

Nonspecific Factors Involved in Attachment of Entomopathogenic Deuteromycetes to Host Insect Cuticle†

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The attachment of the conidia of the insect-pathogenic fungi *Nomuraea rileyi*, *Beauveria bassiana*, and *Metarrhizium anisopliae* to insect cuticle was mediated by strong binding forces. The attachment was passive and nonspecific in that the conidia adhered readily to both host and nonhost cuticle preparations. The hydrophobicity of the conidial wall and the insect epicuticle appeared to mediate the adhesion process. Detergents, solvents, and high-molecular-weight proteins known to neutralize hydrophobicity reduced conidial binding when added to conidium-cuticle preparations. However, these chemicals did not remove the hydrophobic components from the epicuticle or from conidial preparations. The outer surface of the conidium consists of a resilient layer of well-organized fascicles of rodlets. Intact rodlets extracted from *B. bassiana* conidia bound to insect cuticle and exhibited the hydrophobicity expressed by intact conidia. Both electrostatic charges and various hemagglutinin activities were also present on the conidial surface. However, competitive-inhibition studies indicated that these forces played little, if any, role in the adhesion process.

The attachment of spores of the entomopathogenic fungi to host insects represents the initial event in the establishment of mycosis. In terrestrial environments, spores are dispersed passively via air currents or free water over substrata inhabited by host insects. Upon contact, the spores attach preferentially to the cuticle, produce penetrating germ tubes which breach the host cuticle, and invade the host hemocoel. Within the host insect, fungal elements (hyphal bodies, mycelia, or protoplasts) multiply, eventually leading to host death and subsequent mummification. The processes responsible for the initial adhesion of the fungal propagules to the host cuticle are poorly understood. The conidium-cuticle interaction may involve a complex of specific (e.g., glycoprotein) and nonspecific (e.g., electrostatic or hydrophobic) recognition mechanisms (7).

The outer layer of the spore wall, believed to contain the determinants for attachment to the cuticle, varies with the type of spore. The motile spores of the aquatic entomopathogenic fungi *Coelomomyces* spp. have been reported to utilize various cuticle sugars or complex carbohydrates or both as recognition cues for attachment (12, 22). During spore attachment, adhesive materials are secreted actively by the encysting zoospores of these fungi. Similarly, various nematode-trapping fungi use the carbohydrate residues on the nematode cuticle as recognition cues (15). The spores of terrestrial entomopathogenic fungi, such as *Verticillium lecanii* and *Hirsutella thompsonii*, as well as those of certain species of the family *Entomophthorales*, are covered by an amorphous mucus which facilitates adhesion of the spore to the host cuticle. Recent work has shown that the mucus layer of the ballistospores of the entomophthoran *Conidiobolus obscurus* is composed of fibrillar glucans associated with amorphous proteins, one of which is a hemagglutinin having specificity for glucose and *N*-acetylglucosamine residues (13; J. P. Latge, M. Monsigny, and M. C. Prevost, J. Histochem. Cytochem., in press). Upon impact with a

substratum, the mucus layer of *C. obscurus* spreads out over the cuticle, adhering the spore to the integument. Whether the hemagglutinins detected in the mucus layer are involved in the adhesion of conidia to cuticle is not known.

Currently, much less is known about the forces responsible for the attachment of the terrestrial fungi *Nomuraea rileyi*, *Metarrhizium anisopliae*, and *Beauveria bassiana* to host insect cuticle. These fungi, known to produce dry phialoconidia, are currently being researched and developed as microbial pesticides against a spectrum of insect pests (4). In this report, we have analyzed the attachment of the dry phialoconidia to host insect cuticle. Comparative studies have been directed at analyzing the role of both the outer surface of the conidia and the outer epicuticular surface of the insect. By using sodium dodecyl sulfate (SDS)-produced cuticle ghosts as a model substratum, we have been able to determine by light microscopy the effects of various chemical and enzymatic treatments on the adhesion process. These studies, in combination with scanning and transmission electron microscopic examinations of the cuticular and conidial surfaces, have indicated that hydrophobic forces play an important role in adhesion to the epicuticle.

MATERIALS AND METHODS

Cultivation of fungi and recovery of spores. The fungal species used in these studies were *N. rileyi* (UF1 74-6), *M. anisopliae* (UF1 5507), and *B. bassiana* (UF1 5477), isolated from the host insects *Anticarsia gemmatalis* (Noctuidae), *Scapteriscus acletus* (Gryllotalpidae), and *Scapteriscus vicinus*, respectively. These fungi were propagated at 25°C on either Sabouraud dextrose agar with 2% yeast extract (*N. rileyi*) or glucose-yeast extract-basal salts agar medium composed of 10 g of glucose, 5.0 g of yeast extract, 1.05 g of Na₂HPO₄ · 7H₂O, 0.36 g of KH₂PO₄, 1.0 g of KCl, 0.7 g of NH₄NO₃, 0.6 g of MgSO₄, and 20 g of agar per liter of H₂O (*M. anisopliae* and *B. bassiana*). At 10 to 14 days postinoculation, progeny conidia were collected by gently scraping cultures with a spatula. The harvested conidia were suspended in deionized H₂O by gentle sonication for 1 min with

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TABLE 1. Effects of various chemicals on attachment of conidia to fourth-instar cuticle ghost preparations

| Chemical treatment | Relative no. of adhering conidia (\pm SD) ^a for: | | |
|--|--|--------------------|----------------------|
| | <i>N. rileyi</i> | <i>B. bassiana</i> | <i>M. anisopliae</i> |
| H ₂ O (control) | 49 \pm 18 | 37 \pm 19 | 30 \pm 8 |
| 0.1 M HCl | 33 \pm 25 | 28 \pm 25 | 19 \pm 3 |
| 1.0 M HCl | 22 \pm 10 | 22 \pm 17 | 6 \pm 4 |
| 0.1 M NaOH | 47 \pm 34 | 54 \pm 38 | 17 \pm 8 |
| 1.0 M NaOH | 18 \pm 15 | 7 \pm 4 | 3 \pm 1 |
| 1% SDS | 6 \pm 3 | 7 \pm 7 | 11 \pm 8 |
| 1% NP-40 | 6 \pm 5 | 9 \pm 5 | 5 \pm 3 |
| CHCl ₃ -methanol (2:1, vol/vol) | 14 \pm 7 | 16 \pm 7 | 3 \pm 2 |
| 0.1 M ethylene glycol | 63 \pm 21 | NT | NT |
| 1.0 M ethylene glycol | 75 \pm 60 | NT | NT |
| Bovine serum albumin (1 mg/ml) | 4 \pm 1 | 8 \pm 6 | 6 \pm 3 |
| Ovalbumin (1 mg/ml) | 10 \pm 7 | 2 \pm 2 | 4 \pm 3 |

^a Mean values of attached conidia counted within a microscope field (\times 600) having an area of 0.013 mm² (8 to 16 fields for each treatment). NT, Not tested.

a Fisher sonic dismembrator fitted with a microtip operating at 90 W.

Preparation of cuticle ghost substrates. Cuticle ghosts were prepared by boiling cohorts of laboratory-reared first- and fourth-instar velvetbean caterpillar larvae (*A. gemmatilis*) in a 1% SDS solution, followed by rinsing six times in deionized H₂O. This detergent treatment, resulting in the removal of internal tissues, produced transparent "cuticle ghosts" (20). Although it causes partial disruption of the endocuticle, this treatment leaves the epicuticle highly receptive to conidial attachment.

