High-Resolution Cell Surface Dynamics of Germinating *Aspergillus fumigatus* Conidia

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ABSTRACT We used real-time atomic force microscopy with a temperature-controlled stage (37°C) to probe the structural and physicochemical dynamics of single *Aspergillus fumigatus* conidia during germination. Nanoscale topographic images of dormant spores revealed the presence of a layer of rodlets made of hydrophobins, in agreement with earlier electron microscopy observations. Within the 3-h germination period, progressive disruption of the rodlet layer was observed, revealing hydrophilic inner cell wall structures. Using adhesion force mapping with hydrophobic tips, these ultrastructural changes were shown to correlate with major differences in cell surface hydrophobicity. That is, the rodlet surface was uniformly hydrophobic due to the presence of hydrophobins, whereas the cell wall material appearing upon germination was purely hydrophilic. This study illustrates the potential of real-time atomic force microscopy imaging and force spectroscopy for tracking cell-surface dynamics.

INTRODUCTION

The human opportunistic pathogen *Aspergillus fumigatus* causes several important respiratory diseases, such as allergic bronchopulmonary and invasive aspergillosis. Although adhesion of the conidia to host cells is thought to be a primary event during the establishment of infection (1), the mechanisms underlying this process remain poorly understood. Electron microscopic and biochemical studies have shown that the outermost cell-wall layer of *Aspergillus* conidia is characterized by the presence of clustered proteinaceous microfibrils called rodlets (2,3). Rodlets are composed of hydrophobins, a family of small, moderately hydrophobic proteins characterized by the conserved spacing of eight cysteine residues. This hydrophobic character may be involved in several functions, including adhesion to host proteins and cells, dispersion by air currents, and protection against chemicals, enzymes, and phagocytic cells. Germination of *A. fumigatus* conidia is associated with a loss of hydrophobibcity. So far, however, tracking this cell surface dynamics in real time and at high resolution has not been possible.

In past years, atomic force microscopy (AFM) has been increasingly used for exploring the nanoscale surface properties of bacterial, yeast, and fungal cells (4). AFM imaging enables investigators to directly visualize fine surface structures on single hydrated cells (5,6). Notably, real-time imaging can be used to follow cell surface dynamics during cell growth and to monitor the effect of molecules like enzymes and drugs (7,8). In addition, AFM force spectroscopy allows one to measure the local physical properties of cell walls, like adhesion (5) and elasticity (9), and to detect single molecular recognition sites (10,11). Chemical force microscopy (12), in which AFM tips are modified with specific functional groups, enables researchers to map the spatial arrangement of chemical groups and their interactions on cellular surfaces (13,14). Here, we used real-time AFM with a temperature-controlled stage (37°C) to track the cell surface dynamics (structure, hydrophobicity) of *A. fumigatus* conidia during germination.

MATERIALS AND METHODS

The strain of *A. fumigatus* Dal CBS144-89 used in this study is a clinical isolate. Conidia were harvested from 1-week-old culture grown at 25°C on 2% malt extract agar. They were rinsed three times in 0.5% Tween 20 aqueous solution and five times in deionized water. For AFM, conidia were immobilized by mechanical trapping into polycarbonate membranes with 3-μm pore size (Millipore, Billerica, MA). After filtering a spore suspension (20 mL; 2.5×10^6 cells per mL), the filter was carefully rinsed in deionized water, cut (1 cm $\times$ 1 cm), and attached to a steel sample puck using a small piece of adhesive tape, and the mounted sample was transferred into the AFM liquid cell.

AFM contact mode images and adhesion maps were obtained in culture medium containing 22 g L^{-1} glucose (Sigma, St. Louis, MO) and 13 g L^{-1} nutrient broth (Oxoid, Basingstoke, UK) at 37°C using a Nanoscope IV Multimode AFM (Veeco Metrology Group, Santa Barbara, CA) equipped with a temperature-controlled stage. Images and adhesion maps were recorded at increasing incubation times, with the AFM tip withdrawn from the cell surface between each recording to minimize tip contamination and/or sample damage. Therefore, images and maps were not always recorded exactly at the same place on the cell surface. Oxide-sharpened microfabricated Si_3N_4 cantilevers with spring constants of 0.01 N/m were used (Microlevers, Veeco Metrology Group). Cantilevers were coated by electron-beam thermal evaporation with a 5-nm-thick Cr layer followed by a 30-nm-thick Au layer, then immersed for 14 h in 1-mM solutions of $HS(CH_2)_{11}CH_3$ in ethanol, and finally rinsed with ethanol.

