

Genetic and Biochemical Characterization of *Nectria haematococca* Strains with Adhesive and Adhesion-Reduced Macroconidia

L. EPSTEIN,* Y. H. KWON, D. E. ALMOND,† L. M. SCHACHED, AND M. J. JONES‡

Division of Entomology, Plant and Soil Microbiology, Department of Environmental Science, Policy and Management, University of California, Berkeley, California 94720

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A previous study reported the isolation of two mutants (LE1 and LE2) of the plant pathogenic fungus *Nectria haematococca* (anamorph, *Fusarium solani* f. sp. *cucurbitae*) with macroconidia with reduced ability to adhere (Att^-) to zucchini fruits and polystyrene. The adhesion-reduced phenotype in LE1 and LE2 macroconidia is temperature sensitive and dependent on the concentration of nutrients. Classical genetic analysis of progeny derived from LE1 identified a mutation in a genetic locus, named *Att1*. The 90-kDa glycoprotein and macroconidial tip mucilage which were previously associated with the development of adhesion competence in Att^+ macroconidia are specifically associated with macroconidia; neither is produced on microconidia, which are relatively nonadherent. However, macroconidia of both Att^+ and Att^- strains produce the 90-kDa glycoprotein and the macroconidial tip mucilage.

Many fungal species produce vegetative and reproductive structures which adhere to a variety of natural and synthetic surfaces (5, 8, 9, 14, 20). Although the importance of adhesion in plant pathogenesis has been acknowledged, the phenomenon has not been well characterized. No compound that mediates attachment of fungi to plants has been fully characterized, and little is known about the genetic and biochemical basis of fungus-substratum binding (14).

As a model system for investigating adhesion of phytopathogenic fungi, we are studying macroconidium-substratum adhesion of the fungus *Nectria haematococca* Berk. & Br. mating population I (anamorph, *Fusarium solani* Appel & Wollenw. f. sp. *cucurbitae* Snyd. & Hans. race 1) (2, 11–13). In nature, *N. haematococca* macroconidia are water dispersed (1) by either rain or overhead irrigation. Macroconidia are nonadherent in water (11, 13). However, they become adhesive within 10 min after incubation in an adhesion-inducing medium such as zucchini fruit extract (ZE) (11, 13). Also within the same 10-min incubation, *N. haematococca* macroconidia adhere specifically at the spore apices, produce a macroconidial tip mucilage at the spore apex which can be labeled with concanavalin A (ConA), and produce a 90-kDa glycoprotein which can be labeled with ConA (13). Interestingly, the production of the material at the site of attachment and the production of the 90-kDa glycoprotein are specifically associated with adhesion and are not generally associated with the process of germination; neither the spore tip mucilage nor the 90-kDa glycoprotein is observed on spores incubated in media that induce germination but not adhesion.

N. haematococca is well suited for genetic analyses (21, 23). It is an ascomycete with haploid hyphae and spores. The fungus is heterothallic, and after karyogamy, the two divisions of meiosis, and one mitotic division, the four meiotic products, present as eight unordered ascospores, are contained within a

sac, the ascus. Each ascospore in the ascus can be isolated, clonally propagated, and induced to form asexual conidia. As an experimental tool to investigate adhesion, Jones and Epstein (12) isolated two independently derived mutants (LE1 and LE2) which have macroconidia with reduced adherence to zucchini (*Cucurbita pepo* L.) fruits and to polystyrene. The macroconidial adhesion phenotype was named *Att*, for attachment. Analysis of the two Att^- strains suggested that adhesion of *N. haematococca* to its host surface is a virulence factor.

In this study, first, we demonstrated that LE1 and LE2 strains are temperature sensitive and that the *Att* phenotype is dependent on the concentration of ZE. Second, we used classical genetic analysis to show that progeny derived from LE1 have a mutation at a genetic locus, named *Att1*. Third, we microscopically characterized several Att^- progeny derived from LE1. Fourth, we demonstrated that the macroconidial tip mucilage and the 90-kDa glycoprotein which were associated with the induction of adhesion competence are produced only in macroconidia, not in microconidia, which do not adhere efficiently. Fifth, we demonstrated that both Att^+ and Att^- strains produce the macroconidial tip mucilage and the 90-kDa glycoprotein.

MATERIALS AND METHODS

Parental strains. All strains were single spore purified. The wild type and two adhesion-reduced mutants were described previously (12). Briefly, microconidia from a wild-type strain (Nhl-2) were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. After an enrichment procedure, two mutants (LE1 and LE2) with macroconidia with reduced ability to adhere (Att^-) were isolated. Nhl-2 and its clonal progeny are mating type 1 (*MATI-1*) and male sterile (*Mal^-*), i.e., female only. Nhl-5, another wild-type strain, has the opposite mating type (*MATI-2*) and is male fertile (*Mal^+*), i.e., hermaphroditic.

Mating. For matings, we used strains with opposite mating types, the requisite gender, and the desired adhesion phenotypes. Crosses are written as female \times male. Fertilization and ascospore isolation were performed essentially as described by Georgopoulos (6), Snyder et al. (21), and Van Etten and Kistler (23). Briefly, dishes of males and females were produced on V8 agar (12). A suspension of washed conidia,

* Corresponding author. Phone: 510-643-9224. Fax: 510-642-3845. Electronic mail addresses: Internet, lepstein@uclink.berkeley.edu; Bitnet, lepstein@ucbuclin.bitnet.

† Present address: Health and Medical Sciences/Joint Medical Program, University of California, Berkeley, CA 94720.

