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Adhesion Pad Formation and the Involvement of Cutinase and Esterases in the Attachment of Uredospores to the Host Cuticle

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We have investigated the basis of adhesion of uredospores of the obligately parasitic rust fungus *Uromyces viciae-fabae* to leaves of its broad bean host. Upon contact with an aqueous environment, spores form a structure that we have termed an adhesion pad. The adhesion pad is formed by both living and autoclaved spores, but only adhesion pads formed by living spores adhered to the cuticle of leaves of the host plant. Treatment of living spores with the serine-esterase inhibitor diisopropyl fluorophosphate prevented the adhesion of the pad to the leaf surface, suggesting a functional role for esterase or cutinase in the process of adhesion. A cutinase and two nonspecific serine-esterases were found to be localized on the surface of spores. These enzymes were released rapidly from the spore surface upon contact with an aqueous environment. The addition of the cutinase and the nonspecific esterases to autoclaved spores restored their ability to adhere to the host cuticle. Thus, whereas pad formation appears to be a passive response to the aqueous environment, the actual adhesion of pads to the host cuticle appears to depend on the cutinase and esterases associated with the spore surface. These results suggest a new role for cutinases and serine-esterases in the fungal infection process.

INTRODUCTION

Adhesion of fungal spores to the host cuticle is an essential prepenetration process that determines the success of infection and disease development (Kunoh et al., 1991; Nicholson and Epstein, 1991). Studies involving infrared monitoring (Tunlid et al., 1991) and proteinase treatment (Epstein et al., 1987) suggest that proteins are involved in the adhesion of fungi to a substratum. Although the phenomenon has been documented to occur across a broad range of fungal species (Hamer et al., 1988; Nicholson and Epstein, 1991), the mechanisms involved in the process have not as yet been elucidated.

Erosion of the host cuticle has also been observed in association with spores and germlings of phytopathogenic fungi (Hau and Rush, 1982; Nicholson and Epstein, 1991). These observations suggest that erosion occurs as the result of enzymatic modification of the host cuticle, possibly by hydrolytic enzymes such as cutinases or esterases. But the importance of such hydrolytic activity to the infection process of biotrophic fungi, i.e., obligate pathogens feeding on living host cells, has not been demonstrated. In the case of some necrotrophic and hemibiotrophic pathogens that penetrate the epidermis directly, evidence suggests that enzymatic dissolution of the cuticle or cuticle components may be a requirement for successful infection and probably is most important at the time of penetration (Shaykh et al., 1977b; Maiti and Kolattukudy, 1979; Dickman et al., 1982; Köller et al., 1982; Kolattukudy, 1985; Dickman et al., 1989; Köller and Parker, 1989).

Some obligately parasitic fungi also erode the host cuticle, especially those fungi that, like necrotrophic pathogens, penetrate the host epidermis directly (Staub et al., 1974). For example, studies of the interaction of conidia of the obligate parasite *Erysiphe graminis* with leaves of barley have shown that conidia release esterase activity upon contact with the host surface (Nicholson et al., 1988) and that the conidial exudate dissolves the uppermost amorphous component of the epicuticular layer of the barley cuticle (Kunoh et al., 1990). The release of esterase occurs prior to conidium germination. Thus, cuticular erosion may be involved in the initial preparation of the infection court by the pathogen, a phenomenon thought to be essential for development of *E. graminis* (Staub et al., 1974; Nicholson et al., 1988; Kunoh et al., 1990). In the case of obligate parasites such as the rust fungi, uredospore germlings usually penetrate through the stomatal pore, thus raising the question of the need for hydrolytic enzymes such as esterases or cutinases in the infection process of these organisms. Such needs may include adhesion to the host surface and/or recognition of surface topography that results in the required phenomenon of a specific thigmotropic response of

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the fungal germ tube (Wynn, 1981; Wynn and Staples, 1981; Hoch et al., 1987). Whether adhesion to, or recognition of, the host surface by rust fungi requires the action of hydrolytic enzymes, either esterases or cutinases, is unknown. If these phenomena do require the action of such enzymes, their localization and the time at which they must be present to assist the infection process must be demonstrated.