To determine which components on the cuticle were serving as receptors to conidial attachment, a series of chemical and enzyme treatments was done on fourth-instar cuticle ghosts before they were exposed to conidial preparations. Cuticle preparations were incubated in chloroform-methanol (2:1, vol/vol) or hexane for 4 h, rinsed twice with their respective solvent systems, air dried, and suspended in deionized H₂O. A second batch of cuticle ghosts was fixed overnight in 2% buffered glutaraldehyde (4°C) and rinsed four times with deionized H₂O. A 24-h digest with an 8.0 M urea-1% mercaptoethanol mixture was performed on the cuticle ghosts to remove proteins associated with the epicuticle. Alkali hydrolysis of the cuticle ghosts was carried out with 1.0 M NaOH at 100°C for 4 h. Enzymatic digests of the cuticle ghosts with proteinase K (15 U/ml) and trypsin (5,000 U/ml) were performed for 24 h at 37°C. After three rinses with deionized water, the cuticle samples were incubated with the sonicated conidial preparations (see the following paragraph). Representative cuticle samples were examined with both the light microscope and the scanning electron microscope.

Adhesion assay. Cuticle ghosts from fourth-instar *A. gemmatilis* larvae were used in the adhesion assays. The cuticles of the fourth to sixth body segments were carefully dissected from the ghosts and rinsed twice with distilled H₂O prior to adhesion assays. Samples of *N. rileyi*, *B. bassiana*, and *M. anisopliae* conidia (10⁷ conidia per ml) were centrifuged, suspended in various chemical solutions (Table 1), and incubated with cuticle samples on a gyratory shaker (200 rpm) for 30 min. The cuticle preparations were removed, rinsed three times with their respective solutions, mounted flat on glass slides, and stained with lactophenol blue. The

TABLE 2. Chemical treatment of conidia and its effect on attachment to cuticle ghost preparations

| Physiochemical treatment of conidia ^a | Length of treatment | Binding to cuticle ghosts ^b of: | | |
|---|---------------------|--|--------------------|----------------------|
| | | <i>N. rileyi</i> | <i>B. bassiana</i> | <i>M. anisopliae</i> |
| Sonication | | | | |
| Setting 30% | 1 min | +(+) | +(+) | +(+) |
| Setting 70% | 1 min ^c | +(+) | +(+) | +(+) |
| Boiling in CHCl ₃ -methanol (2:1, vol/vol) | 4 h | +(+) | NT | NT |
| Detergents^d | | | | |
| Proteinase K | 24 h | +(+) | NT | NT |
| Cell-wall-lytic enzymes | 24 h | +(+) | +(+) | +(+) |
| Chitinase | 24 h | +(+) | NT | NT |
| 8 M urea-1% β -mercaptoethanol | 12 h | +(+) | +(+) | +(+) |
| 0.1 M HCl | 24 h | +(\pm) | NT | NT |
| 0.1 M NaOH | 24 h | +(\pm) | +(\pm) | +(\pm) |
| 1.0 M NaOH | 4 h | +(\pm) | +(\pm) | +(\pm) |
| 1.0 M NaOH (100°C) | 1 h | -(-) | -(-) | -(-) |

^a After being incubated with chemicals, conidial preparations were washed three times with distilled H₂O prior to being assayed for attachment.

^b +, Binding; -, no binding. Symbols in parentheses refer to the presence (+), partial removal (\pm), or absence (-) of normal, intact fascicles of rodlets in replicas of conidial preparations. NT, Not tested.

^c Four 1-min blasts.

^d SDS, 1% NP-40, Triton X-100, acetylcetrimide.

degree of adhesion was assessed by counting the attached conidia under a phase-contrast microscope.

Chemical treatment of conidial samples. Conidial preparations of *N. rileyi*, *B. bassiana*, and *M. anisopliae* were incubated at intervals in various chemical and enzyme solutions (10 mg of conidia per ml; Table 2) at 25°C and collected by filtration on 0.45- μ m-pore-size membrane filters (Millipore Corp.). The treated conidia were washed, suspended in deionized H₂O, and assayed for their ability to both adhere to cuticle ghosts and germinate on Sabouraud dextrose agar with 2% yeast extract. Surface replicas were made from each conidial preparation and were viewed with a transmission electron microscope to determine whether the distinct outer rodlet layer persists after treatment. (See electron microscopy techniques for details.) Filtrates from *N. rileyi* conidial treatments were analyzed for total hexose and protein content by anthrone carbohydrate and Bio-Rad protein assays (Bio-Rad Laboratories) (3). Filtrates from detergent treatments were preincubated in either Bio-Beads SM-2 or Amberlite IR-120-P resins (Bio-Rad) prior to assay.

Detection of analysis of conidial hemagglutinins. Conidia of various strains of *N. rileyi*, *B. bassiana*, and *M. anisopliae* were harvested from plates (0.8 to 2.0 g per strain) and sonicated (four 1-min bursts) in deionized H₂O (100 mg of conidia per ml) at 4°C by using a sonic dismembrator (power, 210 W). The conidial suspensions were centrifuged at 10,000 \times g for 20 min at 4°C (Beckman JH20 rotor), and the resulting supernatants were collected, filtered, and dialyzed overnight against 0.05 M Tris hydrochloride (pH 7.5) containing 0.25 M phenylmethylsulfonyl fluoride. After the supernatants were decanted, the conidial pellets were suspended in H₂O to the original volume. Hemagglutination activity in supernatants and conidial samples was assayed against sheep, rabbit, human type O, and horse erythrocytes in plastic microtiter plates (16). Various hexoses and other carbohydrates were tested for their ability to inhibit hemagglutinin activity in both intact conidia and conidial extracts

(see Table 5). A similar series of sugars was assayed for the ability to inhibit binding of conidial preparations to cuticle ghosts.

Analysis of electrostatic charge on conidia. Noncuticle substrata were assayed to determine the binding specificity of the conidial preparations. Substrata at concentrations of 1 mg/ml for chitin flakes and chitosan (Sigma Chemical Co.), and 50 μ l of beads per ml for Sepharose 4B (Pharmacia Fine Chemicals), fucose-Sepharose (Sigma), fetuin-Sepharose (Sigma), and DEAE-Bio-Gel and CM-Bio-Gel beads (Bio-Rad) were mixed with 10^7 conidia per ml for 1 h at 26°C on a rotary shaker at 200 rpm. After incubation, substrata were washed four to six times with deionized H₂O. In light of the ability of conidial preparations to bind to both chitosan and DEAE-Bio-Gel beads, additional adhesion experiments were performed with conidial preparations that had been preincubated in solutions of *N*-acetylglucosamine (200 mM), glucosamine (200 mM), and poly-L-lysine (10 mg/ml). Conidial suspensions were washed three times in deionized H₂O before being incubated with the tested substrata.

Hydrophobicity of conidial preparations. Two methods, phase exclusion and salt aggregation, were used to measure the relative hydrophobicity of conidial preparations (14). Conidia were gently sonicated into aqueous suspensions, centrifuged at $10,000 \times g$ for 20 min, and suspended in H₂O or buffered solutions. For the phase exclusion assay, conidia were suspended in 3.0 ml of either sodium acetate or Tris buffer (pH 3.0, 6.0, or 9.0) such that the concentrations of preparations had an A_{600} of 0.6 nm (1-cm path length). Equivalent volumes of toluene were added to conidial samples, which were then vortexed for 10 to 15 s. The solvents were allowed to separate, the lower aqueous phase was removed (excluding the interface zone), and the A_{600} was determined. The proportion of cells excluded from the aqueous phase was used as an index of hydrophobicity. The second assay, salt aggregation, involved mixing samples of spores with twofold-decreasing concentrations of (NH₄)₂SO₄ in a 24-well microtiter plate. A final concentration of 1.5×10^8 spores per 500- μ l volume was used in this assay. After 5 min of gentle agitation, the degree of aggregation was visually assessed.