‡ Present address: Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907.

serving as males, was poured onto a culture with protoperithecia, serving as females. After the conidia settled, the excess liquid was decanted. Perithecia were present after dishes were incubated at 24°C for 7 to 10 days under fluorescent lights with an alternating 12-h light/dark cycle.

Ascospore isolation. After perithecia were produced, we isolated either all ascospores from single asci or single, random ascospores from perithecial cirri. For random ascospore analyses, we examined clonal progeny from 10 to 12 ascospores from either four or five perithecia. Random ascospores were named by using an alphanumeric code, RWXY, where R indicates a random ascospore, W designates the cross, X designates the perithecium, and Y designates the ascospore. For tetrad analysis, only asci with seven or eight germinable ascospores, hence all meiotic products, were used. Asci were named by using a similar alphanumeric code, WXY, where W, X, and Y designate the cross, the perithecium, and the ascus, respectively. For example, 721 and 921 were asci from crosses performed at separate occasions and hence from different perithecia. When present, the fourth number in the alphanumeric code designates the ascospore. In the three asci (441, 611, and B22) in which only seven germinable ascospores were recovered from an ascus, the segregation data were inferred for the eighth ascospore.

Determination of mating type and gender. Duplicate dishes of the two hermaphroditic tester strains 38 (*MATI-1*) and 49 (*MATI-2*) and the clonal progeny were used as both female and male.

Assays for macroconidial adhesion. Macroconidia derived from individual ascospore progeny were stored, produced, and harvested essentially as described previously (11, 12). Briefly, cultures were stored as conidial suspensions in an aqueous solution of 30% (wt/wt) glycerol at -70°C. To produce macroconidia, potato dextrose agar dishes were seeded and incubated at 24°C for 7 days under continuous fluorescent lighting. After the culture dishes were flooded with deionized water, the macroconidia were collected, washed twice in water by centrifugation in 6-ml tubes for 3 min and then 6 min at 220 × g, and resuspended in water.

For adhesion assays, macroconidia were incubated in ZE and evaluated for adhesion on polystyrene (11–13). To prepare ZE, 200 g of 1-cm-thick slices of zucchini fruits per liter of water was incubated on a hot plate. When the liquid was 90°C, the fruit was removed by pouring the broth through cheese-cloth. After the broth was cooled to 24°C and the pH was adjusted to 6.5 with 1 N KOH, the broth was filtered through Whatman GF/C glass fiber filters and then autoclaved.

The adhesion assays were conducted with some modifications from previous descriptions (11–13). First, we determined the concentration of the spore suspensions collected from the culture plate. Subsamples were suspended in a counting medium with a final concentration of 0.02% Tween 20 and 100 mM KCl; spore concentrations were determined with a particle counter (Elzone model 80; Particle Data Inc., Elmhurst, Ill.). The counter, equipped with a 150- μ m-diameter orifice and a 1-ml sampling tube, was set to count particles with volumes of >464 μ m³. Second, water was added to each spore suspension so that each suspension had the same concentration. In different trials, there were 2×10^5 macroconidia in 400 to 800 μ l. For the adhesion assay, for each 60-mm-diameter polystyrene petri dish, 2×10^5 macroconidia were added to 7 ml of ZE (11). The assay dishes were incubated at 28°C for 1 h unless indicated otherwise. After the incubation, nonadherent spores were removed; dishes were gently swirled, decanted, and rinsed twice with 10 ml of water. The recovered nonadherent spores were suspended in the Tween 20-KCl counting

medium, and the number of nonadherent macroconidia recovered from each polystyrene dish was quantified with the particle counter.

To ensure that the particle counter results were reliable, we first compared counts of Nhl-2 and LE1 macroconidia obtained with a hemacytometer with those obtained with the particle counter. Second, we used the hemacytometer to adjust the 11 strains of macroconidia to the same concentration. After an adhesion assay, we compared the percent adhesion data obtained by direct microscopic counting of the petri dishes (5) with data obtained from enumeration of nonadherent macroconidia by using the particle counter.

For tetrad analyses of adhesion, each trial included the two parents and all of the ascospore progeny from a single ascus. For the f_1 analyses, each trial also included the wild-type strain Nhl-2. For random ascospore analyses, each trial included the two parents and 10 to 12 strains derived from a single perithecium. When two parents with the same adhesion phenotype were crossed, each trial also included a control strain with the alternative adhesion phenotype. For all adhesion assays, there were two replicates per strain. Each trial was performed at least twice, and percentage adhesion data from two or three trials were analyzed by an analysis of variance (ANOVA) as a randomized complete block design with trial dates as blocks. From an analysis by the Student-Newman-Keuls multiple-range test at $P = 0.05$, ascospore progeny were assigned to a statistical group for the level of adhesiveness.

To determine the effect of microconidia on macroconidial adhesion, Nhl-2 and Nhl-5 microconidia were produced on V8 agar plates; plates were seeded and incubated at 28°C for 7 days in darkness. Microconidia were collected, washed, and enumerated as described above except that a hemacytometer was used to determine the microconidial concentration. To assess microconidial adhesion in water and zucchini extract, 10^7 microconidia were added per assay dish. After incubation, dishes were washed as described above and the number of nonadherent microconidia was determined with a hemacytometer. To determine whether microconidia interfered with adhesion of macroconidia, 2×10^5 macroconidia with or without 2×10^5 microconidia were added to the assay dishes. The number of nonadherent macroconidia was determined with the particle counter as described above. The experiments were performed twice with two replicates per strain.

