

Adhesion of the Entomopathogenic Fungus *Beauveria (Cordyceps) bassiana* to Substrata†

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The entomopathogenic fungus *Beauveria bassiana* produces at least three distinct single-cell propagules, aerial conidia, vegetative cells termed blastospores, and submerged conidia, which can be isolated from agar plates, from rich broth liquid cultures, and under nutrient limitation conditions in submerged cultures, respectively. Fluorescently labeled fungal cells were used to quantify the kinetics of adhesion of these cell types to surfaces having various hydrophobic or hydrophilic properties. Aerial conidia adhered poorly to weakly polar surfaces and rapidly to both hydrophobic and hydrophilic surfaces but could be readily washed off the latter surfaces. In contrast, blastospores bound poorly to hydrophobic surfaces, forming small aggregates, bound rapidly to hydrophilic surfaces, and required a longer incubation time to bind to weakly polar surfaces than to hydrophilic surfaces. Submerged conidia displayed the broadest binding specificity, adhering to hydrophobic, weakly polar, and hydrophilic surfaces. The adhesion of the *B. bassiana* cell types also differed in sensitivity to glycosidase and protease treatments, pH, and addition of various carbohydrate competitors and detergents. The outer cell wall layer of aerial conidia contained sodium dodecyl sulfate-insoluble, trifluoroacetic acid-soluble proteins (presumably hydrophobins) that were not present on either blastospores or submerged conidia. The variations in the cell surface properties leading to the different adhesion qualities of *B. bassiana* aerial conidia, blastospores, and submerged conidia could lead to rational design decisions for improving the efficacy and possibly the specificity of entomopathogenic fungi for host targets.

Under intensive study for use as a biopesticide, the entomopathogenic fungus *Beauveria bassiana* displays a broad host range and is able to target a number of diverse arthropod species (7, 15, 16, 23, 26, 27, 31). Strains of *B. bassiana* have been selected for control of insects and other arthropods that act as disease vectors, including mosquitoes and ticks (6, 22); crop pests, such as whiteflies, caterpillars, grasshoppers, and borers (5, 9, 21, 25, 36); and even ecologically hazardous, invading pests, such as fire ants and termites (4, 8). The varied cuticles of these organisms represent the first barrier to the pathogen, and attachment of fungal propagules to the cuticle is the initial event in establishing mycosis. Air currents, dispersion via water droplets, and saprophytic growth over substrata inhabited by insects are considered the major routes for contact of fungal spores with host cuticles (2). Upon contact, fungal cells bind to the cuticle and initiate a developmental program that includes the production of specialized infection structures, such as germ tubes and penetrant hyphae (2, 17). If the infection is successful, the fungus grows across the cuticle surface and penetrates the host cuticle to invade and proliferate within the hemolymph, which ultimately results in the death of the host.

Fungal cell attachment to the cuticle may involve specific receptor-ligand and/or nonspecific hydrophobic and electrostatic mechanisms (2, 3, 10). A haploid anamorphic fungus, *B. bassiana*, produces a number of mononucleated single-cell

types, including aerial conidia, blastospores, and submerged conidia, which can be isolated from agar plates, from rich broth submerged cultures, and from nutrient-limited submerged cultures, respectively. Although it is well known that culture conditions (and hence the cell type produced) can affect successful virulence for targeted hosts, little is known about the process of adhesion of *B. bassiana* cell types other than conidia. In this report we describe a quantitative assay used to determine the binding qualities and adhesion substratum preferences of *B. bassiana* aerial conidia, blastospores, and submerged conidia.

MATERIALS AND METHODS

Cultivation of fungi. *B. bassiana* ATCC 90517 was routinely grown on potato dextrose agar. Plates were incubated at 26°C for 10 to 14 days, and aerial conidia were harvested by flooding a plate with sterile distilled H₂O. Conidial suspensions were filtered through a single layer of Miracloth, and final spore concentrations were determined by direct counting using a hemocytometer. Blastospores were produced in Sabouraud dextrose–1 to 2% yeast extract liquid broth cultures using conidia harvested from plates at a final concentration of 0.5×10^5 to 5×10^5 conidia/ml as the inoculum. Cultures were grown for 3 to 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 previously (32). For all cell types, Miracloth- or glass wool-filtered cell suspensions were harvested by centrifugation ($10,000 \times g$, 15 min, 4°C), washed two times with sterile distilled H₂O, and resuspended to the desired concentration as indicated below (typically 10^7 to 10^8 cells/ml).