Extraction of rodlets from *B. bassiana* conidia. Conidia from *B. bassiana* were sonicated (five 1-min bursts; power setting, 210 W) and centrifuged (2 cycles of $6,000 \times g$ for 30 min) at 4°C. Supernatants were centrifuged at $68,000 \times g$ (Sorvall AH650 rotor) for 30 min. Replicas of the $6,000 \times g$ pellet (conidia), the $68,000 \times g$ pellet, and the $68,000 \times g$ supernatant fractions were prepared and examined under the transmission electron microscope (see below). Both the $68,000 \times g$ supernatant and the $68,000 \times g$ pellet (intact rodlet fraction) were conjugated to tetraethylrhodamine isothiocyanate (8). Labeled fractions were incubated with cuticle ghosts, rinsed five times with H₂O, and examined with an Olympus BHC microscope fitted with a BH2-RF1 reflected-light fluorescence attachment. The relative hydrophobicity of the extracted rodlet fractions ($68,000 \times g$ pellet) was measured by the phase exclusion assay described previously (14).

Electron microscopy methods. Samples to be examined by scanning electron microscopy were fixed for 96 h in osmium tetroxide vapors (17). After being fixed, samples were allowed to air dry for at least 48 h in a desiccator. They were then gold coated and examined with a Hitachi S-450 scanning electron microscope operating at 20 kV.

Samples to be examined by transmission electron microscopy were fixed for 2 h in either 2% glutaraldehyde in 0.2 M

cacodylate buffer (pH 7.2) or a 2% paraformaldehyde-glutaraldehyde mixture in cacodylate buffer. They were postfixed for 1 to 2 h in 1% buffered OsO₄, dehydrated through a graded ethanol series, and embedded in Spurr medium (21). Thin sections were cut with a diamond knife on a Porter Blum MT-2 ultramicrotome and were poststained with uranyl acetate followed by lead citrate. Sections were viewed with a JEOL JEM 100CX electron microscope operating at 60 or 80 kV.

Replicas were prepared for transmission electron microscopy by placing 1 to 2 drops of an aqueous suspension of the sample on a piece of split mica (6). The samples were allowed to air dry and were then shadowed with carbon-platinum at 45°C in a Balzers BA-360 M vacuum evaporator. The carbon-platinum replicas were attached to mica supports and soaked overnight in 50% chromic acid. The replicas were rinsed twice in deionized water and mounted on Formvar-coated grids.

RESULTS

Scanning electron microscopy demonstrated that homogeneous preparations of *N. rileyi*, *B. bassiana*, and *M. anisopliae* conidia adhered to cuticle ghost preparations as efficiently as they did to the cuticle of live *A. gemmatilis* larvae. Comparative assays revealed that many more conidia appeared to be trapped by and tightly adsorbed to the cuticular surfaces of first-instar larvae than to those derived from fourth-instar larvae (Fig. 1). Nondiscriminate attachment of conidia was observed over the entire cuticle surface. However, conidia appeared to be most frequently trapped by and tightly bound to areas on the cuticle which contained small spines.

Adhesion assays with fourth-instar cuticle ghost preparations that had been pretreated with various chemicals demonstrated that the epicuticle contained the receptors for conidial adhesion. Treatment of the cuticle ghosts with solvents, chloroform-methanol (2:1, vol/vol), or hexane neither reduced conidial attachment nor increased the wettability of the cuticle ghost preparations. Similarly, treatment of cuticle ghosts with glutaraldehyde, chitinase, or 8 M urea-1% mercaptoethanol failed to reduce either the hydrophobicity of the cuticle or the level of conidial adhesion. Scanning and transmission electron microscopy of these preparations demonstrated that the epicuticle layer remained intact. Conversely, both alkali (1.0 M NaOH) and proteinase K treatments disrupted the epicuticle layer and reduced conidial attachment. Scanning electron microscopy revealed that conidia attached to the epicuticle but not to areas of exposed underlying endocuticle (Fig. 2).

Attempts to displace conidia that had attached to cuticle samples by such treatments as boiling in detergents (1% SDS, Nonidet P-40 [NP-40]) or chloroform-methanol (2:1, vol/vol) or incubation (1 to 4 h) at 25°C in 0.1 M NaOH, 0.1 M HCl, or cell-wall-lytic enzymes failed to remove significant numbers of attached conidia. However, various chemicals, when added to conidial suspensions prior to attachment, did influence the adhesion process (Table 1). Detergents (1% SDS, 1% NP-40), chloroform-methanol (2:1, vol/vol), alkali (1.0 M NaOH), acid (1.0 M HCl), and the proteins bovine serum albumin and ovalbumin (1 mg/ml) caused a significant reduction in the number of conidia adhering to cuticle ghosts. While it did not influence the number of attached conidia, ethylene glycol (0.1 and 1.0 M) did cause aggregation of conidia into large clumps on the cuticle surface.

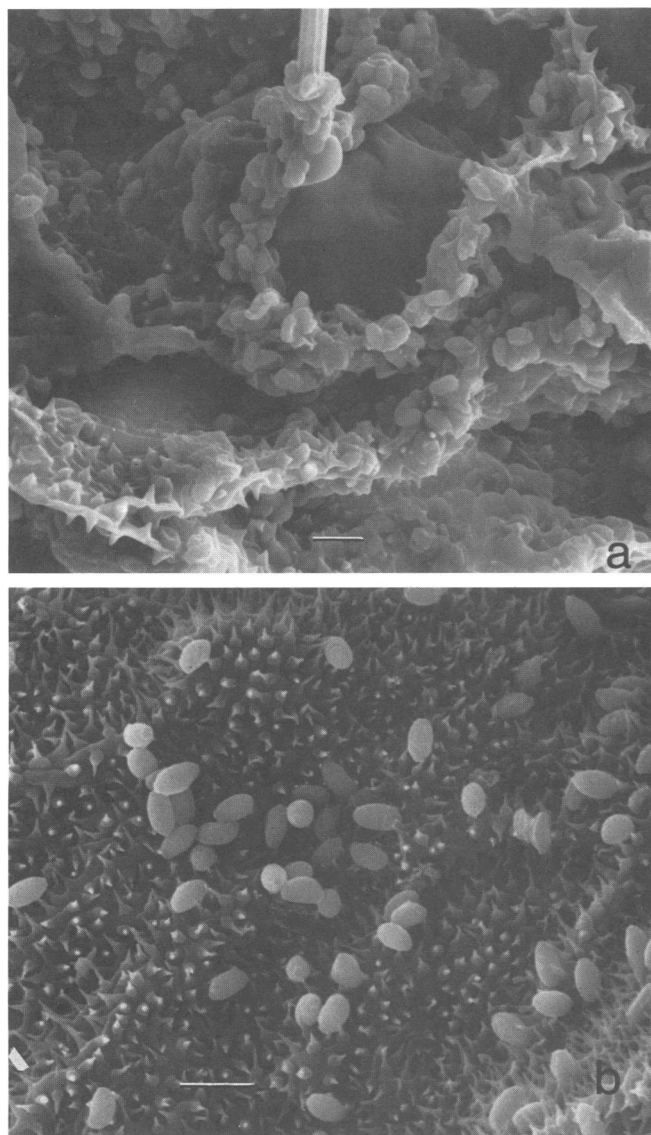


FIG. 1. Scanning electron micrographs of *N. rileyi* conidia attached to (a) first-instar and (b) fourth-instar cuticle ghost preparations. Bars = 5 μ m.