Assays for macroconidial germination. In addition to quantifying adhesion of the macroconidia, we quantified germination as a check on viability. After macroconidia were incubated in ZE for 5.5 to 6.5 h at 28°C in 20- μ l droplets on multiwell slides (Carlson Scientific, Peotone, Ill.), percent germination for three replicates per strain was determined.

Microscopic examination of conidia. To quantify the number of micro- and mesoconidia present in the assay dishes, conidia were harvested and washed as described for the adhesion assay. Conidial suspensions were mounted for light microscopy, and the percentages of one- and two-celled microconidia, three-celled mesoconidia, and four- or more-celled macroconidia were determined at $\times 200$ magnification. One hundred conidia were evaluated in each of two determinations. To determine the average macroconidial length, 25 conidia in each of two determinations were measured at $\times 200$. The experiment was performed four times.

To visualize macroconidial tip mucilage, macroconidia were incubated in ZE on glass coverslips for 30 min. After the ZE was wicked away, we deposited a 50- μ l droplet containing 2 mg of fluorescein isothiocyanate (FITC)-conjugated ConA (Sigma) per ml, 1 mM Na₂HPO₄, 1 mM KH₂PO₄, 6.2 mM NaCl, 0.3 mM KCl, 12.5 μ M CaCl₂, and 12.5 μ M MnCl₂. After

incubation for 30 min in darkness, macroconidia were examined immediately by fluorescence microscopy on a Nikon Optiphot (13). Experiments were performed three times with similar results.

Gel electrophoresis and Western blot (immunoblot) analysis. Macroconidia were harvested as indicated above except that the spore suspensions were centrifuged in 50-ml tubes at $200 \times g$ for 2 min. The supernatant was enriched with microconidia and was discarded except when indicated otherwise. The spore preparations were washed a total of three times. Whenever macroconidia were prepared for a biochemical analysis, a subsample was assayed for adhesion as described above. *N. haematococca* macroconidia ($2 \times 10^6/8.5$ -cm-diameter dish) were incubated at 28°C in 14 ml of ZE for 50 min. Ten dishes were used for each experimental treatment. Adherent macroconidia were gently dislodged with a camel hair brush. Both nonadherent and detached conidia were collected; examination of dishes under a dissecting microscope indicated that adherent spores were efficiently detached and collected. After the spores were collected from the assay dishes, they were pelleted by centrifugation at $1,000 \times g$ for 5 min. The supernatant was discarded, the pellet was wicked dry, and a 100- μ l conidial pellet was incubated at 100°C for 5 to 10 min in an equal volume of 625 mM Tris-HCl (pH 6.8)–2% sodium dodecyl sulfate (SDS)–5% β -mercaptoethanol–10% glycerol–0.05% bromophenol blue (10). Biotinylated molecular weight standards (Bio-Rad) and 10- and 50- μ l aliquots of sample supernatants for the silver staining and blots, respectively, were loaded onto SDS–10% polyacrylamide gels. As a control for any residual ZE in the pellet, an aliquot containing the equivalent of 500 μ l of freeze-dried ZE also was loaded. Electrophoresis gels were run overnight at a constant 50 V. Gels were either stained with periodic acid and silver (3, 15) or electroeluted onto nitrocellulose at a constant 450 mA for 3 h at 23°C and probed as described previously (13). Briefly, blots were developed by using ConA-biotin, streptavidin-conjugated alkaline phosphatase, and 5-bromo-4-chloro-3-indolylphosphate and *p*-nitroblue tetrazolium chloride. In selected trials, to confirm that ConA was binding α -mannose or α -glucose residues, 10 μ g of ConA-biotin per ml was incubated with 100 mM α -methyl mannoside for 30 min before use as a probe. All biochemical experiments were performed at least three times with similar results.

RESULTS

Adhesion assay. With the particle counter set so that only particles of $>464 \mu\text{m}^3$ were counted, we determined that counts of macroconidia of NHI-2 and LE1 on the hemacytometer were statistically ($P > 0.05$) indistinguishable from total spore counts on the particle counter. Thus, at this setting, the particle counter counted macroconidia but not microconidia. Also, we compared the percent adhesion data obtained by direct microscopy of the dishes with the data obtained by using the particle counter to quantify nonadherent macroconidia; the data were highly correlated ($r = 0.98$).

Temperature sensitivity of adhesion in the mutant strains. Percent adhesion data for macroconidia of the two wild-type strains, NHI-2 and NHI-5, were statistically indistinguishable (Table 1). At all three incubation temperatures, LE2 macroconidia adhered significantly less than the wild-type strains; LE1 macroconidia adhered significantly less than either LE2 or the wild-type strains. Increases in assay temperature caused a relatively small but significant decrease in adhesion of wild-type macroconidia. In contrast, increases in assay temperature caused a large decrease in adhesion of LE1 and LE2

TABLE 1. Effect of temperature on adhesion of macroconidia from different strains of *N. haematococca*

Strain ^b	Adhesion (% \pm SD) ^a at:		
	16°C	24°C	28°C
NHI-2	82 \pm 2	82 \pm 1	72 \pm 2
NHI-5	84 \pm 1	84 \pm 1	73 \pm 3
LE1	67 \pm 3	45 \pm 2	16 \pm 2
LE2	75 \pm 2	51 \pm 7	27 \pm 9

^a Data from three replicates were analyzed by a two-way ANOVA. There were highly significant ($P < 0.00001$) strain effects, temperature effects, and a strain-temperature interaction. Least significant difference ($P = 0.05$) is 3 for both strains and temperatures. The experiment was repeated with similar results.

