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Surface characteristics of the entomopathogenic fungus *Beauveria* (*Cordyceps*) *bassiana*

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Marked differences in surface characteristics were observed among three types of single-cell propagules produced by the entomopathogenic fungus *Beauveria bassiana*. Atomic force microscopy (AFM) revealed the presence of bundles or fascicles in aerial conidia absent from *in vitro* blastospores and submerged conidia. Contact angle measurements using polar and apolar test liquids placed on cell layers were used to calculate surface tension values and the free energies of interaction of the cell types with surfaces. These analyses indicated that the cell surfaces of aerial conidia were hydrophobic, whereas those of blastospores and submerged conidia were hydrophilic. Zeta potential determinations of the electrostatic charge distribution across the surface of the cells varied from +22 to −30 mV for 16-day aerial conidia at pH values ranging from 3 to 9, while the net surface charge ranged from +10 to −13 mV for submerged conidia, with much less variation observed for blastospores, +4 to −4 mV, over the same pH range. Measurements of hydrophobicity using microbial adhesion to hydrocarbons (MATH) indicated that the surfaces of aerial conidia were hydrophobic, and those of blastospores hydrophilic, whereas submerged conidia displayed cell surface characteristics on the borderline between hydrophobic and hydrophilic. Insect pathology assays using tobacco budworm (*Heliothis virescens*) larvae revealed some variation in virulence among aerial conidia, *in vitro* blastospores and submerged conidia, using both topical application and haemocoel injection of the fungal cells.

INTRODUCTION

As an alternative to chemical pesticides, the entomopathogenic fungus *Beauveria* (*Cordyceps*) *bassiana* is currently under intensive study as a promising biocontrol agent for insects and other arthropod pests (Kirkland *et al.*, 2004a; Leathers *et al.*, 1993; McCoy, 1990). The range of target organisms spans the classes of the Arthropoda, and practical biocontrol applications have ranged from agricultural pests to human disease agent-carrying organisms such as mosquitoes, sand flies and ticks (Reithinger *et al.*, 1997; Samish *et al.*, 2004; Scholte *et al.*, 2005). The *B. bassiana* lifestyle is unusual in that in addition to being a necrotrophic parasite of a broad range of arthropods, it is a facultative saprophyte and can exist as a plant endophyte (Bing & Lewis, 1991, 1992). An opportunistic pathogen, *B. bassiana* does not require any specialized route of entry towards susceptible host targets, and an array of depolymerases (proteases, chitinases) and secondary metabolites participate in the infection process (Charnley & St Leger, 1991; El-Sayed *et al.*, 1993; Fuguet *et al.*, 2004; Kirkland *et al.*, 2005). Different infectious *B. bassiana* propagules can be isolated and selected for host targeting (Alves *et al.*, 2002; Kirkland *et al.*, 2004b; Thomas *et al.*, 1987; Viaud *et al.*, 1998). Thus, in addition to mycelial and hyphal growth, *B. bassiana* produces a number of mono-nucleated single-cell types, including aerial conidia, blastospores and submerged conidia, which can be isolated from agar plates, rich-broth submerged cultures and nutrient-limited submerged cultures, respectively (Boucias *et al.*, 1988; Holder & Keyhani, 2005). These cells display distinct morphological, biochemical and pathological characteristics, and attempts are being made to exploit these properties in pest targeting and/or the enhancement of virulence. Expressed sequence tag (EST) analysis of cDNA libraries derived from aerial conidia, blastospores and submerged conidia indicates robust and stage-specific gene-expression profiles among these cells (Cho *et al.*, 2006).

The varied cuticles of target organisms represent the initial barrier to the pathogen, and attachment of infective fungal propagules is essential in establishing mycosis (Boucias & Pendland, 1991; Fargues, 1984; Pendland *et al.*, 1993). In this regard, the surface properties of the fungal cells form the basis for the host–pathogen interaction. The production and development of *B. bassiana* aerial conidia,

Abbreviations: AFM, atomic force microscopy; HI, hydrophobicity index; HIC, hydrophobic-interaction chromatography; MATH, microbial adhesion to hydrocarbons.

in vitro blastospores and submerged conidia has been well defined (Bidochka *et al.*, 1987; Hegedus *et al.*, 1990; Thomas *et al.*, 1987). These cells display different cell wall surface characteristics, as exemplified by differences in hydrophobicity and lectin-binding properties (Hegedus *et al.*, 1992; Jeffs *et al.*, 1999). A study of the attachment properties of the *B. bassiana* cell types revealed that aerial conidia adhere poorly to weakly polar surfaces, and rapidly to both hydrophobic and hydrophilic surfaces, although attachment to the latter surface type cannot be consolidated, and the cells can be rapidly washed off (Boucias *et al.*, 1988; Holder & Keyhani, 2005). *In vitro* blastospores, however, bind poorly to hydrophobic surfaces, moderately to weakly polar surfaces, and rapidly to hydrophilic surfaces. Submerged conidia display the broadest binding spectrum, and can attach to hydrophobic, weakly polar and hydrophilic surfaces. Additionally, a rodlet layer, presumably consisting of hydrophobin(s) proteins, was detected on the surfaces of aerial conidia, but not on those of either blastospores or submerged conidia under the conditions tested (Holder & Keyhani, 2005).