FITC labeling of *B. bassiana* cells. Fluorescein isothiocyanate (FITC) (100 μ l of a 1-mg/ml stock solution per ml of fungal cells) was added to washed fungal cells (0.5×10^8 to 1×10^8 cells/ml) resuspended in 50 mM calcium carbonate buffer, pH 9.2. Each reaction mixture was incubated for 20 min in the dark, after which the cells were extensively washed (four or five times with an equal volume) with TB (50 mM Tris-HCl, pH 8.0). The final cell pellets were resuspended in TB to obtain the desired concentrations, as indicated below. The final cell suspension spore concentrations were checked by direct counting using a hemocytometer.

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Adhesion assay. Two assays were used to assess adhesion to substrata. In the first (qualitative) assay, solutions of fungal cell suspensions ($100\ \mu\text{l}$, 1×10^6 to 20×10^6 cells/ml) were placed in slide chambers (treated and untreated glass surfaces; Lab Tech chamber slide system; Nalgene Nunc, Naperville, IL) and incubated at 25°C and 100% humidity for various times. Adhesion was assessed microscopically after one wash or three washes. Digital images were taken using a Nikon Optiphot-2 microscope equipped with a digital camera. Adhesion was also assessed quantitatively using FITC-labeled cells incubated on various black microtiter plate test substrata. Fungal cell suspensions ($100\ \mu\text{l}$, 1×10^6 to 20×10^6 cells/ml) were placed in (black) microtiter plate wells and incubated at 25°C in the dark for various times. Unbound cells were removed by aspiration of the liquid from the wells, followed by up to three washes with $450\ \mu\text{l}$ TB. Fluorescence was measured using a Spectra Max Gemini XS microplate fluorometer (excitation wavelength, $495\ \text{nm}$; emission wavelength, $530\ \text{nm}$; cutoff wavelength, $515\ \text{nm}$; Molecular Devices Corp., Sunnydale, CA). For each experiment a standard curve of fluorescence intensity versus cell number (as measured by direct counting) was prepared. Typically, the fluorescence intensity was measured before washing (total number of cells) and after each wash. Weakly polar microtiter plates (Fluorotrac F200) and hydrophilic microtiter plates (Fluorotrac F600) were purchased from Greiner Biotech (Longwood, FL) and were used unmodified. Hydrophobic substrata were prepared by addition of a thin layer of silicone using Sigmacote (Sigma Corp., St. Louis, Mo.) to glass slides or to Fluorotrac F200 microtiter plate wells. Typically, substrata were treated up to three times with Sigmacote, and the treated plates or slides were placed in a fume hood overnight in order to ensure evaporation of all solvent.

Enzyme treatments. Aerial conidia, blastospores, and submerged conidia (0.5×10^7 to 1.0×10^7 cells/ml) were washed twice and resuspended in the enzyme reaction buffers suggested by the manufacturer. Portions ($100\ \mu\text{l}$) of 10-mg/ml stock solutions of amylase (catalog no. A6255; Sigma), cellulase (catalog no. C9422; Sigma), or laminarinase (catalog no. L5272; Sigma) in $0.01\ \text{M}\ \text{KPO}_4$ (pH 6.8 for α -amylase and pH 5.6 for cellulase and laminarinase) were added to $0.9\ \text{ml}$ of cells resuspended in the same buffer. For protease treatments, $100\ \mu\text{l}$ and $50\ \mu\text{l}$ of stock solutions (10 mg/ml and 1 mg/ml) of proteinase K (catalog no. p6911; Sigma) and pronase E (catalog no. 300140; Stratagene), respectively, in buffer ($0.1\ \text{M}\ \text{Tris}\ \text{HCl}$, pH 7.8, 0.5% sodium dodecyl sulfate [SDS], $1\ \text{mM}\ \text{CaCl}_2$) were added to cells resuspended in the same buffer. Glycosidase reaction mixtures were incubated for 4 h at 25°C , and protease treatments were performed for 4 h at 37°C . After incubation, cells were extensively washed in $50\ \text{mM}$ calcium bicarbonate buffer (pH 9.2) (seven or eight times, $1\ \text{ml}$ each) by centrifugation ($10,000 \times g$, 5 min). Treated, washed cells were then FITC labeled and used in adhesion assays as described above.

Effect of pH on attachment. FITC-labeled cells (0.5×10^7 to 1.0×10^7 cells/ml) were washed twice and resuspended in one of the following physiological buffers ($0.1\ \text{M}$) before they were used in adhesion assays: acetate (pH 4 and 5), morpholineethanesulfonic acid (MES) (pH 6 and 7), HEPES (pH 7 and 8), and TB (pH 8). Control wells with cells suspended in TB (pH 8) were used to determine initial cell concentrations due to the pH sensitivity of FITC fluorescence intensity measurement. Normalization due to pH effects on the FITC intensity was performed by allowing adhered cells (i.e., cells after the adhesion assay incubation and washing steps) to equilibrate in TB (pH 8) until the fluorescence intensity of the signal of the cells stopped increasing.