^b NHI-2 and NHI-5 are wild types of opposite mating types. LE1 and LE2 were clonal progeny from chemically mutagenized NHI-2.

macroconidia. Thus, in comparison with wild-type strains, LE1 and LE2 were least reduced in adhesion at 16°C, more reduced at 24°C, and most reduced at 28°C. While increased temperature reduced adhesion of LE1 and LE2 macroconidia, it had no effect on germination of these macroconidia; after incubation at 28°C for 5.5 h, $>95\%$ of the macroconidia germinated in all strains. Thus, reduced adhesion in LE1 and LE2 at 28°C is not due to a general debilitation at higher temperatures. Our interpretation of the data is that adhesion of LE1 and LE2 macroconidia is temperature sensitive. All subsequent adhesion assays were conducted at 28°C, since the numerical difference between percent adhesion data for the Att⁺ and Att⁻ strains was greatest at this temperature.

ZE concentration dependence. ZE was lyophilized and reconstituted at the indicated concentration (Table 2). After incubation on polystyrene for 1 h in either $1 \times$ or $0.2 \times$ ZE, the Att⁻ strain LE1 and LE2 macroconidia adhered significantly less ($P = 0.05$) than the Att⁺ wild-type strain NHI-2 and NHI-5 macroconidia. However, in $4 \times$ ZE, none of the strains adhered (2 to 7%), even though macroconidia of all of the strains germinated 100% after 6 h. In $0.02 \times$ ZE, macroconidial adhesion in all strains was intermediate (46 to 50%). Thus, while $\geq 97\%$ of the macroconidia of all strains germinated in all concentrations of ZE tested, $4 \times$ ZE inhibited adhesion in all four strains, $1 \times$ and $0.2 \times$ ZE induced greater adhesion in the two Att⁺ strains than in the two Att⁻ strains, and $0.02 \times$ ZE induced an intermediate level of adhesion in all strains.

Genetic analysis of LE1. Using a quantitative adhesion assay, we used an ANOVA and a multiple-range test to classify the adhesiveness of ascospore progeny. In comparison with the parental and control strains, all progeny were parental Att⁺, parental Att⁻, or in four cases, a nonparental intermediate type. The multiple-range test never indicated additional non-

TABLE 2. Effect of concentration of ZE on adhesion of macroconidia from different strains of *N. haematococca*

Strain	Adhesion (% \pm SEM) ^a at concn of:			
	$4 \times$ ZE	$1 \times$ ZE	$0.2 \times$ ZE	$0.02 \times$ ZE
NHI-2	4 \pm 1	76 \pm 4	85 \pm 5	48 \pm 6
NHI-5	7 \pm 2	81 \pm 3	91 \pm 2	50 \pm 5
LE1	2 \pm 1	25 \pm 7	44 \pm 9	40 \pm 8
LE2	2 \pm 1	31 \pm 5	48 \pm 6	46 \pm 7

^a Data from four trials, each with two replicates, were analyzed by a two-way ANOVA as a randomized complete block design with trial dates as blocks. There were highly significant ($P < 0.00001$) strain effects, concentration effects, and a strain-concentration interaction. Least significant difference ($P = 0.05$) is 6 for both strains and concentrations.

TABLE 3. Genetic analyses of adhesion of clonally propagated macroconidia

Parental strains ^a	Segregation ratios of adhesion ^b			Comment
	Att ⁺	Att ¹	Att ⁻	
NhI-2 (Att ⁺) × NhI-5 (Att ⁺)	46	0	0	Random ascospores ^c
LE1 (Att ⁻) × NhI-5 (Att ⁺)	4	2	2	Two asci (413, 421) ^d
	4	0	4	Two asci (441, 481)
4815 (Att ⁺) × 4811 (Att ⁻)	4	0	4	Five asci (721, 722, 911, 921, A21)
4817 (Att ⁺) × 4811 (Att ⁻)	4	0	4	Three asci (611, B11, B21)
NhI-5 (Att ⁺) × 7226 (Att ⁻)	25	0	25	Random ascospores

^a NhI-2 and NhI-5 are wild-type strains. LE1 is a chemically induced mutant of NhI-2. 4815 are f_1 strains from a tetrad that segregated 4 Att⁺:4 Att⁻. 7226 is an F_2 strain from 4815 × 4811.

^b After the percentages of adhesion of macroconidia from the two parental strains and either all of the ascospore progeny from a single ascus or 10 to 12 random ascospores from a single perithecium were determined and the experiment was repeated, the data were analyzed by an ANOVA. Adhesion phenotypes of the ascospore progeny were assigned as statistical groups according to a Student-Newman-Keuls multiple-range test at $P = 0.05$. Data analysis indicated that all strains segregated as either parental Att⁺, parental Att⁻, or a nonparental intermediate (Att¹).

^c Data from random ascospores are from 10 to 12 ascospores from either four or five perithecia.

^d The intermediate phenotype was observed only in two f_1 asci; these asci were not used in subsequent crosses. See text for discussion.

parental types, such as an exceptionally high Att⁺ or an exceptionally low Att⁻. Typically, there was a 55 percentage point difference between adhesion of the wild type and LE1 (Tables 1 and 2) and a least significant difference ($P = 0.05$) of 13. Thus, the Att⁺ and Att⁻ categories appear to represent discrete phenotypes.