This report describes the surface characteristics of *B. bassiana* aerial conidia, *in vitro* blastospores and submerged conidia in terms of the factors that contribute to mediating the adhesion process, including electrostatic charge and hydrophobicity. Each cell type displayed distinct surface features in terms of morphology and biophysical parameters, including surface charge and hydrophobicity. Insect pathology assays revealed that all three cell types were infectious, although some variation was noted. These data indicate that *B. bassiana* produces distinct infectious cell types which allow it to interact with a variety of substrata, and may help account for the broad invertebrate host range of the fungus.

METHODS

Cultivation of fungi. *B. bassiana* (ATCC 90517) was routinely grown on either Potato dextrose agar (PDA) or Sabouraud dextrose + 1 % yeast extract agar plates (SDAY). Plates were incubated at 26 °C for 10–12 days and aerial conidia were harvested by flooding the plate with sterile deionized water (dH_2O). Conidial suspensions were filtered through a single layer of Mira cloth and final spore concentrations were determined by direct counting using a haemocytometer. Blastospores were produced in Sabouraud dextrose + 1 % yeast extract liquid broth cultures (SDY) using conidia harvested from plates to a final concentration of $0.5–5 \times 10^5$ conidia ml^{-1} as the inoculum. Cultures were grown for 3–4 days at 26 °C with aeration. Cultures were filtered (twice) through glass wool to remove mycelia, and the concentration of blastospores was determined by direct counting. Submerged conidia were produced in TKI broth using fructose as the carbon source, as described by Cho *et al.* (2006). For all cell types, Mira cloth- or glass wool-filtered cell suspensions were harvested by centrifugation (10 000 ***g***, 15 min, 4 °C), washed two times with sterile dH_2O, and resuspended to the desired concentration (typically $10^7–10^8$ cells ml^{-1}).

Atomic force microscopy (AFM). Atomic force micrographs were made using a Digital Instruments Multimode SPM atomic force microscope (model MMAFM-2) placed on a marble stone platform. Images were taken in either tapping or contact mode using a Si_3N_4 probe (Digital Instruments, model NP-20, spring constant = 0.12 N m^{-1}). Fungal cells were placed on 1.2 μm pore-size Millipore filters and air-dried for 1–4 h before examination. Images were collected at 512 lines per scan with a scan rate of 0.96 Hz and tip velocity of 30.6 μm s^{-1}. Data from the micrographs were analysed using Nanoscope SPM v4.42 and SPM Image Magic demo v1.10.

Determination of cell surface hydrophobicity

Microbial adhesion to hydrocarbons (MATH) assay. Cell surface hydrophobicity was determined essentially as described by Smith *et al.* (1998). Briefly, aerial conidia, blastospores and submerged conidia were washed into PUM buffer (per litre: 22.2 g K_2HPO_4, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4.7H_2O$, final pH 7.1). Fungal cell suspensions were adjusted to OD_{470} 0.4 and dispensed (3 ml) into acid-washed glass tubes (12 × 75 mm). Hexadecane (300 μl) was then added to each tube and the tubes were vortexed three times for 30 s. The vortexed tubes were allowed to stand at room temperature for 15 min before the hexadecane phase was carefully removed and discarded. Tubes were then cooled to 5 °C and any residual solidified hexadecane removed. The tubes were then returned to room temperature and the A_{470} of the resultant cell suspensions was determined. The hydrophobic index was calculated using the following equation: $(A_{470,\ control} - A_{470,\ hexadecane\ treated})/(A_{470,\ control})$.

Hydrophobic-interaction chromatography (HIC) assay. Fungal cells (1 ml of $1–2 \times 10^7$ cells ml^{-1}) washed in PUM buffer were loaded onto 1 ml columns containing either phenyl-Sepharose or unmodified (CL4B) Sepharose (Sigma) pre-equilibrated in PUM buffer. Columns were subsequently washed with PUM buffer (4 ml) and the number of fungal cells recovered in the eluate was determined using a haemocytometer. The hydrophobicity index (HI) was calculated using the following equation: [(percentage cells eluted from unmodified Sepharose) − (percentage cells eluted from phenyl-Sepharose)]/(percentage cells eluted from unmodified Sepharose).

Contact angle determination. Contact angle measurements of the *B. bassiana* cell types were determined using a Ramehart model 500 Advanced goniometer with automated drop dispenser and tilting plate, and DropImage Advanced software. Advancing angle measurements were determined just prior to movement of the water drop. Briefly, a 10 μl drop of solution was placed onto the surface of the substrata to be tested. The stage and the camera were tilted at 10° increments until the drop was on the verge of movement. The leading edge (dynamic) contact angle was then determined. Experiments were performed with sterile dH_2O, 2-bromonaphthalene and glycerol at room temperature and 50–55 % relative humidity. Each experiment was performed using at least 10 drops on triplicate samples, using three different batches of each cell type.