In this study, we demonstrate the formation of an adhesive pad by uredospores of *Uromyces viciae-fabae* upon their contact with a substratum. We also show that hydrolytic enzyme activity is localized on the uredospore surface. The hydrolytic enzymes are released from spores upon contact with an aqueous environment and prior to their germination. The enzymes released included both cutinase and esterase, and they are shown to assist adhesion of spores to the surface of the host leaf.

RESULTS

Low-Temperature Scanning Electron Microscopy of Adhesion Pad Formation

The surface surrounding echinulations on dry spores appeared smooth in comparison to the surface of spores that had been misted with water, as shown in Figures 1A and 1B. A slimelike material, which often stretched between echinulations, was present on the surface of spores that had been misted and incubated at high relative humidity (Figure 1B). When misted spores were subsequently dried, the slimelike material was still visible at the interface of contact between the spore and leaf surface (Figure 1C) or between spores that were in contact with each other (Figure 1D).

The presence of the material at the spore–cuticle interface indicates that it could function as an adhesive. This suggestion is strengthened by observations that when spores were allowed to dry on the leaf and then removed from the leaf with tape, the material remained attached to the leaf surface (Figure 1E). We have designated the material that remains on the leaf surface as an "adhesion pad." As shown in Figure 1E, the adhesion pad exhibits an imprint of the spore surface, even revealing fine details of the surface such as the ring that surrounds the base of echinulations. Autoclaved uredospores also exhibited a material at the interface of the spore and the leaf (Figure 1F). However, when autoclaved spores were removed from the leaf, the material failed to stick to the leaf cuticle, and it remained attached to the surface of the spore, as shown in Figure 2A. Living spores that had been misted with water containing the esterase inhibitor diisopropyl fluorophosphate (DIPF) also showed the presence of adhesion pads. However, as with autoclaved spores, these pads failed to attach to the leaf cuticle and remained attached to the spore surface (Figure 2B).

Living spores that had been distributed on Mylar or Teflon films also formed adhesion pads at the interface with the substratum. Removal of spores from these films showed that the adhesion pad, or portions of it, remained attached to the Mylar film but failed to attach to the Teflon film (Figures 2C and 2D). Importantly, this pattern of pad adhesion did not change whether spores were autoclaved before application or whether the spores were misted with the esterase inhibitor (data not shown).

Localization of Esterase Activity on the Uredospore Surface

Preliminary investigations demonstrated that uredospores released hydrolytic activity upon exposure to water. This phenomenon suggests that hydrolytic enzymes may be necessary during the initial stages of the infection process.

The deposition of uredospores onto the surface of gelatin that contained indoxyl acetate as an esterase substrate resulted in the formation of crystals of indigo blue on the surface of spores, as shown in Figure 3A. Crystals were formed within 10 min of incubation of spores on the gelatin medium and were often prominently associated with, but not restricted to, surface echinulations (Figure 3A). In contrast, no crystals appeared on the surface of spores that had been washed to remove enzyme activity (Figure 3B). Both washed and unwashed spores showed the accumulation of crystals of indigo blue intracellularly within fat bodies (Figures 3A and 3B). Autoclaved spores failed to accumulate crystals of indigo blue either in fat bodies or on the spore surface, regardless of the time of incubation on the gelatin medium (Figure 3C). To further ascertain the association of crystal formation with the spore surface, spores were removed from the gelatin substratum with an adhesive tape. This resulted in the visualization of clusters of crystals beneath sites where spores had been deposited (Figure 3D), again indicating that enzyme activity is localized on the spore surface.