For the genetic analysis, first we crossed the two Att⁺ wild-type strains (NhI-2 [Att⁺ *MAT1-1 Mal*⁻] × NhI-5 [Att⁺ *MAT1-2 Mal*⁺]) and analyzed 46 progeny from a total of four perithecia (Table 3). All progeny were in a single Att⁺ group ($P = 0.05$). Second, we mated a chemically derived mutant of NhI-2, LE1 (Att⁻ *MAT1-1 Mal*⁻) × NhI-5 (Att⁺) and recovered four tetrads, each from a different perithecium. Our analysis of the clonal progeny for adhesion indicated that two of the f_1 asci (441 and 481) segregated 4 Att⁺:4 Att⁻. The other two f_1 asci (413 and 421) segregated 4 Att⁺:2 nonparental, intermediate-adhesion type: 2 Att⁻. Third, for f_2 analyses, we crossed Att⁺ and Att⁻ f_1 progeny from a single ascus (481) that had segregated 4 Att⁺:4 Att⁻. For these crosses, either 4815 (Att⁺ *MAT1-2 Mal*⁺) or 4817 (Att⁺ *MAT1-2, Mal*⁻) was used as the Att⁺ female and 4811 (Att⁻ *MAT1-1 Mal*⁺) was used as the Att⁻ male; 4815 and 4817 are not a mitotic pair. A total of eight asci from seven different perithecia were recovered. Progeny from all eight f_2 asci segregated 4 wild-type Att⁺:4 mutant-type Att⁻. Fourth, we mated wild-type NhI-5 (Att⁺) with the f_2 7226 (Att⁻ *MAT1-1 Mal*⁺) and examined a total of 50 random ascospores from five perithecia. The progeny segregated 25 wild-type Att⁺:25 mutant-type Att⁻, a perfect 1:1 segregation.

After a 5.5-h incubation, germination of macroconidia (presented as the mean ± standard deviation) from Att⁺ (95% ± 9%) and Att⁻ (98% ± 3%) strains was uniformly high. Thus, low adhesion was not correlated with low viability.

We determined mating type and male fertility of the progeny from the eight f_2 asci and the two f_1 asci that segregated 4 Att⁺:4 Att⁻. In all of the asci, mating type segregated 4:4, consistent with a trait previously determined to be conferred by alleles at a single locus (22). With regard to cosegregation of *Att* and *MAT1*, four of the asci (481, 721, 921, and B11) were parental ditypes, three (441, A21, and B22) were nonparental ditypes, and three (611, 722, and 911) were tetratypes. Male fertility (*Mal*) also was determined. As expected, in a cross in which both the female and male parents were hermaphroditic (4815 × 4811), all of the progeny (721, 722, 911, 921, and A21) were *Mal*⁺. In a *Mal*⁻ × *Mal*⁺ (4817 × 4811) cross, as expected (4), male fertility segregated 4:4. With regard to

cosegregation of *Att* and *Mal*, one of the asci (B11) was a parental ditype, none were nonparental ditypes, and four (441, 481, 611, and B22) were tetratypes. Thus, there is no evidence for linkage between *Att1* and either *MAT1* or *Mal*. Mating type and male fertility are not linked in *N. haematococca* mating population I (7).

Comparison of other phenotypes in selected Att⁺ (NhI-2 and NhI-5) and Att⁻ (LE1, 7226, R1CD, and R1EC) strains. On potato dextrose agar at 24°C in the presence of light, the wild-type strains NhI-2 and NhI-5 produced predominately (≥95%) macroconidia (Table 4). In addition to a very low (≤2%) percentage of one- and two-celled microconidia, the wild-type preparations also had a very low percentage (≤3%) of mesoconidia, i.e., three-celled conidia that morphologically resembled microconidia more than macroconidia. LE1 produced a higher ($P = 0.05$) percentage of microconidia and mesoconidia than the wild-type strains did. In addition, average macroconidial length in LE1 was shorter than in NhI-2 and NhI-5. We selected one of the f_2 Att⁻ strains, 7226, that had macroconidia significantly longer than those of LE1. However, 7226 had macroconidia that were shorter ($P = 0.05$) than wild-type macroconidia and produced more micro- and mesoconidia than the wild-type strains did. After backcrossing NhI-5 with 7226, we selected two Att⁻ strains (R1CD and R1EC) which were statistically ($P = 0.05$) indistinguishable

TABLE 4. Characteristics of wild-type, Att⁺ (NhI-2 and NhI-5), and selected Att⁻ (LE1, 7226, R1CD, and R1EC) strains of *N. haematococca*^a

Strain ^b	Type of conidia (%) ^c			Macroconidial length (μm)
	Macroconidia	Microconidia	Mesoconidia	
NhI-5	95 y	2 y	3 y	59 w
NhI-2	97 y	1 y	2 y	57 wx
LE1	61 z	29 z	10 z	48 z
7226	65 z	26 z	9 z	53 y
R1CD	97 y	1 y	2 y	56 x
R1EC	97 y	2 y	1 y	57 wx

^a Data from four trials were analyzed by a one-way ANOVA. Values within the same column followed by the same letter are not significantly different by the Student-Newman-Keuls multiple-range test ($P = 0.05$).

^b NhI-2 and NhI-5 are wild-type strains; LE1 is a chemically induced mutant of NhI-2; 7226 is an f_2 strain; R1CD and R1EC are products of NhI-5 × 7226.

^c Macroconidia had four or more cells, microconidia had one or two cells, and mesoconidia had three cells.

from NhI-2 with regard to macroconidial length. Also, R1CD and R1EC were statistically ($P = 0.05$) indistinguishable from wild-type strains in the percentages of macro-, meso-, and microconidia produced in light-grown cultures. The two Att^- strains that produce negligible percentages of meso- and microconidia demonstrate that these conidia are not causally associated with the Att^- phenotype in macroconidia.