Competition assays. Cells were FITC labeled, and the final cell pellets resulting from the washing steps of the labeling reaction were resuspended in TB containing either $0.3\ \text{M}$ carbohydrate (added as a competitor), 0.1% detergent (SDS, Tween 80, or cetyltrimethylammonium bromide [CTAB]) or $1\ \text{M}\ \text{NaCl}$. Cells were then immediately used in adhesion assays.

Contact angle determination. Contact angle ($c\angle a$) measurements for the surfaces used to evaluate the adhesive properties of the fungal cell types were obtained by using a Ramehart model 500 Advanced goniometer with an automated drop dispenser and tilting plate, using the DropImage Advanced software. Dynamic angle measurements were obtained just prior to movement of the water drop. Briefly, a $10\text{-}\mu\text{l}$ drop of sterile water was placed onto the surface of the substratum to be tested. The stage and the camera were tilted at 10° increments until the drop was on the verge of movement. The advanced (dynamic) contact angle was then determined.

Rodlet layer extraction. The rodlet layer proteins were removed from the surface of the spores as described by Paris et al. (29). Briefly, aerial conidia, blastospores, and submerged conidia were prepared as described above, resuspended in water, and sonicated at $140\ \text{W}$ (3-mm-diameter microtip, 50% duty cycle) twice for 10 min using a Sonifier cell disrupter B-30 (Branson Ultrasonics, Rungis, France). Unlysed cells and cell debris were removed by low-speed centrifugation ($10,000 \times g$, 10 min), and the supernatant was centrifuged for 1 h at $50,000 \times g$. The resultant pellet was boiled in SDS-polyacrylamide gel electro-

phoresis (PAGE) sample buffer (2% SDS, 5% β -mercaptoethanol, and 10% glycerol in $62\ \text{mM}\ \text{Tris-HCl}$, pH 6.8) and washed twice with sample buffer and three times with distilled water. The final pellet was lyophilized and then treated with 100% trifluoroacetic acid for 10 min at room temperature. The acid was removed under a stream of nitrogen, and dried extracts were stored at room temperature under dry air and resuspended in water prior to analysis. Aliquots of a protein sample were mixed with $4\times$ lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) plus dithiothreitol and run on a 10% bis-Tris Nu-PAGE gel with MES-SDS gel running buffer together with standards. Protein bands were visualized using either Sypro Ruby red (Bio-Rad, Hercules, CA) or Coomassie blue.

RESULTS

A quantitative assay was developed in order to measure the kinetics of fungal cell adhesion to various substrata. Fungal cells chemically treated with the fluorescent reagent FITC appeared to be uniformly labeled, with clear halo rings defining the cell envelope. Labeling of all three cell types, aerial conidia, blastospores, and submerged conidia, revealed a linear relationship between cell number (as measured by cell counting using a hemocytometer) and fluorescence intensity. Little variation was observed within experiments; however, some variation (up to a twofold difference in fluorescence intensity) was observed between experiments (i.e., between separate FITC labeling reactions). Therefore, a standard curve of fluorescence intensity versus cell number as determined by cell counting was determined and used for each experiment.

In order to determine the effects of the labeling reaction on the adhesive qualities of the cells, a series of preliminary qualitative experiments were performed using untreated and siliconized glass slides with both unlabeled and labeled cells. In all instances no difference was observed between using FITC-labeled cells and using unlabeled cells. These data demonstrated (i) that aerial conidia were able to bind to hydrophobic surfaces but not to hydrophilic surfaces; (ii) that blastospores bound uniformly to hydrophilic surfaces but bound poorly to hydrophobic surfaces, forming small clumps on the latter; and (iii) that submerged conidia bound equally well to both hydrophilic and hydrophobic surfaces, forming large clumps that appeared to become more evenly distributed over time. These patterns were identical for FITC-labeled and unlabeled cells.

For the quantitative assays, the following three types of black polystyrene-based microtiter plates with different surface characteristics were used as substrata: (i) siliconized Fluorotrac F200 plates, which were highly hydrophobic; (ii) F200 untreated polystyrene surface plates, which were weakly polar; and (iii) F600 plates, which were treated polystyrene and had hydrophilic, polar surfaces containing hydroxyl, carbonyl, and amino groups with a small net negative charge. Dynamic advanced water droplet contact angle measurements for the three substrata were consistent with the decreasing hydrophobicities; the silinated F200 plates displayed a $c\angle a$ of 104.7° , the untreated F200 plates displayed a $c\angle a$ of 95.6° , and the F600 plates displayed a $c\angle a$ of 85.6° (the contact angles for cleaned polished glass, the glass chamber slides, and silinated glass were determined to be 73.1° , 87.4° , and 109.7° , respectively).