The localization of esterase on the spore surface was also shown by experiments in which uredospores were washed for increasing lengths of time, and the aqueous washes were assayed for esterase activity with the substrate *p*-nitrophenyl butyrate. The release of activity occurred rapidly within the first 1 to 2 min of washing, and the removal of activity was essentially complete after 10 min, as shown in Figure 4. Incorporation of the inhibitor DIPF into reaction mixtures completely inhibited esterase activity, indicating that surface-localized esterases are of the serine-esterase class. Washing spores had no effect on their germinability because germination rates of both washed and unwashed spores were >98%. The inclusion of cycloheximide (6 μg/mL) in the wash medium had no effect on the release of esterase from spores, suggesting that enzyme release does not involve protein synthesis (data not shown).

To ensure that the esterase released from spores was from the spore wall and did not represent enzyme that had leaked

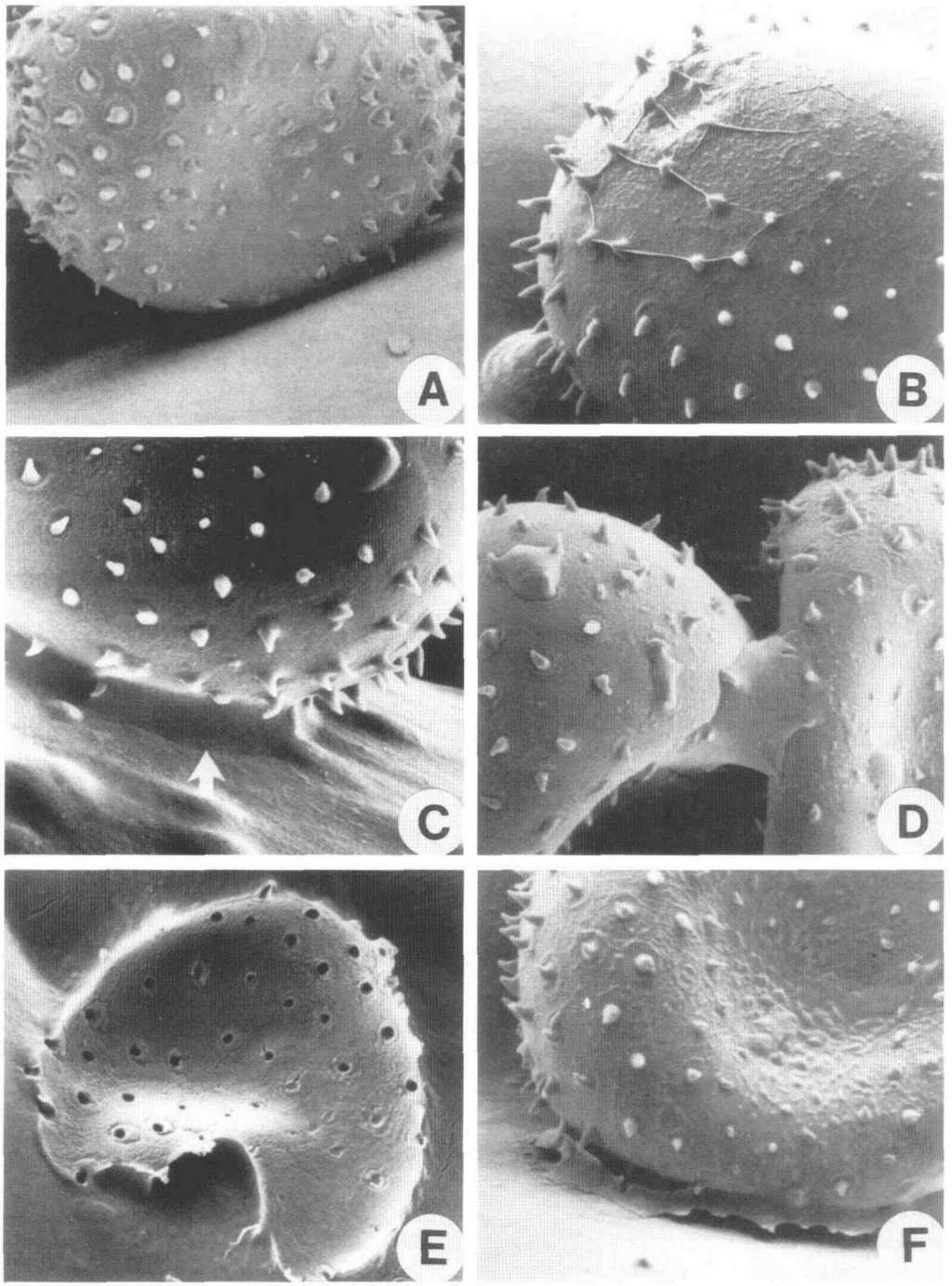

Figure 1. LTSEM Micrographs of Uredospores of *U. viciae-fabae* Showing Adhesion Pad Formation.

(A) Dry uredospore, immediately after contact with the surface of a broad bean leaf (×2900).
(B) Uredospore on the leaf surface misted with water and incubated at high humidity for 1 hr. Note the presence of a material that stretches between echinulations on the spore surface (×3000).
(C) Uredospore misted with water, incubated at high humidity, and subsequently dried at ambient room conditions for 20 min. Note that the material that accumulated between the spore and the leaf surface (arrow) is still visible (×4280).
(D) Spores treated as in **(C)**. Note the presence of a material that appears to connect two adjacent spores (×3300).
(E) Sample treated as indicated in **(C)** except that a spore was removed from the leaf surface with an adhesive tape revealing that an adhesion pad remains attached to the leaf surface (×4000).
(F) Autoclaved uredospore treated as in **(C)** also exhibits the presence of an adhesion pad. Note the presence of a material between the spore and the underlying leaf surface (×2680).