Insect bioassays. Second- to fourth-instar *Heliothis virescens* (Fabricius) (tobacco budworm) were used in bioassays testing the virulence of the *B. bassiana* cell types. Aerial conidia, *in vitro* blastospores and submerged conidia were isolated as described above. Aerial conidia were harvested from agar plates directly in sterile dH_2O, whereas *in vitro* blastospores and submerged conidia were washed twice in sterile dH_2O before use. Two different assay conditions were tested for each cell type. (1) Topical application: the larvae were dipped in a solution of 10^8 fungal spores ml^{-1}, and the excess liquid on the insect body was removed with dry paper towel; control larvae were treated with sterile dH_2O. (2) Intrahemocoel injection: the larvae were injected with 5 μl of 10^8 fungal spores ml^{-1} into the haemocoel cavity; controls were injected with 5 μl sterile dH_2O. Experimental and control larvae were placed in individual plastic chambers containing modified wheatgerm-based

insect media (Greene *et al.*, 1976) and incubated at 24 °C. The number of dead insects was recorded daily. For each experimental condition, 20–40 larvae were used, and all experiments were repeated three times.

RESULTS

AFM

Detailed surface topological features of live *B. bassiana* cells could be distinguished by AFM (Fig. 1). AFM allows the visualization of live cells without fixation, and was used to provide resolution at the micrometre level of surface features of freshly harvested cells. Fascicle bundles, presumably composed of assembled hydrophobin(s) protein rodlets, were clearly visible on *B. bassiana* aerial conidia (Fig. 1a, d), and could be removed by sonication (Boucias *et al.*, 1988). Rodlet filaments could faintly be distinguished within the fascicles (Fig. 1g). In contrast, no bundles or fascicles were visible on either blastospores or submerged conidia. The blastospore surface appeared smooth (Fig. 1b, e), whereas the submerged conidial surface was rough, and a circular ring was apparent on some of the latter cells (Fig. 1c, f). In several instances, bipolar germination was noted for *B. bassiana* (Fig. 1h), and this has been reported to correlate with its infectious nature (Talaei-Hassanloui *et al.*, 2006). No fascicles were visible on the germ tubes or hyphae of germinating aerial conidia, although fascicles appeared to remain on the conidium during germination (Fig. 1i, j). AFM images of the germinated conidium revealed fascicles throughout the mother cell, and the lack of fascicles on the slopes of the images presented is due to cantilever artefacts at the resolution employed.