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RESULTS AND DISCUSSION

Tracking structural dynamics in real-time

For in vivo, real-time imaging, conidia were immobilized by mechanical trapping in porous membranes. Fig. 1 shows a series of low-resolution AFM images of a single spore during germination in culture medium at 37°C. Due to the large curvature of the specimen, the height images (*left*) have fairly poor resolution, whereas the deflection images (*right*) are much more sensitive to the surface relief. Using small imaging forces (<0.1 nN), images of the same cell were obtained repeatedly without detaching the cell or altering significantly the surface morphology. The cell, located at the center of the image, was surrounded by a flat area representing the polymer membrane. In the deflection images, the cell border showed artifactual structures resulting from the contact between the AFM probe and the pore edges.

Images of the same spore after 20 min, 60 min, and 120 min clearly reveal significant swelling, the spore growing and protruding more and more from the pore. Spore growth also led to ultrastructural alterations, which were better visualized by recording high-resolution images on the spore surface (Fig. 2). At short germination times (0–60 min), the surface was covered with a crystalline-like array of rodlets ~10 nm in diameter, consistent with earlier electron microscopy data (15,16). Such regular arrays of rodlets are known to cover the surface of airborne dry conidia of all fungi (17). Notably, dramatic changes of cell surface structure were observed after 2 h germination corresponding to the swelling of the conidium, the rodlet layer changing into a layer of amorphous material, presumably reflecting the underlying old or neosynthesized layer. These nanoscale observations are in good agreement with previous structural and chemical studies, which showed that germination of *Aspergillus* spores results in the disruption of the proteinaceous rodlet layer and reveals inner spore walls that are essentially composed of polysaccharides (3). We note that streaks were seen in the scanning direction, suggesting that soft, loosely bound material was pushed away by the scanning tip.

Cell surface structure correlates with hydrophobicity

We then used spatially resolved force measurements with hydrophobic tips to probe the local hydrophobic character of the spore surface. Fig. 3 shows adhesion force maps recorded with a hydrophobic tip on a single germinating spore. Before germination started (0 min), the map showed bright contrast, 96% of the force curves showing strong adhesion forces of 2858 ± 1010 pN magnitude. Comparison with data obtained on organic surfaces (data not shown) indicates that the spore surface is markedly hydrophobic, corresponding to a surface

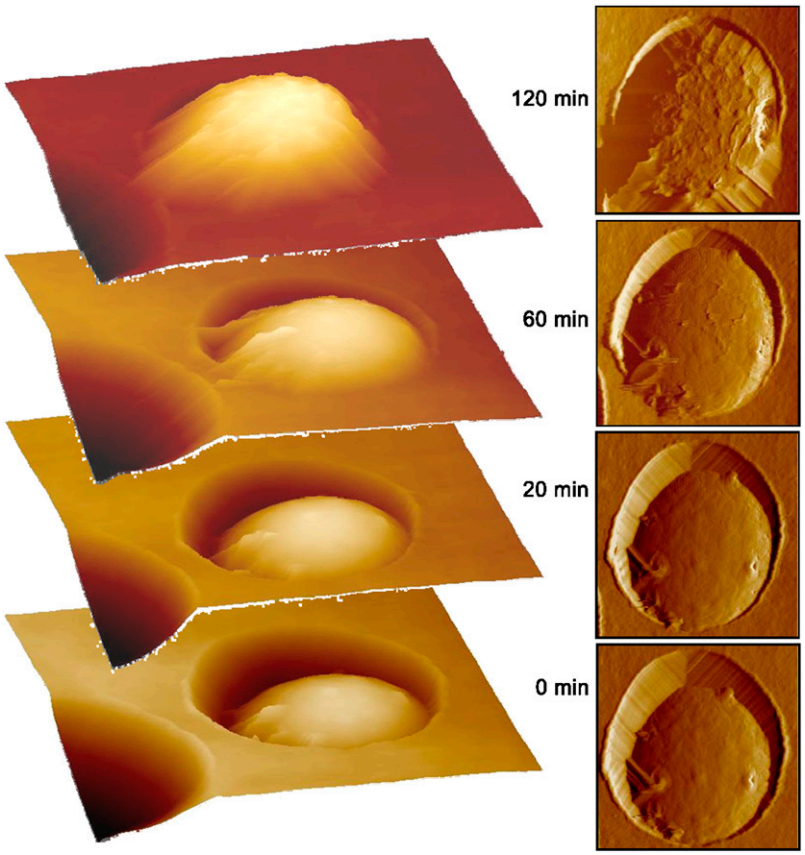

FIGURE 1 Real-time imaging of a single *Aspergillus fumigatus* conidium during germination. Series of height (6 μm × 6 μm) and deflection (4 μm × 4 μm) images in culture medium showing a single spore trapped into a pore, as a function of germination time. During germination, spore swelling and ultrastructural alterations were clearly noted.