Examination of the replicas of conidia revealed that all three species possessed an outer layer composed of fascicles of rodlets (Fig. 3). The morphological arrangement of rodlets on *N. rileyi* and *B. bassiana* conidia could easily be distinguished from that observed on *M. anisopliae* conidia. The relative stability of this rodlet layer appeared to vary with the species. For example, sonication (four 1-min bursts) of *N. rileyi* and *M. anisopliae* conidia released only amorphous material with very few, if any, intact rodlets. In contrast, replicas of similar preparations derived from *B. bassiana* spores contained numerous intact rodlets (Fig. 4a). The rhodamine-rodlet conjugates prepared from these preparations bound to cuticle ghosts, whereas treatment of the cuticle with control rhodamine-bovine serum albumin and rhodamine-*B. bassiana* soluble fraction conjugates (68,000 \times g) resulted in only very low background fluorescence (Fig. 4b and c).

A series of chemical and enzymatic treatments was per-

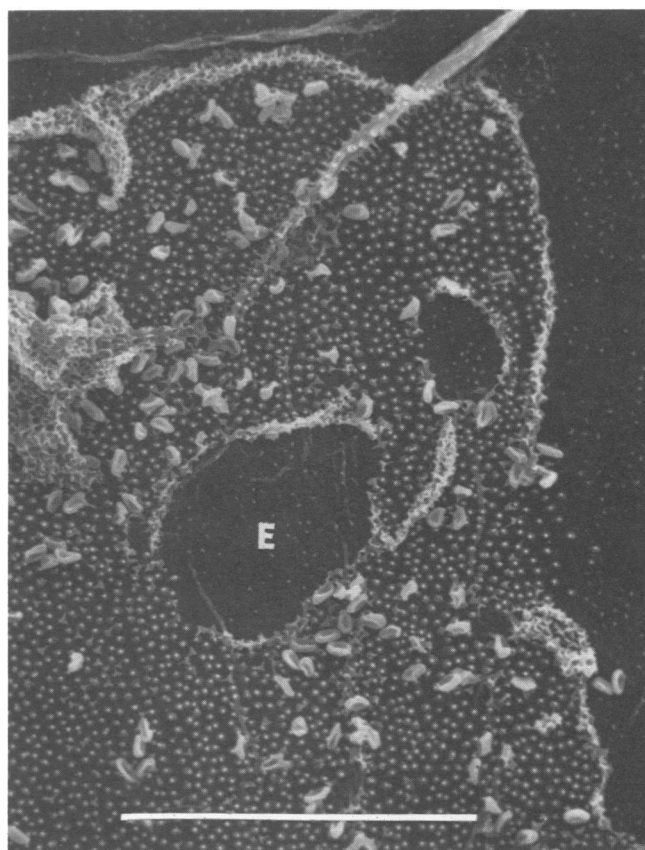


FIG. 2. Selective attachment of *N. rileyi* conidia to the epicuticle of proteinase K-digested cuticle ghost preparations. Note the lack of conidia attaching to underlying endocuticle (E). Bar = 50 μ m.

formed on conidial preparations in an attempt to digest the rodlet layer and prevent adhesion to cuticle ghosts (Table 2). Multiple sonication bursts at a power setting of 70%, while removing soluble and insoluble components from conidia, did not produce conidial preparations void of rodlets. In fact, replicas prepared from sonicated conidial suspensions were indistinguishable from replicas prepared from untreated conidial preparations. Extended incubation with organic solvents, various detergents, and urea-mercaptoethanol, as well as enzymatic treatments, also failed to remove the rodlet layer and to prevent attachment to cuticle preparations. Mild acid and alkali treatment of the conidia, although resulting in partial removal of the rodlet layer (Fig. 5), did not result in a marked reduction of conidial attachment to cuticle ghosts. Analysis of the material removed by 0.1 M alkali "hydrolysis" of *N. rileyi* demonstrated that alkali treatment was the only one that removed detectable levels of protein from sonicated conidia (Table 3). Prolonged treatment (12 to 14 h) or boiling (1 h) in 1 M NaOH did remove the rodlet layers from conidial preparations. Detergent treatments, when compared with H₂O controls, appeared to inhibit the release of proteins and sugars from *N. rileyi* conidia. These results are different from those of Cole and Pope (5), who reported that nonionic-detergent treatment of *Aspergillus* spores removed significant amounts of wall components.

Sonication of conidial samples resulted in the release of hemagglutinins for all of the strains assayed (Table 4). Low