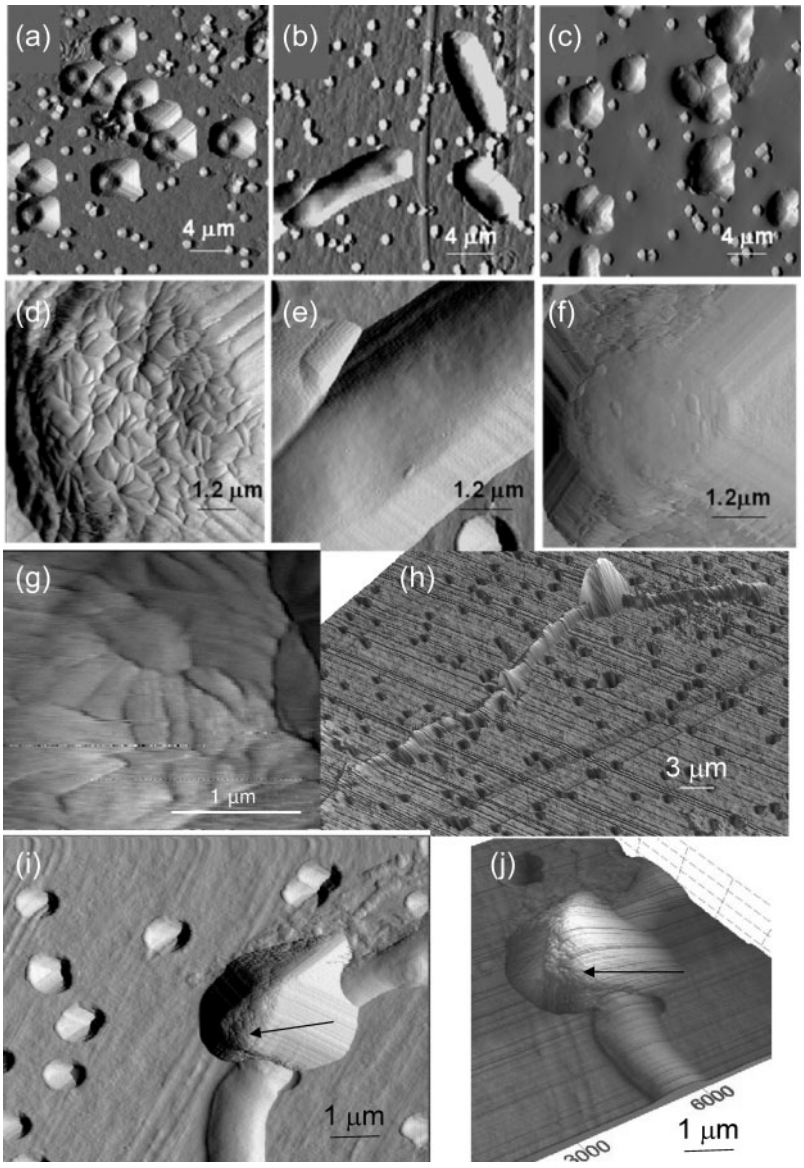

Fig. 1. Atomic force micrographs of *B. bassiana* spore types and germinating conidia. (a, d, g) Aerial conidia: note surface fascicles presumably composed of hydrophobin rodlets [rodlet filaments faintly visible in (g)]; (b, e) *in vitro* blastospores; (c, f) submerged conidia; (h) bipolar germination of aerial conidia. (i, j) Higher resolution of germinating aerial conidia: note the fascicles still present on the germinated conidia (arrows).

Contact angle measurement

Microbial lawns were generated by depositing fungal cells onto glass coverslips. In the case of aerial conidia the glass was treated with Sigmacote to siliconize the surface, allowing efficient attachment of the cells. Microscopic visualization of deposited cells was used to gauge the homogeneity of the microbial lawn. Cell densities ranged from 1×10^6 to 5×10^6 fungal cells mm^{-2}, equivalent to microbial lawns 50–100 cells thick. Samples were dried in covered Petri dishes for 2–4 h before use. Contact angle measurements were taken for each of the *B. bassiana* cell types using water, α-bromonaphthalene and glycerol droplets (Fig. 2, Tables 1 and 2). These determinations revealed critical differences in cell wall physicochemical surface properties, including surface tension, charge and hydrophobicity, among aerial conidia, blastospores and submerged conidia. Aerial conidia displayed greater affinity for the apolar liquid (lower contact angle with α-bromonaphthalene) than for either water or glycerol. Intriguingly, although both blastospores and submerged conidia displayed overall greater affinity for water than for the apolar solvent, the relative affinity of the latter two cell types for the apolar solvent was lower than that of aerial conidia. Overall analysis (of the three tested liquids) revealed that the apolar component (γ_s^{LW}) of aerial conidia was much greater than that of submerged conidia and blastospores, indicating that the surfaces of aerial conidia are more apolar than those of the other two cell types.

The hydrophobicity of the *B. bassiana* cell types was calculated using the surface tension values (Leckband & Israelachvili, 2001; Strevett & Chen, 2003) and evaluated as the interfacial free energy of interaction (ΔG_{iwi}^{IF}). This value represents the degree to which the attraction of the cells to water is greater or smaller than the attraction of water molecules to each other. When the value for this free energy of interaction of the solid molecule in water is repulsive ($\Delta G_{iwi} > 0$), the molecule is considered hydrophilic and will tend to disperse in water. In contrast, the more negative the ΔG_{iwi}, the greater the hydrophobicity of the entity, and the greater the likelihood that the particles will aggregate in aqueous solution. According to this analysis, aerial conidia were the most hydrophobic, and are likely to aggregate in water, consistent with observations of the behaviour of these cells in aqueous solution, as low concentrations of detergent are often added to decrease conidial aggregation. In contrast, blastospores and submerged conidia were hydrophilic, with the latter somewhat less hydrophilic than the former.

Zeta potential

The surface charges of the three cell types were evaluated by means of zeta potential values (Strevett & Chen, 2003; Wilson *et al.*, 2001). Mean zeta potential values for aerial conidia, blastospores and submerged conidia were obtained over a pH range from 3 to 10 (Fig. 3). Since the surface charge of aerial conidia of other fungi are known to differ depending upon the age of the spores, both 16-day- and 20-day-old aerial conidia were examined. The results revealed that 16-day aerial conidia displayed the highest positive zeta potential ($+22\pm2$ mV) at low pH (3), which rapidly became negative by pH 5, reaching a net negative surface charge of -30 ± 4 mV at pH 8–9. In order to obtain the zero potential, i.e. the pH at which the net surface charge of the cells is zero, the data were fitted using polynomial equations. For 16-day aerial conidia, regression analysis yielded the equation, $y=-0.09x^3+2.3x^2-25.3x+80.6$, $r^2=0.999$, resulting in a zero potential of 4.9. Older conidia (20 days) displayed a more negative shift across the pH spectrum starting at $+10\pm2$ mV at pH 3.0 and decreasing to -50 ± 2 mV at pH 9.0. The data were fitted to the equation $y=-0.2x^3+3.4x^2-24.2x+55$, $r^2=0.9986$, yielding a zero potential of 3.9. Submerged conidia also displayed a net positive zeta potential ($+10\pm2$ mV) at low pH that decreased to -13 ± 2 mV at pH 9, whereas blastospores displayed the smallest pH-dependent surface-charge variation, from a small net positive charge ($+4\pm1$ mV) at pH 3.0 to a small net negative charge (-4 ± 1 mV) at pH 9. Regression analyses for the latter two datasets yielded the equations $y=-0.17x^3+3.3x^2-23x+51.1$, $r^2=0.9963$, and $y=-0.11x^3+2.1x^2-13.7x+28.7$, $r^2=0.9543$, with zero potentials calculated to be 4.1 and 4.2 for submerged conidia and blastospores, respectively. At a physiological pH value of 7.0, 16- and 20-day-old aerial conidia displayed net surface charge values of -15 and -22 mV, respectively. In contrast, the surface charges on submerged conidia and blastospores were much smaller, -8 and -2 mV, respectively, at pH 7.0.