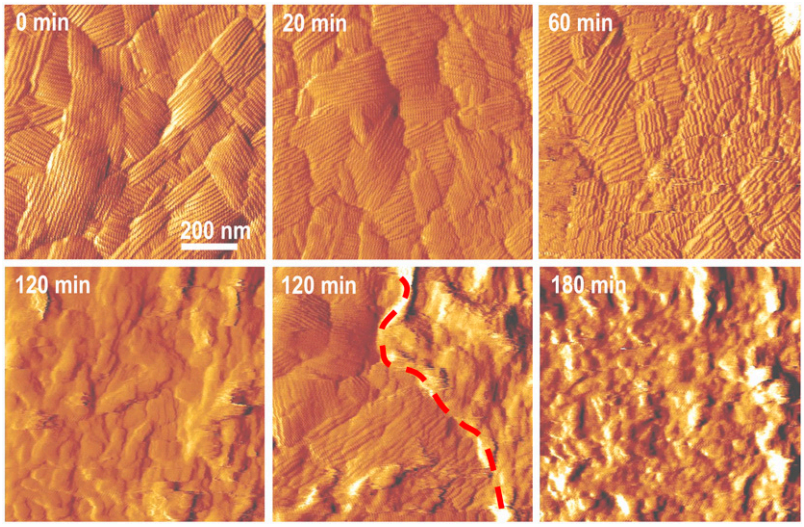

FIGURE 2 Structural dynamics of single germinating conidia. Series of high-resolution deflection images recorded on a single spore during germination. Within <3 h, the crystalline rodlet layer changes into a layer of amorphous material, presumably reflecting inner cell wall polysaccharides. After 2 h, both rodlet and amorphous regions were found to coexist (*lower middle panel, left* and *right, respectively*, of *dashed line*).

composed of 40% hydrophobic methyl groups, thereby confirming the hydrophobic character of hydrophobins. We also note the fairly homogeneous contrast, suggesting that the surface was uniformly hydrophobic.

Upon germination, the adhesion contrast became progressively darker and the number of nonadhesive events increased continuously (Fig. 4), indicating progressive loss of surface hydrophobicity. After 3 h, only 3% of the force curves showed adhesion events, indicating that the surface was uniformly hydrophilic. This finding, fully consistent with the ultrastructural data, points to the exposure of hydrophilic cell wall material that is either amorphous carbohydrates or proteins secreted during germination. Interestingly, after 2 h germination, the adhesion map was heterogeneous, with hydrophobic patches observed in a hydrophilic sea. Based on the heterogeneous surface morphology (Fig. 2), we believe this heterogeneous hydrophobic contrast reflects the coexistence of hydrophobic rodlets and hydrophilic polysaccharides. Thus, AFM is capable of resolving nanoscale structural and chemical heterogeneities on live fungal cells as they grow.

Finally, it is worth comparing our data with earlier results obtained on *A. oryzae* (17,18). We found that, as with *A. fumigatus*, the surface of dormant spores of *A. oryzae* is covered with a layer of rodlets, whereas the surface of germinating spores consists of soft granular material, attributed to polysaccharides. Despite these similarities, our work brings an important new breakthrough in that, for the first time to our knowledge, time-dependent structural changes could be observed on the same single growing cell owing to the use of a temperature-controlled stage. We also found that major changes of adhesion forces occur upon germination of *A. oryzae*: although the surface of dormant spores showed poor adhesion, germinating spores displayed strong adhesion