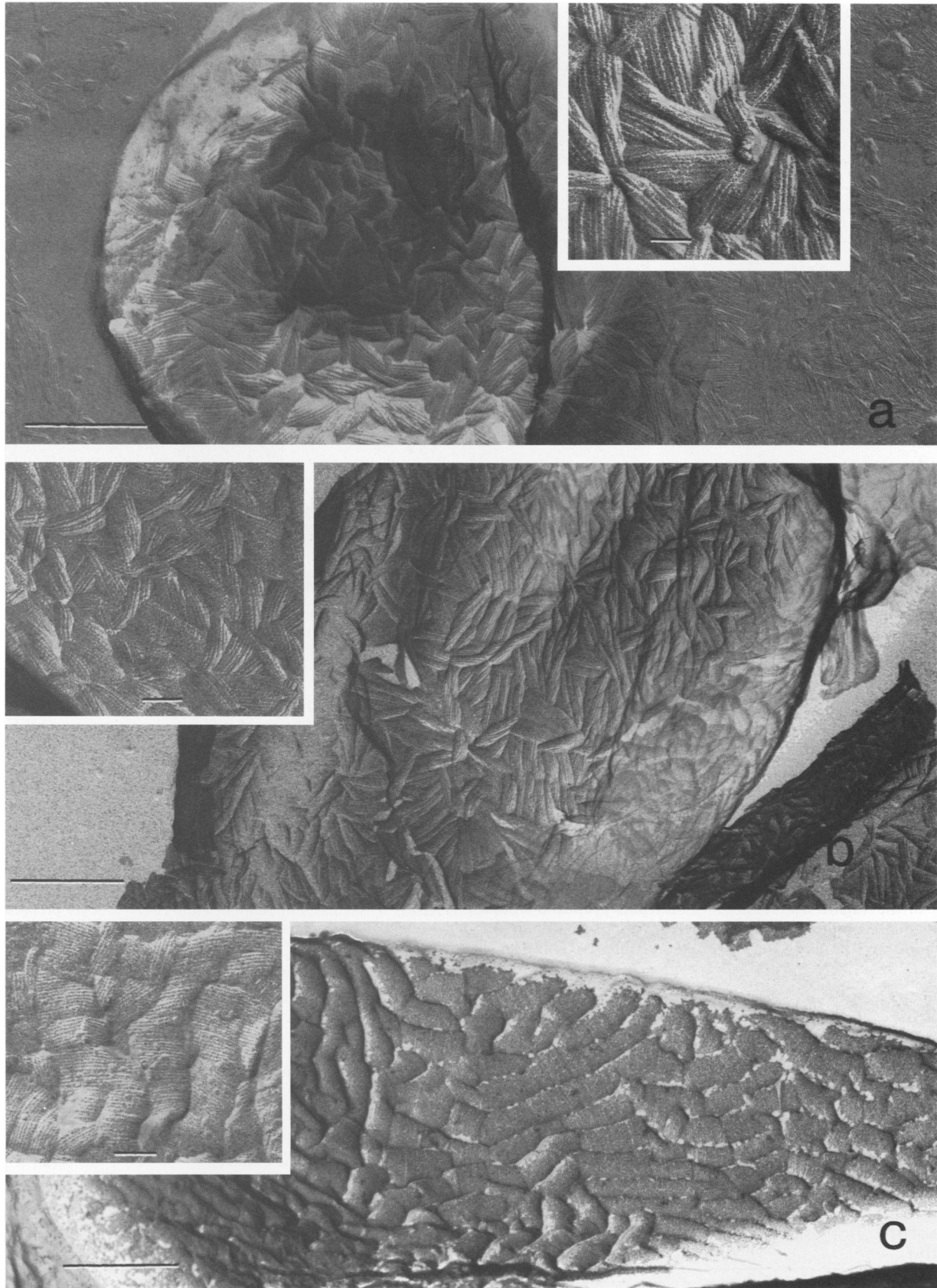


FIG. 3. Surface replicas of (a) *B. bassiana*, (b) *N. rileyi*, and (c) *M. anisopliae* water-sonicated conidial preparations (bars = 0.5 μm). Insets show rodlet fascicles (inset bars = 0.1 μm).

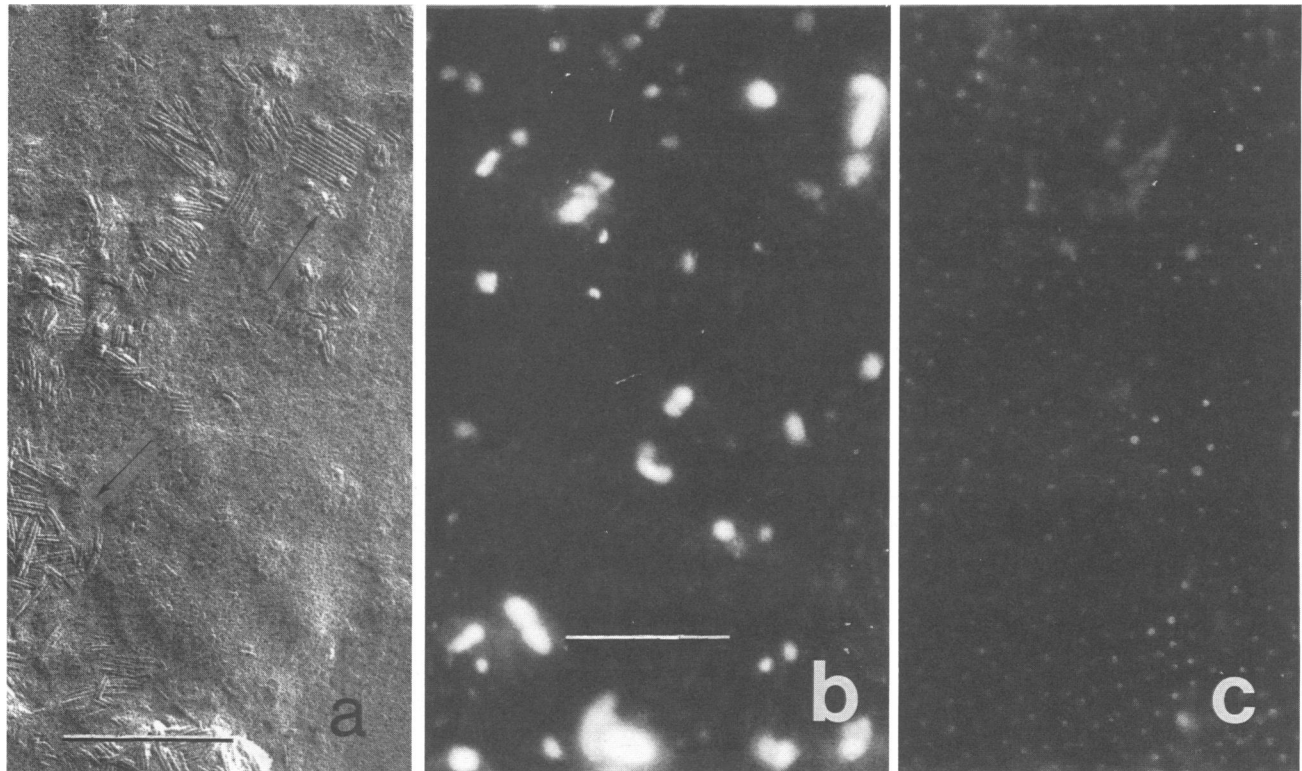


FIG. 4. (a) Replica of isolated *B. bassiana* rodlets (arrows). Bar = 0.5 μm . (b) Light micrograph showing rodlet-rhodamine conjugate (bright fluorescent areas) binding to cuticle ghosts. Bar = 10 μm . (c) Light micrograph of cuticle ghost incubated in bovine serum albumin-rhodamine conjugate. Only background fluorescence is visible.

titers of hemagglutinin activity (HA) were detected in both intact conidia and supernatants of conidial sonic extracts from *N. rileyi* and *B. bassiana*. Unlike *N. rileyi* and *M. anisopliae* extracts, which had the highest HA against rabbit and human O erythrocytes, *B. bassiana* extract was most effective against sheep erythrocytes. The results of competitive-inhibition tests with various simple and complex carbohydrates demonstrated that the *B. bassiana* agglutinin could only be inhibited (one well) by *N*-acetylglucosamine (200 mM), whereas the *N. rileyi* HA could not be inhibited by any of the sugars used (Table 5). Extracts prepared from *M. anisopliae* conidia contained high levels of HA against both rabbit and human O erythrocytes (Table 4). This HA,

TABLE 3. Relative amounts of sugars and protein released from *N. rileyi* conidia after chemical treatment

| Chemical treatment | Amt of protein ^a after: | | Amt of sugars ^b after: | |
|------------------------------------|------------------------------------|------|-----------------------------------|------|
| | 4 h | 17 h | 4 h | 17 h |
| H ₂ O (control) | 5 | 7.5 | 30 | 22 |
| Chloroform-methanol (2:1, vol/vol) | 4 | 4 | 9 | 11 |
| 1% NP-40 | 4 | 0 | 4 | 0 |
| 1% Tween 20 | 0 | 0 | 9 | 4 |
| 1% SDS | 2 | 2 | 6 | 4 |
| 0.1 N NaOH | 19 | 16 | 31 | 30 |
| 0.1 N HCl | 2 | 2 | 29 | 35 |

^a Bio-Rad protein microassay (micrograms of protein per 10 mg of conidia).