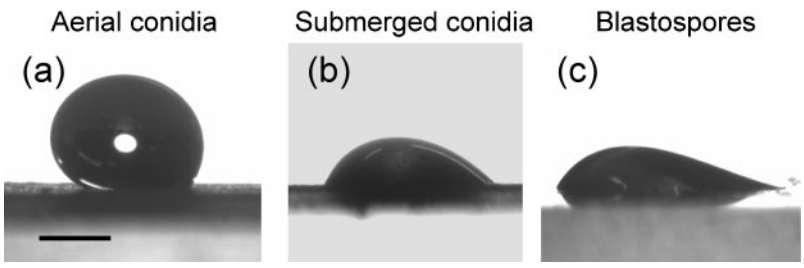

Fig. 2. Contact angle measurements. Microbial lawns of aerial conidia, submerged conidia and blastospores were prepared on glass slides as described in Methods. Images are of water droplets placed on respective *B. bassiana* cell types. Bar, 2 mm.

Table 1. Contact angle and calculated interfacial (ΔG_{iwi}^{IF}) and polar (ΔG_{iwi}^{AB}) free energy values for the *B. bassiana* cell types

B. bassiana cell type	Contact angle			Hydrophobicity	
	dH_2O	1-Bromo-naphthalene	Glycerol	ΔG_{iwi}^{AB}	ΔG_{iwi}^{IF}
Aerial conidia	120.3±2.5°	66.2±0.5°	112.7±0.9°	47.2	−34.8
Blastospores	23.3±1.8°	33.6±0.4°	59.4±1.8°	187.4	74.4
Submerged conidia	30.6±1.0°	56.4±0.8°	53.6±1.1°	167.1	46.0

Measurement of cell surface hydrophobicity

Two methods were used to assess the cell surface hydrophobicity of the different *B. bassiana* cell types. In the first, a MATH assay in which cells partition between two immiscible solutions (water and hexadecane) was used (Fig. 4). In this assay, entities with HI >0.7 are considered hydrophobic (HI=no. cells in organic phase/total no. cells). Aerial conidia were clearly hydrophobic and distributed into the organic phase (HI=0.88), whereas blastospores were hydrophilic, predominantly localizing to the aqueous phase (HI=0.4). Interestingly, submerged conidia partitioned to a slightly greater extent into the organic phase rather than the aqueous phase (HI=0.72), with cell surface characteristics apparently on the borderline between hydrophobic and hydrophilic. A second assay, involving HIC, in which the binding of cells to phenyl-Sepharose and unmodified Sepharose is used as an indicator of the hydrophobic nature of particle surfaces, confirmed the results of the MATH assay (Fig. 4).

Insect bioassays

Second- to fourth-instar *H. virescens* (tobacco budworm) larvae were used to assess the infectivity of the *B. bassiana* cell types. Bioassays were performed by either topical application or haemocoel injection of the fungal cells. Topical application is the method used in biological control applications and reflects the natural route of infection, since *B. bassiana* does not require any specialized route of entry, and fungal cells will germinate, grow and eventually penetrate the insect cuticle essentially anywhere on the surface of the host. Upon topical application, *in vitro* blastospores gave rise to a slightly earlier onset of disease and mortality than either submerged conidia or aerial conidia (Fig. 5a). Onset of disease after application of aerial conidia took ~3–4 days, eventually resulting in similar levels of mortality by day 8 as those seen with blastospores and submerged conidia. The early onset of mortality seen using blastospores is likely due to the faster growth rate observed with these cells as compared to submerged conidia and aerial conidia. In the case of aerial conidia, germination can take between 16 and 24 h, whereas blastospores and submerged conidia can produce additional cells (depending upon the availability of nutrients) within a few hours.

During the infectious cycle, fungal germ tubes eventually penetrate into the host haemocoel. In the haemocoel, the fungus produces distinct single cells termed *in vivo* blastospores (dissimilar to the *in vitro* blastospores) that are able to evade the host immune system. In order to test whether the three *in vitro B. bassiana* cell types were competent in differentiating into *in vivo* blastospores and evading the host immune system, fungal cells were injected directly into the haemocoel of *H. virescens* (Fig. 5b). These data revealed that *in vitro* blastospores were the most robust, resulting in approximately 75 % mortality 2 days post-injection. Submerged conidia were slightly less virulent, resulting in ~40 % mortality 2 days post-infection, although by days 7–8, the overall mortality was similar between the two cell types. Aerial conidia were noticeably less virulent, resulting in only 10 % mortality 2 days post-infection, reaching ~55 % by day 10. A large variation using aerial conidia was noted, however, which could be due to variation in the cultivation conditions (i.e. cell production and harvesting from agar plates), and which does not occur when cells are harvested from liquid

Table 2. Calculated surface energy values

All units are mJ m^{-2}.

