be seen in the cytoplasm.

**FIG. 19.** Transverse sections of hyphae fixed in KMnO₄. These sections (a) show the approach of the vesicles to the plasmalemma, and (b) suggest that the vesicles may cross the surface of the plasmalemma (arrows).

**FIG. 20.** Transverse section of hypha showing vesicles between the plasmalemma and the wall.
Fig. 21. Transverse sections of hyphae showing lomosomes (arrows). Note the tubular structure of some of these organelles (c and d) and their position outside the cytoplasmic membrane.
matrix of the wall of *Pythium* presents an interesting biochemical problem.

The presence of two layers of wall having different orientations of microfibrils in mature hyphae and the absence of an inner layer at the tips of the hyphae may indicate that at least two different processes are responsible for the deposition of wall in *Pythium*. If, as in most fungi, hyphal wall extension in *Pythium* occurs solely through the apical deposition of newly synthesized wall material, this mechanism may be applicable to the formation of the thicker outer layer of the cell wall. After formation of this outer layer, the thin, longitudinally oriented inner layer may be deposited. Similar modes of extension and similar structures have been observed in the primary walls of differentiating tracheids of conifers, where growth is localized at the cell tips (15). However, the deposition of longitudinal microfibrils along the length of the wall immediately raises the question of the relation of cytoplasmic structure to such deposition. Ledbetter and Porter (7) suggested that microtubules beneath the surface of the plant cell protoplast somehow influence the orientation of the cellulose microfibrils of the adjacent cell wall. However, we were unable to observe any such microtubules.

In view of the frequent presence of Golgi bodies and lomasomes in *P. debaryanum*, this fungus seems to resemble the green algae more than any other member of *Mycota* (5). The role of Golgi bodies in the deposition of secondary wall material is now well supported (11), and it is tempting to suggest that, in *P. debaryanum* the vesicles associated with Golgi bodies fuse with the plasma membrane as a part of the process of contributing to the inner layer of the cell wall. The absence of an inner layer of parallel microfibrils in *N. crassa* may then be attributed to the absence of Golgi bodies. However, there is no direct experimental evidence to support the suggestion.

In addition to Golgi bodies, lomasomes are often present in the hyphae of *Pythium*. At one time, lomasomes were presumed to be exclusive to fungi (10), but recently they have been observed in green plants (6, 9). The function of these structures is not known, but Wilsenach and Kessel (16) suggested that (i) lomasomes may be associated with wall formation and (ii) there is a similarity between fungal lomasomes and Golgi body vesicles. Although Wilsenach and Kessel (16) believe that Golgi bodies may not be observed in fungi containing lomasomes, the above results for *Pythium* show that this opinion needs revision. Crawley (3) and Barton (1) have also published accounts of a cytoplasmic organelle associated with cell walls in *Chara* and *Nitella*, suggesting that this organelle has a function in

Fig. 22. Transverse section of a hypha. It appears that lomasomes may be produced in the cytoplasm and then may migrate to the surface of the plasmalemma.
wall formation. Finally, the apparent resemblance between lomasomes and the mesosomes which are associated with some aspects of the synthesis of bacterial cell walls (12) should be considered. The resemblance is certainly strong but may be fortuitous. The relationship between lomasomes and Golgi bodies in cell wall formation is not yet clear, but the existence of such a relationship seems probable.

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LITERATURE CITED