Nonetheless, since several of the initially isolated Att^- strains produced more microconidia than either of the wild-type strains did (Table 4), we established that wild-type microconidia, in concentrations used in our assay system, did not affect macroconidial adhesion of the wild-type strains. Macroconidial adhesion assays with strains NhI-2 and NhI-5 were statistically indistinguishable ($P = 0.05$) when an equal quantity of microconidia either was or was not added to the assay dish. During the adhesion assay, NhI-2 and NhI-5 microconidia had relatively low percentages of adhesion; in both strains, approximately 18% of the microconidia adhered in water and 25% adhered in ZE.

Macroconidial tip mucilage on Att^+ and Att^- strains. We detected macroconidial tip mucilage with FITC-conjugated ConA. After incubation in ZE, both Att^+ (NhI-2 and NhI-5) and Att^- (LE1 and LE2) strains produced the macroconidial tip mucilage at one or both spore apices (data not shown). In all strains, only material at the apices was labeled with FITC-conjugated ConA. No differences were detected in the intensity of labeling at the apices of the selected strains. No labeling was detected on either the rest of the macroconidial surface or anywhere on microconidia. Tip mucilage was produced on the apices of the three-celled mesoconidia.

Biochemical comparison of Att^+ and Att^- strains. Previously, we examined extracts enriched for spore surface compounds by gel electrophoresis and Western analyses (13). Our results indicated that a 90-kDa glycoprotein which can be labeled with ConA is associated with the development of adhesiveness in wild-type macroconidia. As reported previously for NhI-2 (Att^+) (13), NhI-5 (Att^+) had bands which could be labeled with ConA with molecular masses of 90, 65, 39, 34, 19, and 16 kDa (Fig. 1A). The ConA probe apparently bound to either α -mannose or α -glucose residues on the fungal compounds, since binding of the probe to a macroconidial extract from NhI-2 was completely blocked by preincubation of the ConA with the hapten α -methyl mannoside (data not shown). We compared extracts from Att^+ and Att^- strains. In all strains, the banding patterns on the blots were generally similar, suggesting that the Att^- strains are not grossly pleiotropically affected. No differences between strains were detected in the 90-kDa band. On the Western blots, in contrast to NhI-5 (Att^+), LE1 had two additional bands at 32.5 and 14 kDa. Since, LE1 produced more microconidia than either NhI-2 or NhI-5 did (Table 4), a preparation of LE1 enriched for microconidia was analyzed (Fig. 1A, lane 6). LE1 microconidia did not have the 90-kDa band but did have the 32.5- and 14-kDa bands. Similarly, NhI-2 microconidia did not have a 90-kDa band (data not shown). Thus, the 90-kDa band is apparently specific for macroconidia. The control lane with concentrated ZE had no bands which were labeled with ConA (data not shown), indicating that all of the bands on the blot were of fungal origin and not from the medium.

Also, we compared gels of silver-periodic acid-stained conidial surface-enriched extracts from Att^+ and Att^- strains (Fig. 1B). All strains had similar bands. Predominant bands had molecular masses of 175, 65, 56, 50, 43, 39, 34, 32, and 29 kDa. Whereas the 90-kDa band was a prominent band on the ConA-labeled blots, the 90-kDa band was only a faint, but consistent, band on the silver-periodic acid-stained gels. Thus,

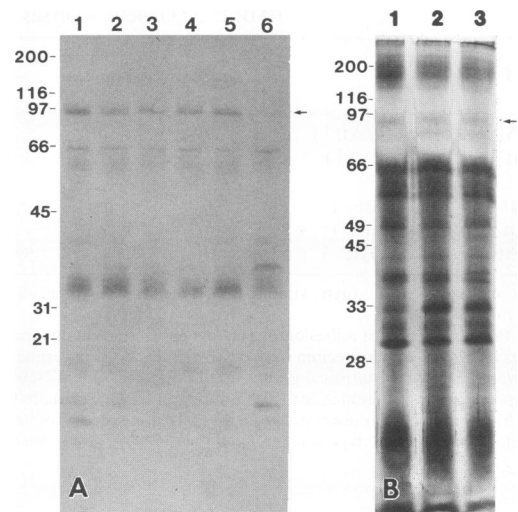


FIG. 1. A cell surface-enriched fraction from *N. haematococca* was separated by SDS-polyacrylamide gel electrophoresis. The molecular masses of standards are shown on the left in kilodaltons. The arrow indicates the 90-kDa glycoprotein. (A) Western blot probed with ConA. Lanes 1 to 5, macroconidial surface-enriched fractions. Lane 1, Att^+ wild-type NhI-5; lane 2, Att^- LE1; lane 3, Att^- LE2; lane 4, Att^- f₂ 7226; lane 5, Att^- f₂ B217; lane 6, microconidial surface-enriched fraction from LE1. (B) Gel stained with periodic acid-silver. Lane 1, Att^+ NhI-5; lane 2, Att^- LE1; lane 3, Att^- LE2.

only half of the major bands (i.e., the 65-, 39-, and 34-kDa bands) on the ConA-labeled blot were prominent on the silver-periodic acid-stained gel. The control lane with concentrated ZE had no distinct bands and a lightly stained smear of compounds with molecular masses of <45 kDa (data not shown). Thus, all of the higher-molecular-mass bands and probably all of the lower-molecular-mass bands on the silver-periodic acid-stained gel are of fungal origin.