B. bassiana cell type	Surface energy				
	Polar, γ^{AB}	Apolar, γ_s^{LW}	Total, γ_s^{total}	Polar components	
				Acid, γ^+	Base, γ^-
Aerial conidia	9.7±1.5	33.3±0.6	43.0±1.1	4.3±0.4	5.5±1.5
Blastospores	10.8±2.3	23.6±0.3	34.4±1.3	0.4±0.2	86.1±3.7
Submerged conidia	23.3±1.1	16.3±0.4	39.6±0.8	2.0±0.3	68.4±2.0

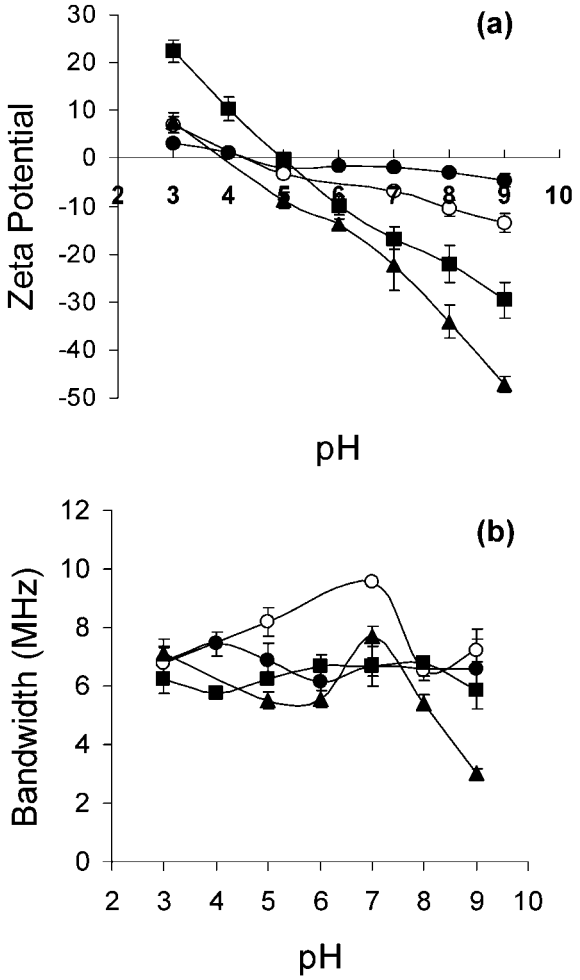

Fig. 3. Zeta potential (a) and spectrum width (b) of *B. bassiana* aerial conidia (■, 16 days; ▲, 20 days). (●) Blastospores; (○) submerged conidia.

cultures (i.e. *in vitro* blastospore and submerged conidia cultivation).

DISCUSSION

Fungal cells display a wide range of surface physicochemical properties that allow them to interact and adhere to substrata. Cell surface hydrophobicity is associated with increased virulence of *Candida* strains, and the hydrophobic rodlet layer of *Aspergillus* conidia appears to confer protection against host immune reactions (Hazen, 2004; Paris *et al.*, 2003; Singleton *et al.*, 2005). In the latter case, the determination of surface biophysical features of *Aspergillus* spores has revealed a role for the rodlet layers and their hydrophobin constituents in contributing to hydrophobicity, adhesion and resistance to killing by alveolar macrophages of the fungal cells (Dynesen & Nielsen, 2003; Girardin *et al.*, 1999; Paris *et al.*, 2003; Stringer & Timberlake, 1995; Thau *et al.*, 1994).

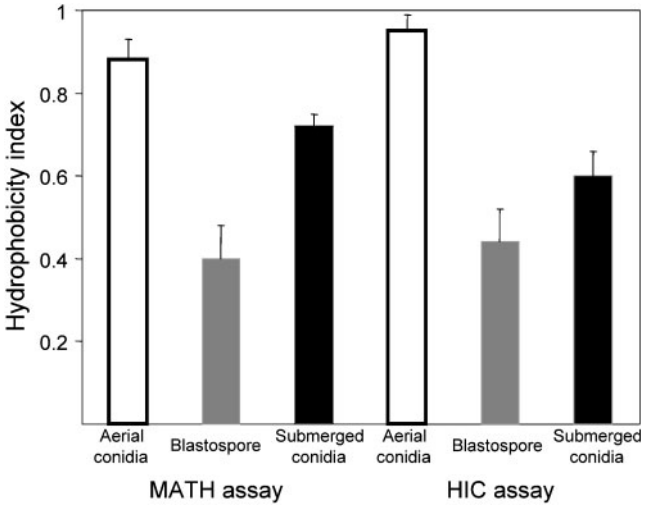

Fig. 4. Cell surface hydrophobicity of the three *B. bassiana* spore types assessed by MATH assay and HIC.