The number of binding sites per microtiter plate well was estimated to be 4×10^5 to 8×10^5 , as determined by the saturation point derived from plots of the percentage of cells bound as a function of the cell concentration (Fig. 1). These data indicated that the linear ranges of the cell types were

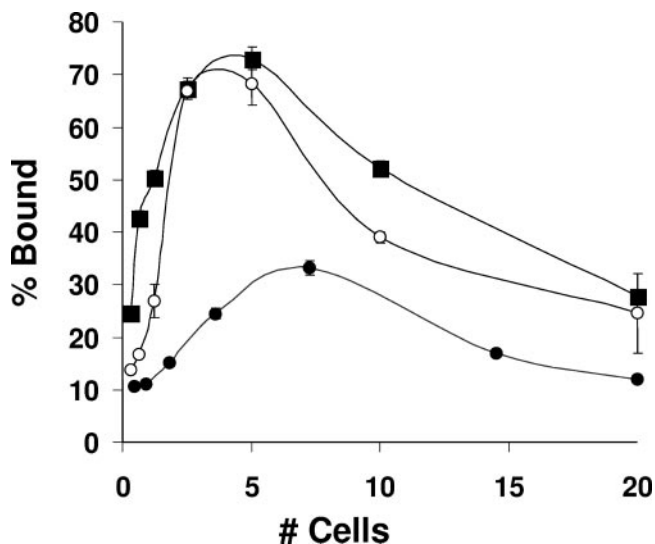


FIG. 1. Saturation points of sites for binding of *B. bassiana* aerial conidia (●), blastospores (■), and submerged conidia (○) to microtiter plates. The error bars indicate the results of at least three independent experiments.

similar, although the saturation points for the cell types varied from approximately 25% of the aerial conidial cells able to bind per well to more than 70% of the submerged conidia bound in wells when $\sim 5 \times 10^5$ cells/well was used. All subsequent experiments were performed using cell concentrations in the linear range of the attachment curve (2×10^5 to 5×10^5 cells/well).

Quantitative adhesion assays using aerial conidia, blastospores, and submerged conidia on hydrophobic, weakly polar, and hydrophilic surfaces with either one or three washes were performed (Fig. 2). Aerial conidia bound rapidly and tightly to hydrophobic surfaces, and there was no decrease in cell binding after up to 10 washes with buffer (data not shown). Aerial conidia bound poorly to weakly polar surfaces even after prolonged exposure (24 h) to the substrata. Interestingly, these cells bound weakly to hydrophilic surfaces and were readily washed off. In contrast, blastospores bound poorly to hydrophobic surfaces, and no more than 10% of the cells were bound even after 24 h. Blastospores bound moderately to weakly polar surfaces, and approximately 1×10^5 to 2×10^5 cells bound/well (30% of the 5×10^5 cells used in the assay) after 4 h of incubation. Blastospores bound more readily to hydrophilic surfaces, and up to 50% of the cells bound within 30 min. Submerged conidia displayed the broadest binding characteristics, adhering to all three surfaces, although with slightly different kinetics. On hydrophobic, weakly polar, and hydrophilic surfaces, up to 60% of the cells used bound to the substrata within 4 h, although in the case of the hydrophobic and weakly polar surfaces, almost one-half of the bound cells could be removed using three washes.

Attachment of aerial conidia to hydrophobic surfaces could not be competed with any of the carbohydrate compounds tested, including glucose, galactose, lactose, maltose, melibiose, or trehalose, and was not sensitive to salt concentrations as high as 1 M NaCl (Table 1). The presence of *N*-acetylglucosamine appeared to promote adhesion (confirmed by micro-

scopic analysis). The effect was not due to any visible growth or mucilage production; i.e., no germination, germ tube, or secretion of an extracellular matrix could be seen. Conidial attachment was, however, sensitive to the presence of detergents, and 80 to 90% inhibition was observed when 0.1% Tween 80 (a nonionic detergent), CTAB (a cationic detergent), or Triton X-100 (a nonionic detergent) was used; SDS (an anionic detergent) also inhibited conidial attachment to hydrophobic surfaces, although a large degree of variation was observed. Adhesion of aerial conidia to surfaces was only slightly affected by pH, and 30% fewer cells were bound at pH 4.0 than at pH 7.0.