DISCUSSION

We previously correlated the appearance of macroconidial tip mucilage and a 90-kDa glycoprotein with the development of adhesion competence of macroconidia in the wild-type strain NhI-2 (13). In this study, we demonstrated that the tip mucilage and the 90-kDa glycoprotein are specific for macroconidia. That is, microconidia, which are relatively nonadherent, do not produce either tip mucilage or the 90-kDa glycoprotein. Also, we demonstrated that the macroconidial tip mucilage and the 90-kDa glycoprotein are present on both Att^+ and Att^- strains. This may be evidence that the 90-kDa glycoprotein is merely associated with the development of adhesion competence and not the adhesive material itself. However, we would not have detected differences in the 90-kDa glycoprotein if the mutants were leaky, e.g., (i) produced slightly less (ca. 50 to 80% of the wild-type quantities) or (ii) produced defective adhesive material with either no change in molecular mass or a change of <5 kDa. Furthermore, it is possible that the 90-kDa glycoprotein is the adhesive compound and that the adhesion-reduced mutants do not adhere as well as the wild type because the mutants overproduce a compound which interferes with adhesion. For example, reduced adhesion of a *Pseudomonas fluorescens* mutant apparently was caused by the overproduction of an extracellular polysaccharide that inhibited adhesion (18, 19). Our ultrastructural observations of macroconidia of NhI-2, LE1, and LE2

were not inconsistent with the hypothesis that the Att^- strains overproduce an antiadhesive (2). LE1 had a statistically significantly ($P = 0.05$) wider electron-dense layer within the wall than the wild type did. LE2 had so much electron-dense material in the wall that wall layers were not resolvable. Our current efforts to identify the excessive electron-dense material in the mutant walls and to chemically characterize the 90-kDa glycoprotein should help to elucidate the biochemistry of adhesion of *N. haematococca* macroconidia.

Our data suggest that f_2 progeny derived from *N. haematococca* LE1 have a mutation at a single genetic locus (*Att1*). In an f_2 tetrad analysis of an $Att^+ \times Att^-$ cross, all eight tetrads segregated 4 $Att^+ : 4 Att^-$. In a random ascospore analysis of the wild-type $Att^+ \times$ an $f_2 Att^-$, the 50 progeny segregated 1 $Att^+ : 1 Att^-$. Analyses of additional progeny are necessary to determine whether this locus contains mutations in one or more linked adhesion genes. Nonetheless, *Att1* is the first adhesion gene described for a filamentous fungus.

We were unable to determine the genetic basis of the intermediate phenotype in the two f_1 asci that segregated 4 $Att^+ : 2$ nonparental intermediate : 2 Att^- (data not shown). However, mutagenized laboratory strains are often genetically heterogeneous, and a variety of phenotypes frequently appear in the first generation (17a). It is common practice to cross a new mutant with a standard strain to obtain f_1 progeny that is free of secondary mutations or modifiers (17). Regardless of the cause of the intermediate phenotype in the two f_1 asci, there is no evidence of any mutation other than *Att1* in the two f_1 strains (4815 and 4811) used to produce the $Att^- f_2$ strain 7226; the intermediate phenotype was not observed in either the 40 f_2 progeny from 4815 \times 4811 or the 50 backcrossed progeny from wild type \times 7226.

Our results confirm that LE1 and LE2 macroconidia incubated at 24°C in 1 \times ZE adhered approximately half as well as the wild-type NHI-2 (12). Here, we show that adhesion reduction in LE1 and LE2 is a temperature-sensitive phenotype and that at 28°C, the two mutant strains adhere only approximately one-third as well as the wild-type strains. On polystyrene, the adhesion-reduced phenotype in LE1 and LE2 is dependent on the concentration of the ZE. Our data suggest that ZE contains compounds which promote adhesion and compounds which inhibit adhesion, at least at high concentrations. Neither the adhesion-inhibiting nor the adhesion-promoting component(s) in ZE is known. The adhesion-promoting component(s) apparently is present on the surface of unwounded squash fruits, since NHI-2 macroconidia incubated in water on unwounded squash fruits adhered well (12); LE1 and LE2 macroconidia incubated in water on unwounded squash fruits adhered significantly less than the wild-type macroconidia. The fact that wild-type macroconidia adhere on the fruit surface suggests that any adhesion-inhibiting compound(s) is present in a very low concentration on the fruit.

Jones and Epstein (12) indicated that macroconidia of LE1 and LE2 were statistically significantly ($P = 0.05$) shorter than NHI-2 macroconidia. Here, we report the isolation of backcrossed Att^- strains which are statistically indistinguishable in length from NHI-2. This result supports our hypothesis that adhesion reduction in the Att^- strains is due to a biochemical abnormality in the compounds that mediate adhesion and not to a physical characteristic such as spore size.

We observed that LE1 and LE2 (data not shown) produced a higher percentage of microconidia and mesoconidia than the wild type did when grown on potato dextrose agar at 24°C in the light. However, the microconidia did not inhibit adhesion of macroconidia, at least in our adhesion assay conditions; we used a low spore density, with the macroconidia covering less

than 3% of the polystyrene substratum. The isolation of backcrossed Att^- strains which produce 97% macroconidia further demonstrates that the Att^- phenotype, which was expressed in macroconidia, is not affected by the presence of microconidia.

In this report, we classified the three-celled conidia as mesoconidia (16) because they did not fit the classical descriptions of either microconidia or macroconidia. Any epidemiological role of the three-celled conidia is unknown. In any case, the three-celled conidia are only a relatively small percentage ($\leq 10\%$) of the total number of conidia in any of the strains.