^b Anthrone reaction (micrograms of glucose equivalents per 10 mg of conidia).

detected in both dry and sonicated intact conidia, could be inhibited by the addition of various simple sugars and EDTA (Table 5) and was destroyed by heat treatment (60°C, 1 h). Competitive-inhibition studies were performed to determine whether the hemagglutinin(s) detected was involved in the attachment process. Conidial samples were preincubated with a variety of hapten sugars, alone and in various combinations, and were then incubated with cuticle ghost preparations. Results demonstrated that none of the carbohy-

TABLE 4. Hemagglutination of various erythrocyte preparations by conidia and sonic extracts of conidia

| Conidial preparation | HA ^a of erythrocytes from: | | |
|--|---------------------------------------|-----------|-----------------|
| | Rabbit | Human O | Sheep |
| <i>N. rileyi</i> (UF1 74-6) | | | |
| Dry conidia | 4 | 0 | 0 |
| Sonicated conidia | 2-16 | 2 | 0 |
| Supernatant from sonic extract | 16 | 32 | 0 |
| <i>B. bassiana</i> (#5477) | | | |
| Dry conidia | 4 | 0 | 16 |
| Sonicated conidia | 4-8 | 8 | 8-16 |
| Supernates from sonic extract | 2 | 2 | 8 |
| <i>M. anisopliae</i> (#5507) | | | |
| Dry conidia | 4-64 | 128 | 4 |
| Supernatants from sonic extract | 2,048-4,096 | 512-4,096 | Tr ^b |
| Supernatants from sonic extracts treated for 1 h at 60°C | 2 | Tr | 0 |

^a HA for horse erythrocytes was zero for all conidial preparations.

^b Tr. Trace levels of HA detected.

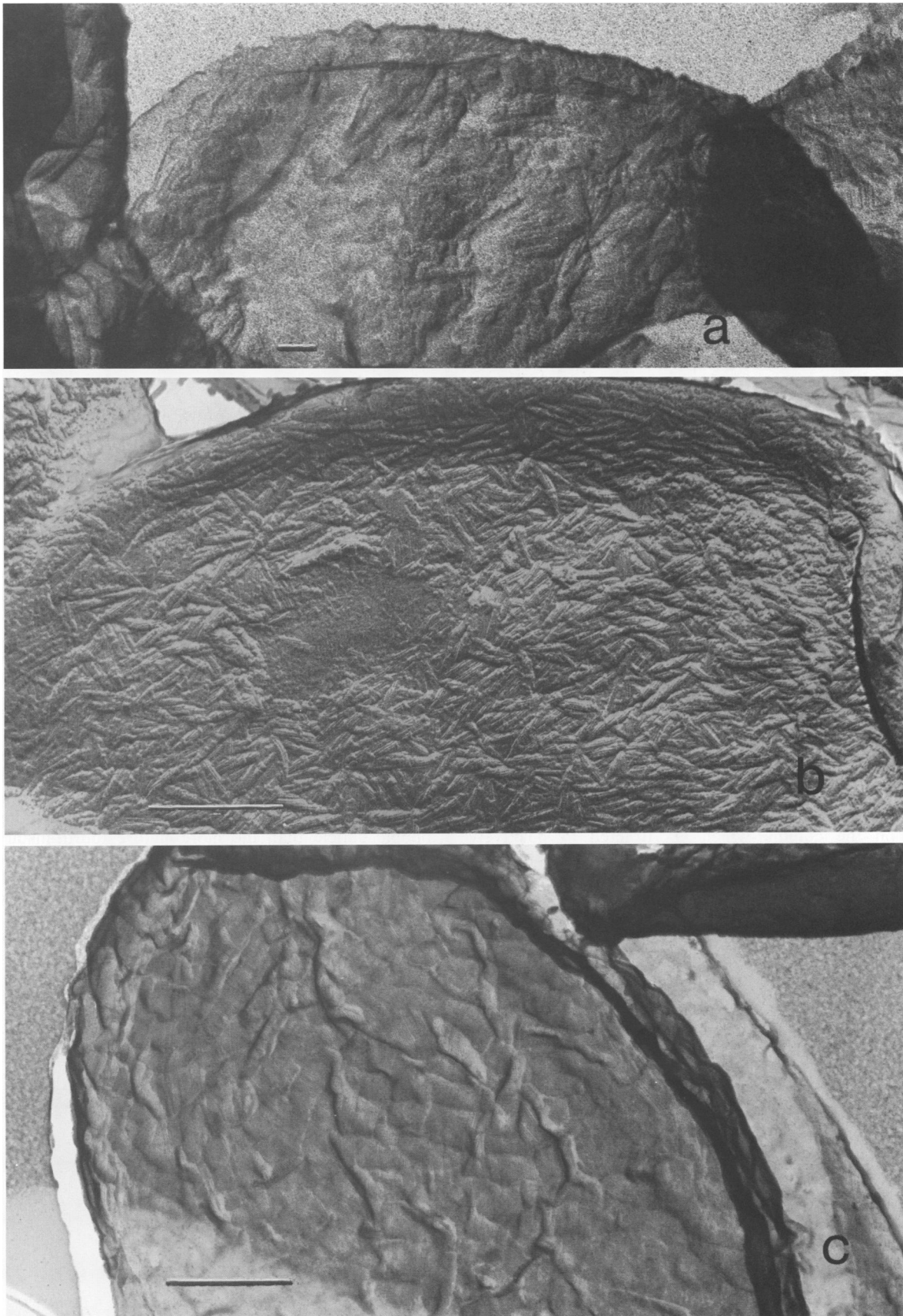


FIG. 5. Replicas of (a) *B. bassiana*, (b) *N. rileyi*, and (c) *M. anisopliae* conidia which had been incubated in 0.1 M NaOH for 17 h at room temperature. Note etching of rodlet layer of *N. rileyi* and *M. anisopliae*, whereas this layer has been almost completely digested from the surfaces of *B. bassiana* conidia. Bar = 0.1 μm (a) or 0.5 μm (b and c).

TABLE 5. Carbohydrate inhibition of *M. anisopliae* (UF1 5507) conidial extract hemagglutinin

| Carbohydrate ^a | Hemagglutination of erythrocytes ^b from: | |
|---------------------------------|---|---------|
| | Rabbit | Human O |
| D-Glucose | + | + |
| D-Mannose (100 mM) | ++ | - |
| α -Methylmannoside | + | - |
| Glucuronic acid | - | - |
| <i>N</i> -Acetylglucosamine | - | - |
| Trehalose | - | - |
| L-Rhamnose | - | - |
| D-Galactose | + | - |
| Lactose | + | - |
| L-Fucose (100 mM) | ++ | ++ |
| Galacturonic acid (100 mM) | ++ | + |
| <i>N</i> -Acetylgalactosamine | + | - |
| <i>N</i> -Acetylneuraminic acid | - | - |
| EDTA (100 mM) | ++ | ++ |

^a Carbohydrate concentration was 200 mM unless otherwise stated.

^b Symbols: -, hemagglutination (HA) not inhibited; +, HA inhibited by 200 mM sugar; ++, HA inhibited by <200 mM sugar.

drates assayed, including those known to inhibit the *M. anisopliae* HA, was effective in suppressing conidial adhesion. Further evidence for the noninvolvement of these hemagglutinins in attachment is indicated by both the failure of *M. anisopliae* conidia to bind to Sepharose beads, which possessed the HA-binding galactose and fucose residues, and the inability of *B. bassiana* to bind to chitin flakes (although *B. bassiana* hemagglutinin was inhibited by *N*-acetylglucosamine).