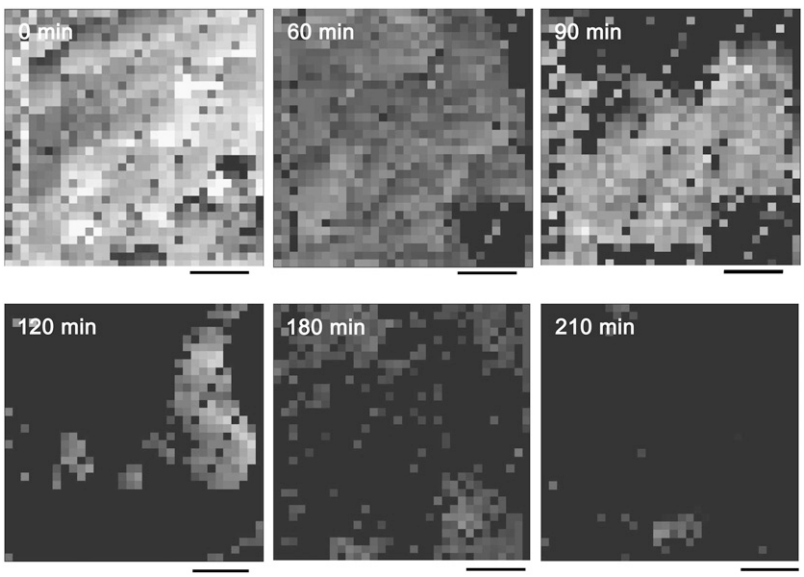

FIGURE 3 Structural dynamics correlates with differences in hydrophobicity. Series of adhesion force maps (scale bars, 100 nm; z range = 5 nN) obtained by recording 32 × 32 force curves using a hydrophobic tip on a single germinating spore. With time, substantial reduction of adhesion contrast was noted, reflecting a dramatic decrease of hydrophobicity. After 2 h, heterogeneous contrast was observed in the form of hydrophobic patches surrounded by a hydrophilic sea.

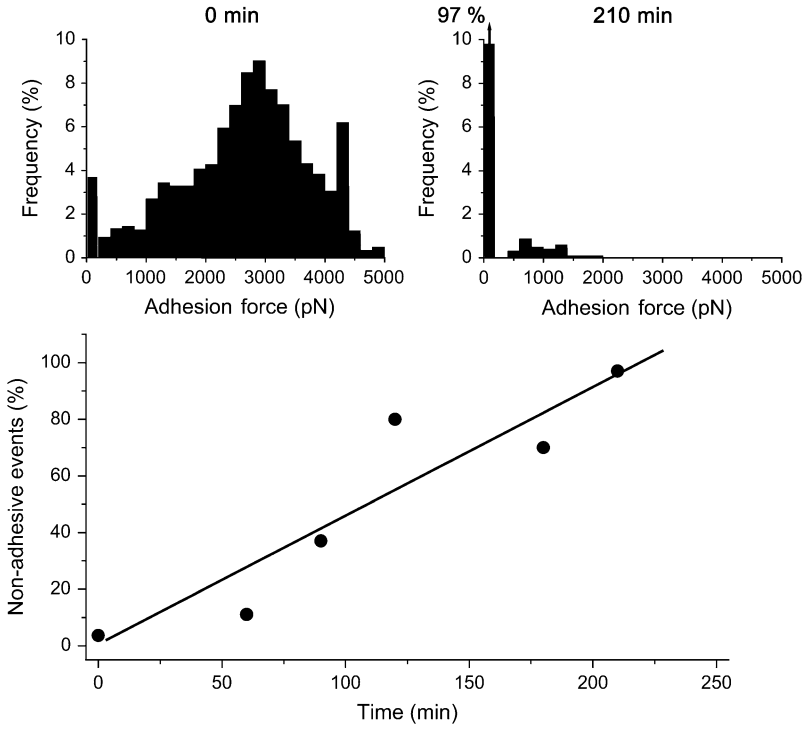

FIGURE 4 Evolution of surface hydrophobicity with germination time. (*Upper*) Adhesion force histograms obtained before germination started (*left*) and after 210 min germination (*right*). (*Lower panel*) Evolution of the number of nonadhesive events, showing the progressive loss of hydrophobicity as a function of germination time.

forces and elongation events, attributed to the stretching of long flexible polysaccharides. By contrast, here we show that germination of *A. fumigatus* conidia leads to a dramatic reduction of adhesion, reflecting a loss of surface hydrophobicity, whereas elongation events are never observed. These differences may reflect differences in cell wall properties as well as in tip chemistry since the *A. oryzae* and *A. fumigatus* studies were performed with silicon nitride and methyl-terminated tips, respectively. Unlike in this study, no conclusion could be drawn regarding the hydrophobic character of the *A. oryzae* surface due to the poorly controlled surface chemistry of silicon nitride tips. Finally, another novelty of this study is the ability to resolve nanoscale chemical heterogeneities in a time-dependent manner, using adhesion force mapping with modified tips.

CONCLUSION

Although AFM is being increasingly used in fungal research, this is the first time that structural and physicochemical dynamics are monitored in real time on a single germinating spore. The data show that dramatic changes of surface properties occur during germination of *A. fumigatus* conidia. Within a few hours, the hydrophobic rodlet layer changes into a hydrophilic layer of amorphous material, presumably cell wall polysaccharides. We believe the observed changes are function-related (19). As a matter of fact, the hydrophobic rodlets are likely to play a central role in promoting spore dispersion by air currents and in mediating adhesion to various surfaces, including host cells. By contrast, the very hydrophilic nature of the germ tube cell wall is expected to favor hyphal growth through moist environments and especially endothelia and epithelia (20).

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