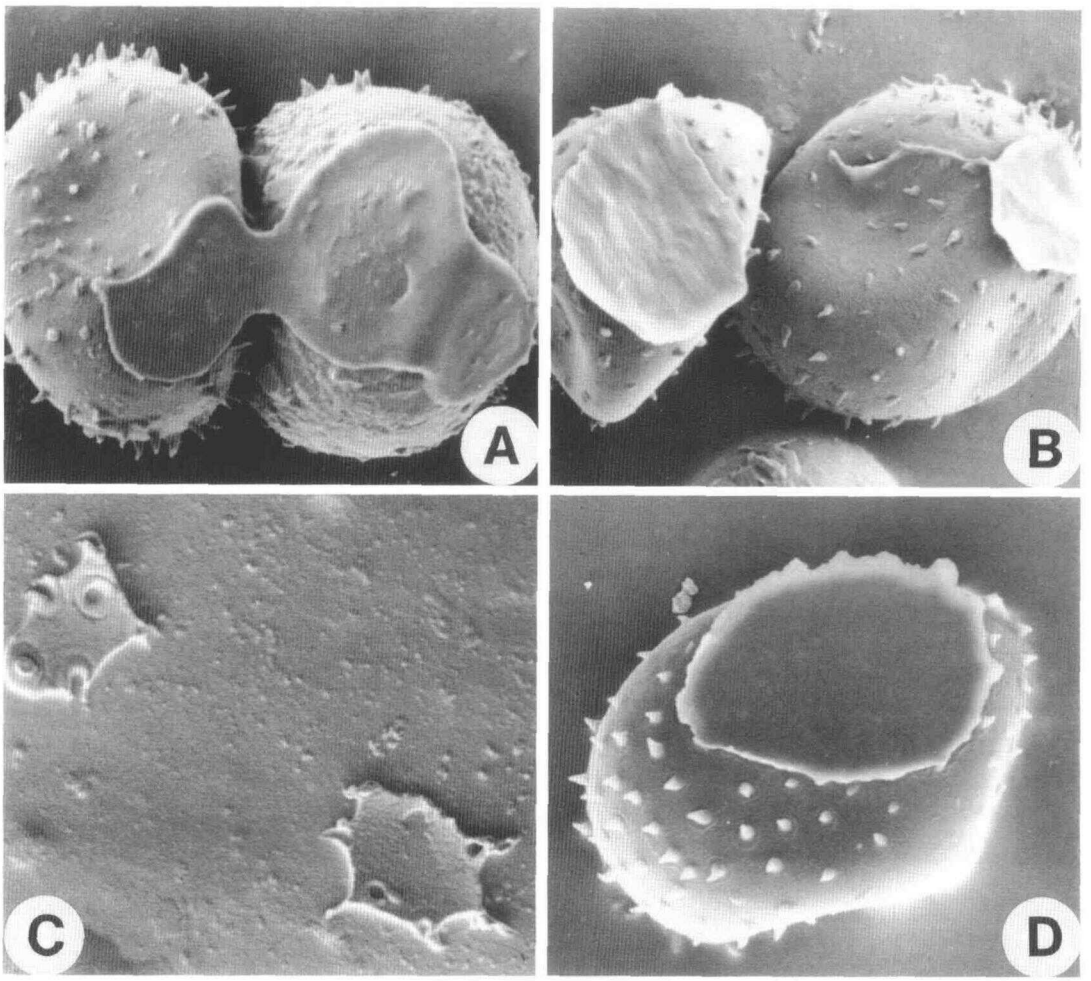

Figure 2. LTSEM Micrographs of Adhesion Pads on Uredospores of *U. viciae-fabae* and of Adhesion Pad Fragments on the Surface of a Substratum.

(A) Autoclaved uredospores treated as in Figure 1C and removed from the leaf with an adhesive tape. Adhesion pads remain attached to the spore surface that had been in contact with the leaf (×2300).

(B) Living uredospores that had been misted with water containing the esterase-cutinase inhibitor DIPF. Removal of spores from the leaf with an adhesive tape reveals that the adhesion pad again remains attached to the surface of the spore (×2300).

(C) Living uredospores distributed on the surface of Mylar film and treated as in Figure 1C. Removal of spores from the Mylar film reveals that fragments of adhesion pads remain attached to the surface of the Mylar film (×3560).

(D) Living uredospores distributed on the surface of Teflon film and treated as in Figure 1C. Removal of spores from the Teflon film reveals that adhesion pads remain attached to the surface of the spore (×2850).

from within spores during hydration, the patterns of intracellular and extracellular esterases were compared by native polyacrylamide gel electrophoresis. Three distinct esterases were detected in preparations that had been washed from the spore surface, as shown in Figure 5. None of these esterases corresponded to bands representing intracellular esterases. As a further confirmation that the esterase isolated by the washing procedure did represent surface-bound enzyme, the extracellular fraction of esterase was compared to that of the cytoplasmic marker malate dehydrogenase. Results