The involvement of electrostatic charge in the attachment was investigated by using various noncuticle substrata (Table 6). Conidial preparations not attaching to chitin or CM-Bio-Gel beads were highly attracted to both chitosan (deacylated chitin) and DEAE-Bio-Gel beads. Adhesion to these latter substrates is mediated by an overall negative charge on the conidia which is attracted to positively charged surfaces (chitosan, DEAE-Bio-Gel beads). Pretreatment of conidia with poly-L-lysine inhibited binding of conidial suspensions to previously mentioned substrata. However, pretreatment of conidia with poly-L-lysine did not inhibit adhesion to cuticle substrata. Likewise, pretreatment of cuticle ghosts with poly-DL-aspartic acid (10 mg/ml) prior

to the addition of conidia also failed to prevent adhesion of sonicated conidia.

The two experiments to measure the hydrophobicity of the conidial preparations demonstrated that all three species were extremely hydrophobic (Table 7). The results of a salt aggregation assay showed that less than 0.01 M (NH₄)₂SO₄ was sufficient to cause visible precipitation in all of the conidial preparations. Results of the phase exclusion assay indicated that *M. anisopliae* and *B. bassiana*, having more than 95% of the conidia excluded from any of the pH regimes assayed, were somewhat more hydrophobic than *N. rileyi* conidia. The phase exclusion assay was also performed on extracted intact rodlets from *B. bassiana*. At pH 6.0, more than 98% of the rodlet material was excluded from the aqueous phase.

DISCUSSION

The attachment of *N. rileyi*, *M. anisopliae*, and *B. bassiana* conidia to insect cuticle is nonspecific. In this study, the cuticle ghost of the *A. gemmatilis* larva, a host to *N. rileyi*, was an attractive substratum for both *B. bassiana* and *M. anisopliae*. Correspondingly, *N. rileyi* conidia have been shown to attach readily to the cuticle of non-host insects, such as the pea aphid *Acrythosiphon pisum* (Boucias and Latge, unpublished data). Firm attachment of the conidial preparations occurred immediately upon contact with the cuticle substrata. Attempts to remove the conidia after a contact period of less than 5 min failed to displace the conidia from the cuticle surface (Fig. 6a). Conidia which were killed by boiling in detergent (1% SDS) or solvents (chloroform-methanol [2:1, vol/vol]) or by incubation in fixatives (2% glutaraldehyde) readily attached to cuticle substrata after being rinsed in H₂O. These findings suggest strongly that conidial attachment is passive, involving neither the synthesis nor the release of adhesive material.

Conidial attachment occurred over the entire epicuticle of the insect cuticle ghost preparations. Qualitative observations indicated that first-instar larvae possess a more hydrophobic cuticle than the more-mature fourth-instar larvae. Furthermore, when incubated with conidial suspensions (10⁷ conidia per ml), cuticle ghosts of first-instar larvae were completely coated with a monolayer of conidia, whereas substantially fewer conidia attached to fourth-instar larvae cuticle ghosts. Examination of conidium-cuticle ghost preparations demonstrated that the presence and spatial arrange-

TABLE 6. Adhesion of conidial preparations to noncuticle substrata

| Substratum | Preincubation treatment | Conidial binding ^a of: | | |
|------------------------|--------------------------------------|-----------------------------------|--------------------|----------------------|
| | | <i>N. rileyi</i> | <i>B. bassiana</i> | <i>M. anisopliae</i> |
| Sepharose 4B beads | H ₂ O | - | NT ^b | - |
| Fucose-Sepharose beads | H ₂ O | - | NT | - |
| Fetuin-Sepharose beads | H ₂ O | - | NT | - |
| Chitin flakes | H ₂ O | - | - | - |
| Chitosan | H ₂ O | + | + | + |
| DEAE-Bio-Gel beads | H ₂ O | + | + | + |
| CM-Bio-Gel beads | H ₂ O | - | - | - |
| Chitosan | <i>N</i> -Acetylglucosamine (200 mM) | + | + | + |
| Chitosan | Glucosamine (200 mM) | + | + | + |
| Chitosan | Poly-L-lysine (1 mg/ml) | - | - | - |
| DEAE-Bio-Gel beads | Poly-L-lysine | - | - | - |
| Cuticle ghosts | Poly-L-lysine | + | + | + |

^a Conidial suspensions (10⁷ to 10⁸ conidia per ml) were incubated in preincubation solution for 60 min, centrifuged, and then resuspended and mixed with substrata as outlined in Materials and Methods. +, Binding; -, no binding.

^b NT, Not tested.

TABLE 7. Relative hydrophobicity of conidial preparations as determined by salt aggregation and phase exclusion assays

| Assay | Hydrophobicity or molarity ^a of conidial preparations of: | | |
|------------------|--|--------------------|----------------------|
| | <i>N. rileyi</i> | <i>B. bassiana</i> | <i>M. anisopliae</i> |
| Phase exclusion | | | |
| pH 3.0 | 69% | 100% | 100% |
| 6.0 | 92% | 100% | 100% |
| 9.0 | 71% | 96% | 100% |
| Salt aggregation | <0.01 M | <0.01 M | <0.01 M |

^a Data for salt aggregation assay represent the molarity of $(\text{NH}_4)_2\text{SO}_4$ causing visible precipitation of assayed conidia. Data for phase exclusion assay represent the percentage of conidia which were removed from the aqueous phase by vortexing with toluene.

ment of cuticular spines facilitated conidial attachment (Fig. 6b). Sets of three to five spines appeared to bind to and trap conidia. Examination of thin sections through the cuticle demonstrated that each spine consisted of a thickened osmophilic epicuticle overlying an amorphous granular core. The presence of these spines was not a requirement for attachment, since conidia were also found to adhere to cuticle surfaces (head capsule, seta, etc.) which were void of these spines. The presence of the epicuticle, however, is essential for conidial attachment. Pretreatment of the cuticle with detergents (SDS), denaturing agents (urea), or solvents (chloroform-methanol, hexane), while removing certain components from the cuticle, did not reduce their hydrophobicity or their affinity for conidial attachment. These results

indicate that the extractable cuticular lipids previously reported to possess antifungal activity (19) or to serve a nutritional role in germination (2) play little, if any, role in the attachment of these conidia to insect cuticle.

The conidial surfaces of the three entomopathogenic fungi were covered by well-organized fascicles of rodlets. The rodlet layers of *B. bassiana* and *N. rileyi*, organized as interwoven bundles, were very similar to those detected on *Neurospora* spores (9). The surfaces of *M. anisopliae* conidia, composed of linear arrays of small short rodlets (>50 rodlets per bundle), were easily distinguished from the observed surfaces of *N. rileyi* and *B. bassiana* conidia. The rodlet layers of all three fungi, being resistant to enzymes, denaturation agents, detergents, and solvents, possessed the chemical stability reported previously for *Penicillium* and *Trichophyton* conidia (10, 11). The rodlet layer of *B. bassiana*, a fungus which produces unpigmented conidia, appeared to be more sensitive to mild alkali treatment and to sonication (which resulted in partial removal of intact rodlets) than were those of *N. rileyi* and *M. anisopliae*. Harsh alkali treatment (1.0 M NaOH, 100°C for 1 h) was the only treatment that completely stripped away the rodlet layer from these three fungal species and caused significant decreases in the level of conidial attachment to the cuticle surface.