A unique feature of blastospore attachment was that adhesion could be competed with maltose (Table 1). No other sugar tested had any effect on blastospore adhesion, nor did maltose affect conidial or submerged conidial adhesion. Blastospore attachment was not sensitive to salt (1 M NaCl), SDS, and CTAB, but it was inhibited by Tween 20 and Triton X-100. In contrast to the adhesion of the other cell types, the adhesion of blastospores appeared to be pH dependent, and there was a 50% decrease in the number of cells bound when assays were performed at pH 4 to 5 compared with the number of cells bound when assays were performed at pH 7 to 8. Submerged conidia behaved like aerial conidia, except that *N*-acetylglucosamine did not increase the number of cells bound and the presence of the detergents CTAB and Triton X-100 (Table 1) and changes in pH had only minor effects on microcycle conidial adhesion.

Removal of carbohydrates (maltose, glucose, or glucuronic acid) from the cell surface of aerial conidia using either α -amylase or laminarinase, but not removal of carbohydrates using cellulase, resulted in decreased conidial adhesion to hydrophobic surfaces but had no effect on conidial adhesion to hydrophilic surfaces (Table 2). Treatment of blastospores with glycosidases appeared to either slightly promote adhesion (α -amylase and to a lesser extent cellulase treatment) to hydrophilic surfaces or to not affect adhesion (laminarinase and/or hydrophobic surface). Glycosidase treatment of submerged conidia resulted in a 25 to 50% decrease in adhesion of cells to hydrophilic surfaces and hydrophobic surfaces except for α -amylase-treated cells, for which great variation was observed for adhesion to hydrophobic surfaces. Some differential effects were observed after protease treatment of the cell types. Aerial conidia treated with pronase E exhibited a more than 50% loss of adhesion to hydrophobic surfaces but no loss of adhesion to hydrophilic surfaces, although great variation was observed. This variation was between experiments (i.e., different cell batches treated with the enzyme) and may have reflected surface heterogeneity or accessibility of target substrates to the enzyme. Protease K treatment of aerial conidia did not result in appreciable changes in adhesion. Similar treatment of blastospores with proteases had no effect or resulted in an almost twofold apparent increase in adhesion. Microscopic analysis (i.e., visual counting) of the number of cells bound indicated that there did not appear to be an actual increase in the number of cells bound; instead, protease treatment appeared to increase the intensity of the fluorescence signal. These conditions were the only conditions tested in which the fluorescent signal was affected, and in all other experiments the results of microscopic analysis were in agreement with the fluorescent intensity measurements. Finally, little or no effect