Entomopathogenic fungal spore surfaces range from hydrophobic, exemplified by fungi such as *Nomuraea rileyi*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus*, all of which possess defined outer rodlet layers, to hydrophilic, as seen in *Hirsutella thompsonii* and *Verticillium lecanii*, often characterized by the lack of a rodlet layer but containing an outer mucilaginous coat produced during spore maturation (Boucias & Pendland, 1991). AFM has been used to visualize the surface features of live fungal cells (Dufrene, 2000; Zhao *et al.*, 2005). High-resolution AFM micrographs of *Aspergillus nidulans* have revealed alterations to the spore rodlet layer during swelling in aqueous solutions, and ultrastructural features of newly deposited walls at hyphal tips as well as in mature walls (Ma *et al.*, 2005, 2006). In the present study, AFM was used to examine gross morphological differences among the live spore types that were briefly air-dried before imaging. Initial experiments using an aqueous AFM cell chamber resulted in images that were not as clear as the ones presented, although future experiments imaging the cell types in an aqueous environment are warranted. *B. bassiana* aerial conidia contained fascicle bundles that were not present on either submerged conidia or blastospores. Surfaces of blastospores were smooth, whereas those of submerged conidia were more granulated in appearance. These results are consistent with the lack of SDS-insoluble, trifluoroacetic acid (TFA)-soluble proteins in the latter two cell types and their presence in aerial conidia (Holder & Keyhani, 2005). Interestingly, from our results, no fascicles were visible on germ tubes emanating from aerial conidia, suggesting little membrane fluidity between spore and growing germ tube.

Hydrophobicity and zeta potential measurements have been used to predict the binding preferences of the fungal cells. Analysis of the cell surface properties of

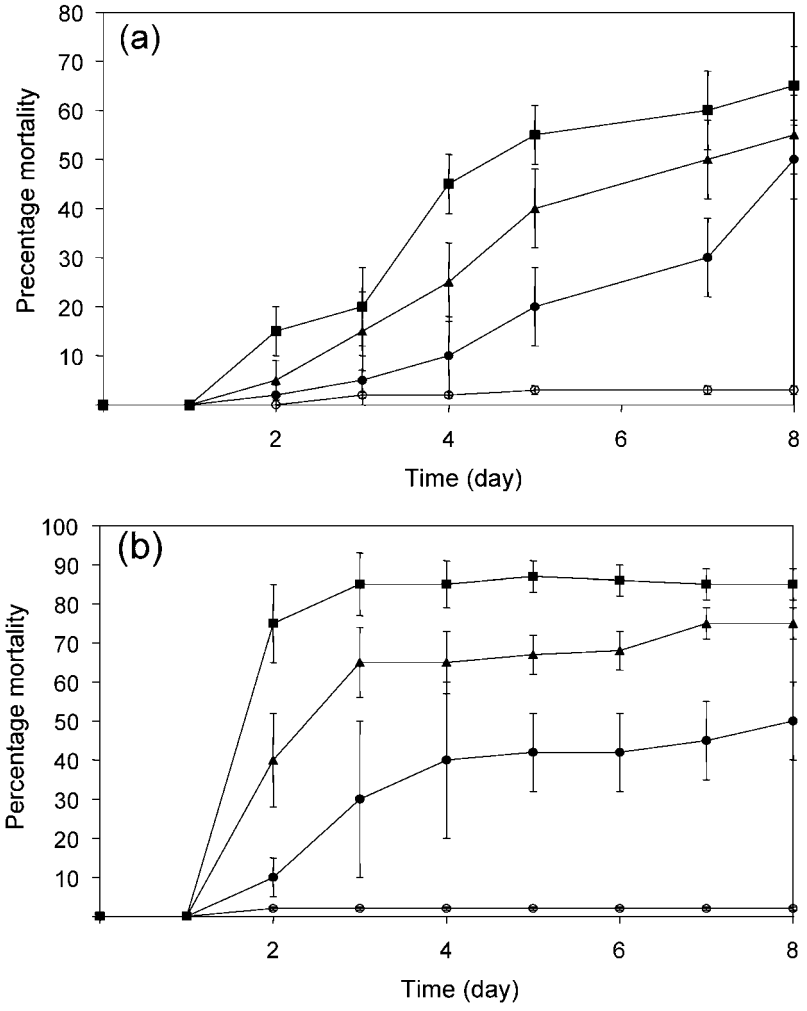

Fig. 5. Insect bioassays. *H. virescens* larvae were treated either topically (a) or via intra-haemocoel injection (b) with *B. bassiana in vitro* blastospores (■), submerged conidia (▲), aerial conidia (●) or mock-treated controls (○), as described in Methods. The percentage mortality over the indicated time-course is presented.