The objective of this study was to identify the force(s) responsible for conidial adhesion to insect cuticle. Initial experiments revealed the presence of various hemagglutinins on intact conidia and in supernatants from sonicated conidia. The failure of various hapten sugar combinations to competitively inhibit conidial binding suggests that these

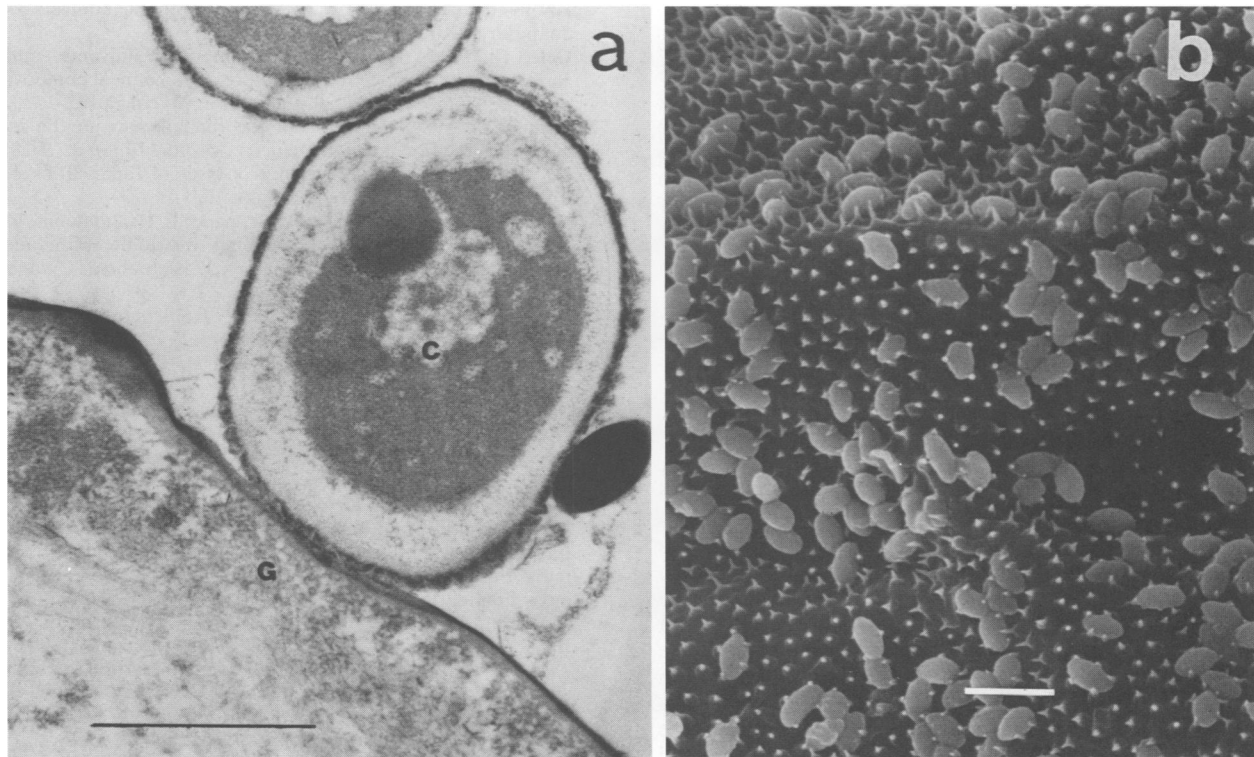


FIG. 6. (a) Thin section through a conidium (c) allowed to attach to a cuticle ghost (G). Cuticle ghosts were subsequently boiled in 1% SDS in an attempt to remove conidia. Bar = 1 μm . (b) Scanning electron micrograph of *N. rileyi* conidia on a cuticle ghost. Note the association between spines and conidia. Bar = 5 μm .

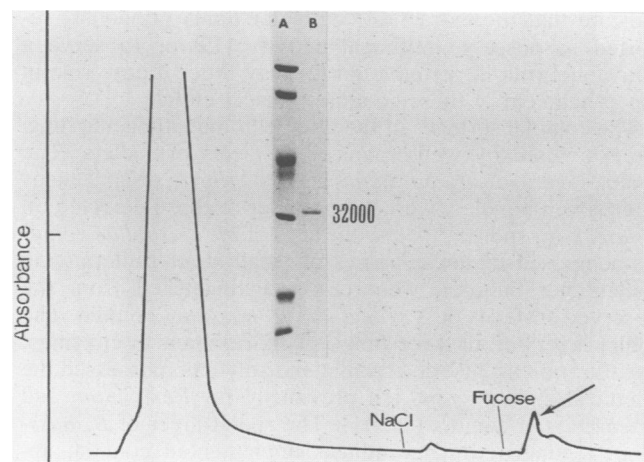


FIG. 7. Extraction of *M. anisopliae* lectin by using fucose-agarose affinity resin. Fucose-binding protein was selectively eluted from resin with 200 mM fucose in 0.05 M Tris hydrochloride (pH 7.8) buffer. Fucose-binding protein (lane B) had a calculated molecular weight of 32,000 when run in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular weight standards phosphorylase BB, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme are shown in lane A.

compounds are not involved in the attachment process. These findings are supported by recent studies on the isolated *M. anisopliae* hemagglutinin. With a fucose-agarose resin, the major lectin molecule (M_w , 32,000) has been isolated from *M. anisopliae* conidial sonic extracts (Fig. 7). Rhodamine and ferritin conjugates of this lectin failed to bind to the surfaces of cuticle ghosts. The role of the hemagglutinins detected in the conidia remains to be elucidated.

The conidia of all three species bound readily to DEAE ion-exchange resins, indicating the presence of a net negative charge on the conidial surfaces. This electrostatic charge could be effectively neutralized by preincubating conidia in poly-L-lysine prior to incubation with either chitosan or DEAE-resin substrata. A similar poly-L-lysine pretreatment did not influence the binding of conidia to cuticle substrata. Additionally, various combinations of poly-L-lysine and hapten sugars were tested (data not shown) but also failed to inhibit conidial binding to cuticle.

The hydrophobicity of these conidia is responsible for their adhesion to insect cuticle. The salt aggregation assay, reported to be very effective for measuring very hydrophobic microorganisms (14), demonstrated that all three entomopathogenic fungi produce conidia which are extremely hydrophobic [flocculation at 0.01 M $(\text{NH}_4)_2\text{SO}_4$]. Neutralization of these hydrophobic properties with detergents, solvents, or high-molecular-weight proteins resulted in significant reductions in the numbers of conidia adhering to cuticle preparations. At present, the component(s) responsible for the hydrophobicity expressed by these conidia has yet to be identified. Intact rodlets extracted from *B. bassiana* spores were extremely hydrophobic, as demonstrated by the results of the phase exclusion assay. Harsh alkali treatment (boiling for 1 h in NaOH) of conidial samples, despite effectively stripping away the outer rodlet layer, resulted in only a partial reduction (4 to 18%) in the relative hydrophobicity of treated conidia. These results suggest that the components responsible for hydrophobicity are located throughout the cell wall of these conidia.

It has been suggested previously that the rodlet layer present on fungal spores functions to protect against dehy-

dration (18), provide water-repellent properties (11), or serve in the aerial dispersal of conidia (1). Our studies demonstrate that the conidia of the entomopathogens *B. bassiana*, *M. anisopliae*, and *N. rileyi* also possess a hydrophobic rodlet layer which may provide protection against dehydration (and microbial attack) and a means of dispersal in air currents. However, a major role of the rodlet layer present on these fungi is the attachment of conidia to insect cuticle, a process which is mediated by a hydrophobic interaction. While this interaction does not appear to be specific to a particular host, it does provide a means for preferential binding to insect epicuticle, which is less hydrophobic than other substrata (soil particles, etc.).

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