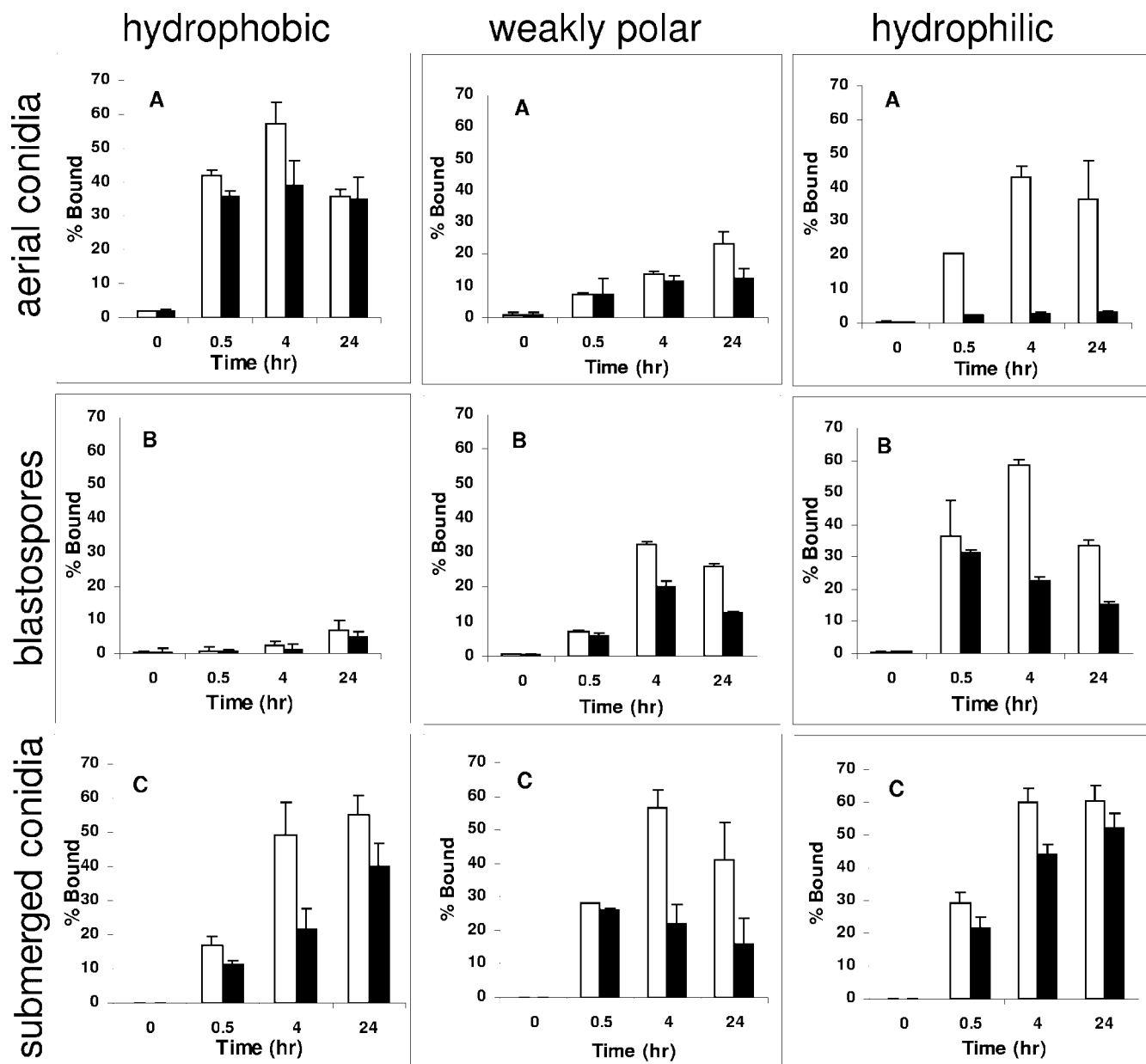


FIG. 2. Quantitative adhesion and influence of washing on adhesion of aerial conidia (A), blastospores (B), and submerged conidia (C) to silanated F200 (hydrophobic), F200 (weakly polar), and F600 (hydrophilic) microtiter plates. The percentages of cells bound after one wash (open bars) and after three washes (solid bars) are presented versus assay incubation time. The error bars indicate the results of at least three independent experiments.

was observed on the adhesion properties of protease-treated submerged conidia.

Hydrophobic interactions are thought to be mediated (at least in part) by low-molecular-weight proteins termed hydrophobins that form a rodlet layer on the surface of fungal cells. Hydrophobins are highly resistant to extraction by detergents but can be solubilized by (100%) trifluoroacetic acid. *B. bassiana* aerial conidia, blastospores, and submerged conidia were examined for the presence of hydrophobins by using cell extracts prepared as described in Materials and Methods and were analyzed by SDS-PAGE (Fig. 3) (gels stained with Sypro Ruby red gave essentially the same results). Protein bands

corresponding to putative hydrophobins were extracted only from aerial conidia, and no bands were formed by extracts derived from either blastospores or submerged conidia.

DISCUSSION

Conidial adhesion has been examined in a number of plant- and insect-pathogenic fungi (2, 28). Adhesion of entomopathogenic fungi has been considered to involve an initial binding interaction followed by a consolidation step, resulting in firm attachment to the cuticle (2, 13, 14). Similarly, studies on the phytopathogenic fungus *Botrytis cinerea* revealed a two-stage

TABLE 1. Effects of various competitors and chemicals on *B. bassiana* cell adhesion

Compound	Attachment ratio ^a		
	Aerial conidia ^b	Blastospores ^c	Submerged conidia ^c
Glucose	1.0 ± 0.1	1 ± 0.1	1 ± 0.1
<i>N</i> -Acetylglucosamine	2.0 ± 0.1	1 ± 0.1	1 ± 0.1
Fucose	1.0 ± 0.2	1 ± 0.2	1 ± 0.1
Melibiose	1.0 ± 0.2	1 ± 0.2	ND ^d
Maltose	1.0 ± 0.2	0.1 ± 0.1	1 ± 0.3
Trehalose	0.8 ± 0.1	0.9 ± 0.1	1 ± 0.1
1 M NaCl	1.0 ± 0.2	0.9 ± 0.2	1 ± 0.2
0.2% Tween 20	0.2 ± 0.1	0.1 ± 0.1	ND
0.2% SDS	0.4 ± 0.5	0.8 ± 0.3	0.3 ± 0.1
0.2% CTAB	0.2 ± 0.1	0.6 ± 0.3	1 ± 0.2
0.2% Triton X-100	0.1 ± 0.1	0.3 ± 0.1	0.6 ± 0.1

^a Attachment ratio = (% cells bound under conditions tested)/(% cells bound under control conditions). The data are the means ± standard deviations for at least three independent experiments.

^b Cells were tested on siliconized F200 (hydrophobic) microtiter plates.

^c Cells were tested on F600 (hydrophilic) microtiter plates.