Cryptosporidium oocytes has revealed a preference for adhesion to glass rather than hydrophobic plastic materials, although cell surface hydrophobicity increases with increasing ionic strength of the medium (Drozd & Schwartzbrod, 1996). In contrast, conidia of the mycoparasite *Coniothyrium minitans* are hydrophobic, although in this case, conidial hydrophobicity decreases with culture age for some isolates (Smith *et al.*, 1998). Similarly, a comparison of two cell types of *Trichoderma harzianum*, a potential biological control agent of phytopathogenic fungi, reveals that aerial conidia display higher UV resistance and longer viability, and are more hydrophobic than submerged conidia, which are hydrophilic (Munoz *et al.*, 1995). Contact angle measurements, microbial adhesion to solvents and zeta potential determinations of blastospores of the entomopathogenic fungus *P. fumosoroseus* indicate that these cells have a hydrophilic, basic monopolar surface, and are negatively charged under neutral conditions (Dunlap *et al.*, 2005). Our results indicate that *B. bassiana* produces cells with divergent surface properties that are likely to confer distinct selective advantages in response to varying environmental conditions, although the nature of these advantages remains to be explored. Hydrophobicity was examined using three different methods: partitioning of cells in organic vs aqueous solvents (MATH), HIC and contact angle measurement. The first two methods gave almost identical HIs for the cells tested. Contact angle measurements using non-polar and polar probe liquids allowed the estimation of surface energy parameters. The polar component of aerial conidia (γ^{AB}) was equally distributed into the base (γ^-) and acid (γ^+) surface energy parameters, being 4.3 and 5.5 mJ m^{-2}, respectively. Since monopolar surfaces with $\gamma^- > 28$ mJ m^{-2} are repulsive when dissolved or suspended in water (van Oss *et al.*, 1987), these results suggest that aerial conidia will aggregate in aqueous solutions. In contrast, the large base (86.1 and 68.4 mJ m^{-2}) as compared to acid (0.4 and 2.0 mJ m^{-2}) surface energy components of blastospores and submerged conidia, respectively, indicate that these cells would be repulsive when suspended in water.

Overall, aerial conidia are hydrophobic and represent the more resistant spore type, but are slower growing than

blastospores and submerged conidia. Hydrophobic interactions predominate in the case of aerial conidia and are likely to be the most important force in the host–pathogen interaction. Although they are hydrophobic, the net negative surface charge of aerial conidia at neutral pH may help account for their ability to bind hydrophilic surfaces weakly (Holder & Keyhani, 2005). A negative shift in the surface electrostatic charge distribution was noted for aerial conidia as they aged. This could be due to the production of increasing amounts of surface anionic species over time, or the unmasking of negative charges as the spores dry. Similar experiments could not be performed with blastospores and submerged conidia, as these cells are not stable and will either grow (i.e. germinate or microcycle conidiate) if sufficient nutrients are present or lose viability over a similar time-course.

Although further experimentation is needed, blastospores are fast growing and seem to be able to evade the host immune system better than the aerial conidia. In contrast to the aerial conidia, electrostatic charge appears to play a significant role in adhesion for the *in vitro* blastospores. The surface charge distribution of these cells is consistent with their ability to bind weakly polar and hydrophilic substrata (Holder & Keyhani, 2005). Finally, submerged conidia display intermediate surface properties in terms of hydrophobicity and electrostatic charge. These cells can grow under nutrient-limiting conditions and are likely to exist on insect surfaces and during host–parasite competition for nutrients. The intermediate surface hydrophobicity values reported in this study again may help account for the adhesive nature of these cells, which were able to bind to hydrophobic, weakly polar and hydrophilic surfaces (Holder & Keyhani, 2005).

The different *B. bassiana* cell types also express differential lectin-binding properties (Hegedus *et al.*, 1992). All three cell types bound concanavalin A (Con A); however, whereas both aerial conidia and submerged conidia bound wheatgerm agglutinin (WGA), the *Phaseolus vulgaris* phytohaemagglutinin P (PHA-P) and the *Ricinus communis* agglutinin 1 (RCA) blastospores did not bind the first two substrates and interacted only weakly with the third. Furthermore, only aerial conidia showed a weak reaction against soybean agglutinin. Similar to our results with *H. virescens*, in topical applications all three cell types are pathogenic towards the migratory grasshopper *Melanoplus sanguinipes*, with the highest virulence reported for grasshoppers infected with blastospores (Hegedus *et al.*, 1992). Direct haemocoel challenge of *H. virescens* with the different cell types did reveal that blastospores and submerged conidia were better able to respond to immune reactions than aerial conidia. Further experimentation, however, is required in order to determine whether the observed effects are due to the greater immune-evasion properties of the first two cell types or simply to the fact that they are able to grow faster than the aerial conidia.

The production of different infectious propagules and the wide range of their surface properties imply a diversification of adaptations evolved by fungal pathogens in mediating attachment and adhesion to target insect surfaces. The ability to produce more than one spore type with different surface properties can be expected to increase the possibility of binding diverse ranges of substrata. Biological control applications of entomopathogenic fungi, including *B. bassiana*, often employ aerial conidia as the infective agent; however, the use of other single-cell propagules, such as blastospores and submerged conidia, has also been attempted. Knowledge concerning the forces that mediate interactions between *B. bassiana* and its host targets can be used to develop formulation conditions for specific targets, with future experiments defining key molecular players in this process likely to yield a wealth of information concerning host–pathogen interactions.

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