Although different strains had variable percentages of microconidia present in the spore suspensions, the particle counter assessed the number of macroconidia in the original spore suspension and the percentage of nonadherent macroconidia in the adhesion assay accurately; parameters on the particle counter were set so that macroconidia, but not microconidia, were counted. The particle counter had several advantages for quantification of the adhesion assay. The earlier radiolabeling assay (11, 12) did not completely discriminate between microconidia and macroconidia. However, even in the radiolabeling assay, the percentage of radiolabeled counts contributed by the microconidia is lower than their percentage of total conidia, since microconidia have a smaller volume than macroconidia. Obviously, the particle counter has none of the contamination and waste disposal problems associated with radioisotopes. Use of the particle counter is approximately three to four times faster than use of either the hemacytometer or microscopic counts. Coefficients of variability of data from the particle counter were typically lower than from microscopic counts and often lower than from hemacytometer counts. Thus, the particle counter seems well suited for quantifying adhesion of fungal spores.

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REFERENCES

- Burgess, L. W. 1981. General ecology of the fusaria, p. 225–235. In P. E. Nelson, T. A. Toussoun, and R. J. Cook (ed.), *Fusarium: diseases, biology, and taxonomy*. Pennsylvania State University Press, University Park, Pa.
- Caesar-TonThat, T. C., and L. Epstein. 1991. Adhesion-reduced mutants and the wild type *Nectria haematococca*: an ultrastructural comparison of the macroconidial walls. *Exp. Mycol.* 15:193–205.
- Dubray, G., and G. Bezard. 1982. A highly sensitive periodic acid-silver stain for 1,2,-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. *Anal. Biochem.* 119:325–329.
- El-Ani, A. S. The genetics of sex in *Hypomyces solani* f. *cucurbitae*. *Am. J. Bot.* 41:110–113.
- Epstein, L., L. Laccetti, R. C. Staples, and H. C. Hoch. 1987. Cell-substratum adhesive proteins involved in surface contact responses of the bean rust fungus. *Physiol. Mol. Plant Pathol.* 30:373–388.
- Georgopoulos, S. G. 1963. Tolerance to chlorinated nitrobenzenes in *Hypomyces solani* f. *cucurbitae* and its mode of inheritance. *Phytopathology* 53:1086–1093.
- Georgopoulos, S. G. 1963. Genetic markers and linkage relationships in *Hypomyces solani* f. *cucurbitae*. *Can. J. Bot.* 41:649–659.
- Gubler, F., A. R. Hardham, and J. Duniec. 1989. Characterizing adhesiveness of *Phytophthora cinnamomi* zoospores during encystment. *Protoplasma* 149:24–30.

9. **Hamer, J. E., R. J. Howard, F. G. Chumley, and B. Valent.** 1988. A mechanism for surface attachment of spores of a plant pathogenic fungus. *Science* **239**:288–290.
10. **Hames, B. D.** 1990. One-dimensional polyacrylamide gel electrophoresis, p. 1–147. *In* B. D. Hames and D. Rickwood (ed.), *Gel electrophoresis of proteins: a practical approach*. IRL Press, Oxford.
11. **Jones, M. J., and L. Epstein.** 1989. Adhesion of *Nectria haematococca* macroconidia. *Physiol. Mol. Plant Pathol.* **35**:453–461.
12. **Jones, M. J., and L. Epstein.** 1990. Adhesion of macroconidia to the plant surface and virulence of *Nectria haematococca*. *Appl. Environ. Microbiol.* **56**:3772–3778.
13. **Kwon, Y. H., and L. Epstein.** 1993. A 90 kDa glycoprotein associated with adhesion of *Nectria haematococca* macroconidia to substrata. *Mol. Plant-Microbe Interact.* **6**:481–487.
14. **Nicholson, R. L., and L. Epstein.** 1991. Adhesion of fungi to the plant surface: prerequisite for pathogenesis, p. 3–23. *In* G. T. Cole and H. C. Hoch (ed.), *Fungal spore and disease initiation in plants and animals*. Plenum Press, New York.
15. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105**:361–363.
16. **Pascoe, I. G.** 1990. *Fusarium* morphology. I. Identification and characterization of a third conidial type, the mesoconidium. *Mycotaxon* **37**:121–160.
17. **Perkins, D. D.** 1986. Hints and precautions for the care, feeding and breeding of *Neurospora*. *Fungal Genet. Newsl.* **33**:35–41.
- 17a. **Perkins, D. D.** Personal communication.
18. **Pringle, J. H., and M. Fletcher.** 1986. Adsorption of bacterial surface polymers to attachment substrata. *J. Gen. Microbiol.* **132**:743–749.
19. **Pringle, J. H., M. Fletcher, and D. C. Ellwood.** 1983. Selection of attachment mutants during the continuous culture of *Pseudomonas fluorescens* and relationship between attachment ability and surface composition. *J. Gen. Microbiol.* **129**:2557–2569.
20. **Sela-Buurlage, M. B., L. Epstein, and R. J. Rodriguez.** 1991. Adhesion of ungerminated *Colletotrichum musae* conidia. *Physiol. Mol. Plant Pathol.* **39**:345–352.
21. **Snyder, W. C., S. G. Georgopoulos, R. K. Webster, and S. N. Smith.** 1975. Sexuality and genetic behavior in the fungus *Hypomyces (Fusarium) solani* f. sp. *cucurbitae*. *Hilgardia* **43**:161–185.
22. **Snyder, W. C., and H. N. Hansen.** 1954. Species concept, genetics and pathogenicity in *Hypomyces solani*. *Phytopathology* **44**:338–342.
23. **Van Etten, H. D., and H. C. Kistler.** 1988. *Nectria haematococca*, mating populations I and VI. *Adv. Plant Pathol.* **6**:189–206.