^d ND, not determined.

adhesion process; immediate adhesion occurred upon hydration and was characterized by relatively weak attachment, and stronger delayed adhesion was observed as the conidia germinated (10, 11). Immediate adhesion of *B. cinerea* was passive (nonmetabolic), and although no specific structures were visible on the conidia, adhesion was characterized as dependent (in part) on hydrophobic interactions. Hydrophobic interactions have also been implicated in the attachment of conidia of the insect-pathogenic fungi *Nomuraea rileyi*, *Metarhizium anisopliae*, and *B. bassiana* to both host and nonhost cuticle preparations (3).

B. bassiana produces at least three single-cell types that can be distinguished based on morphological and adhesive characteristics. Qualitative studies assessing entomopathogenic fungal adhesion to various surfaces, including insect cuticles (2, 3, 10, 11, 17), have almost exclusively addressed conidial binding to surfaces and have not examined the adhesion properties of either blastospores or submerged conidia. We used a quantitative adhesion assay, and our results demonstrate that there are complex interactions between various cell types and substrata with different surface properties. All three *B. bassiana* single-cell types studied, aerial conidia, blastospores, and sub-

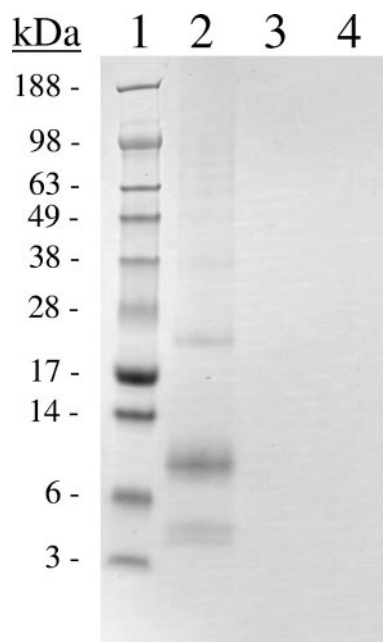


FIG. 3. Analysis of SDS-insoluble trifluoroacetic acid-soluble material from ultrasonic extracts of *B. bassiana* aerial conidia, blastospores, and microcycle conidia. An SDS-PAGE gel (10% polyacrylamide, bis-Tris) was stained with Coomassie blue. Lane 1, protein molecular weight standards; lane 2, aerial conidia; lane 3, blastospores; lane 4, submerged conidia.

merged conidia, displayed different adhesion properties that appeared to be mediated by different cell-specific mechanisms.

B. bassiana aerial conidia were able to bind to both hydrophobic and hydrophilic surfaces, although adhesion to the latter was weak and the cells could readily be washed off. Aerial conidia binding to hydrophobic surfaces could not be competed off with any carbohydrate tested, although addition of *N*-acetylglucosamine, the monomeric constituent of chitin, the major carbohydrate (polymer) found in arthropod cuticles, appeared to increase adhesion. The hydrophobic nature of *B. bassiana* conidial spores, as well as conidial spores from other entomopathogens, such as *N. rileyi*, *M. anisopliae*, and *Paecilomyces fumosoroseus*, has been correlated with the presence of an outer cell layer comprised of rodlets or fascicles, as visualized

TABLE 2. Effects of various enzymatic treatments on *B. bassiana* cell adhesion

Enzyme	Attachment ratio ^a					
	Aerial conidia		Blastospores		Submerged conidia	
	Hydrophobic ^b	Hydrophilic ^c	Hydrophobic	Hydrophilic	Hydrophobic	Hydrophilic
α-Amylase	0.3 ± 0.05	1.0 ± 0.3	1.2 ± 0.2	1.5 ± 0.15	1.4 ± 0.2	0.5 ± 0.15
Cellulase	0.8 ± 0.15	1.1 ± 0.2	1.0 ± 0.7	1.3 ± 0.05	0.4 ± 0.2	0.6 ± 0.15
Laminarinase	0.3 ± 0.05	0.9 ± 0.3	0.9 ± 0.1	1.0 ± 0.2	0.4 ± 0.2	0.8 ± 0.15
Pronase E	0.4 ± 0.3	1.2 ± 0.3	0.9 ± 0.1	1.7 ± 0.2 ^d	1.1 ± 0.2	1.1 ± .05
Protease K	0.9 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	1.8 ± 0.2 ^d	2.8 ± 0.4	1.0 ± 0.15

^a Attachment ratio = (% cells bound under conditions tested)/(% cells bound under control conditions). The data are the means ± standard deviations for at least three independent experiments, each consisting of triplicate determinations.

^b Cells were tested on siliconized F200 (hydrophobic) microtiter plates.

^c Cells were tested on F600 (hydrophilic) microtiter plates.

^d Apparent increase (see text for details).

by electron microscopy (2, 3). The rodlet layers are presumably formed by assembly of specific proteins termed hydrophobins, which in turn are thought to passively mediate adhesion to hydrophobic surfaces (34, 35). Consistent with this model, extraction of the *B. bassiana* cell types revealed the presence of putative hydrophobins (a rodlet layer) in aerial conidia that were absent from both blastospores and submerged conidia. Although hydrophobins may account for some of the observed adhesion qualities, our data also indicate that the interaction of aerial conidia with hydrophobic surfaces may be more complex. Since amylase and laminarinase treatments, as well as protease treatments, reduced adhesion (but had no discernible effects on the rodlet layer [B. H. Kirkland, D. J. Holder, and N. O. Keyhani, unpublished results]), both carbohydrates on the cell surface and proteins may be involved in mediating adhesion of aerial conidia. Some caution, however, should be taken in any interpretation of enzymatic treatments of cells since amylase, laminarinase, and cellulase are enzyme mixtures which contain various additional enzymes, often including proteases.

In contrast, blastospores, which are cells that lack any visible rodlet layer (2, 3) and from which no (putative) hydrophobins were extracted under the conditions tested, bound poorly to hydrophobic surfaces, forming small aggregates or clumps, but they displayed high levels of binding to hydrophilic substrata. Blastospores were also able to bind to weakly polar substrata, although the incubation time required was greater than that required for hydrophilic substrata. Intriguingly, blastospore attachment could be specifically competed with maltose. No other carbohydrate tested appeared to compete with adhesion of blastospores or the other fungal cell types; this included trehalose, the major carbohydrate constituent found in insect hemolymph. In vivo-generated blastospores (distinct but similar to the rich broth-produced blastospores), produced during fungal proliferation in the insect hemolymph after penetration of the cuticle, are able to evade recognition by insect hemocytes and display altered membrane characteristics (19, 20, 30); however, the physiological significance of potential maltose inhibition of adhesion of these cells is unclear.

Submerged conidia displayed the broadest binding characteristics of the *B. bassiana* single-cell types (they also appeared not to contain a rodlet layer or hydrophobins), and they were able to bind to hydrophobic, weakly polar, and hydrophilic surfaces. Spore tips or mucilage-covered appendages and adhesive knobs have been implicated as structures that mediate conidial adhesion of several fungi (1, 18, 33). The mucosal coat of nematophagous fungi not only appears to mediate adhesion but also is attractive to host insects, and a wide variety of arthropod mycopathogens appear to produce exocellular mucilage during germ tube or appressorial formation (2). Similarly, the hydrophilic nature of conidia of the Entomophthorales is thought to be mediated by a mucilaginous coat released upon attachment to cuticle surfaces, which acts as a glue mediating attachment (12, 24). Although blastospores and submerged conidia attached to hydrophilic surfaces, no obvious mucilaginous coat was visible in either cell type, and scanning electron microscopy did not reveal any specific structures in either conidia, blastospores, or submerged conidia of *B. bassiana* that appeared to be involved in mediating adhesion (unpublished data). It is possible, however, that extracellular ma-

trix components or mucilage located between the inner and outer walls that may not be readily detectable could be involved in mediating adhesion.

Although aerial conidia are considered easily dispersible via air currents and due to their spore-like cell walls are more resistant to adverse environmental conditions, such as desiccation and extreme temperatures, microcycle conidiation and blastospore formation may occur under a variety of environmental conditions, as well as during the host-pathogen interaction. The production of multiple cell types with different adhesive properties may occur in response to specific environmental conditions, allowing fungal cells to bind to a broad range of host targets and providing the fungus a way to adapt to substratum conditions (13, 14). It is unlikely, however, that alteration of adhesion can account for the emergence of the restricted-host-range *B. bassiana* strains since these strains may have altered (cuticle-degrading) enzyme production or be unable to penetrate and/or respond to surface cues of certain hosts but still retain the means to initiate binding or adhesion interactions. Indeed, there is some evidence that when entomopathogens specialize, they lose structures rather than gain them, although it would be interesting to see whether any alteration in the adhesion kinetics of general and specialized strains of *B. bassiana* occurs. Our data do indicate that certain practical considerations should be taken into account during application of *B. bassiana*. For instance, if blastospores are to be used, formulations should probably contain aqueous or nonpolar liquids that may prove to be more successful in biocontrol of certain hosts compared to aerial conidia. In contrast, the use of detergents in order to avoid aggregation of aerial conidia may prove to be detrimental during application since the presence of such detergents can decrease adhesion to surfaces. Future research correlating the virulence of the fungal cell types to specific insect targets may lead to rational design decisions for the selection of fungal strains with greater specificity for desired targets.

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