showed that 4.9% of the total esterase was attributable to enzyme associated with the spore surface, whereas <0.05% of the total malate dehydrogenase leaked out of spores during washing. These results suggest that there is insignificant leakage of intracellular proteins from spores during the procedure of washing. The amount of protein that could be washed from the surface of uredospores was equivalent to 233 ± 73 μg/g of spores.

Uredospore Adhesion

Low-temperature scanning electron microscopy (LTSEM) studies suggested that adhesion is in part an active process, possibly involving enzymes. This is based on the observation that adhesion pads failed to attach to the leaf cuticle when spores had been autoclaved or treated with DIPF. Measurements of spore adhesion showed that all spore treatments, including autoclaved spores, exhibited approximately 30% adhesion after incubation for 30 min, as shown in Figure 6. This background level of adhesion, which represents a purely physical phenomenon and does not depend on cutinase and/or esterase activity on the spore surface, is consistent with LTSEM observations that showed that adhesion pad formation, regardless of the treatment, occurred on leaves as well as artificial membranes. From 30 min on, the percentage adhesion of native spores increased progressively over a 2-hr incubation period. In contrast, autoclaved spores and native spores incubated in the presence of the esterase inhibitor DIPF failed to exhibit significant increases in adhesion from 30 min to 2 hr. Early in the incubation period, washed spores did not adhere to a greater extent than autoclaved or DIPF-treated spores

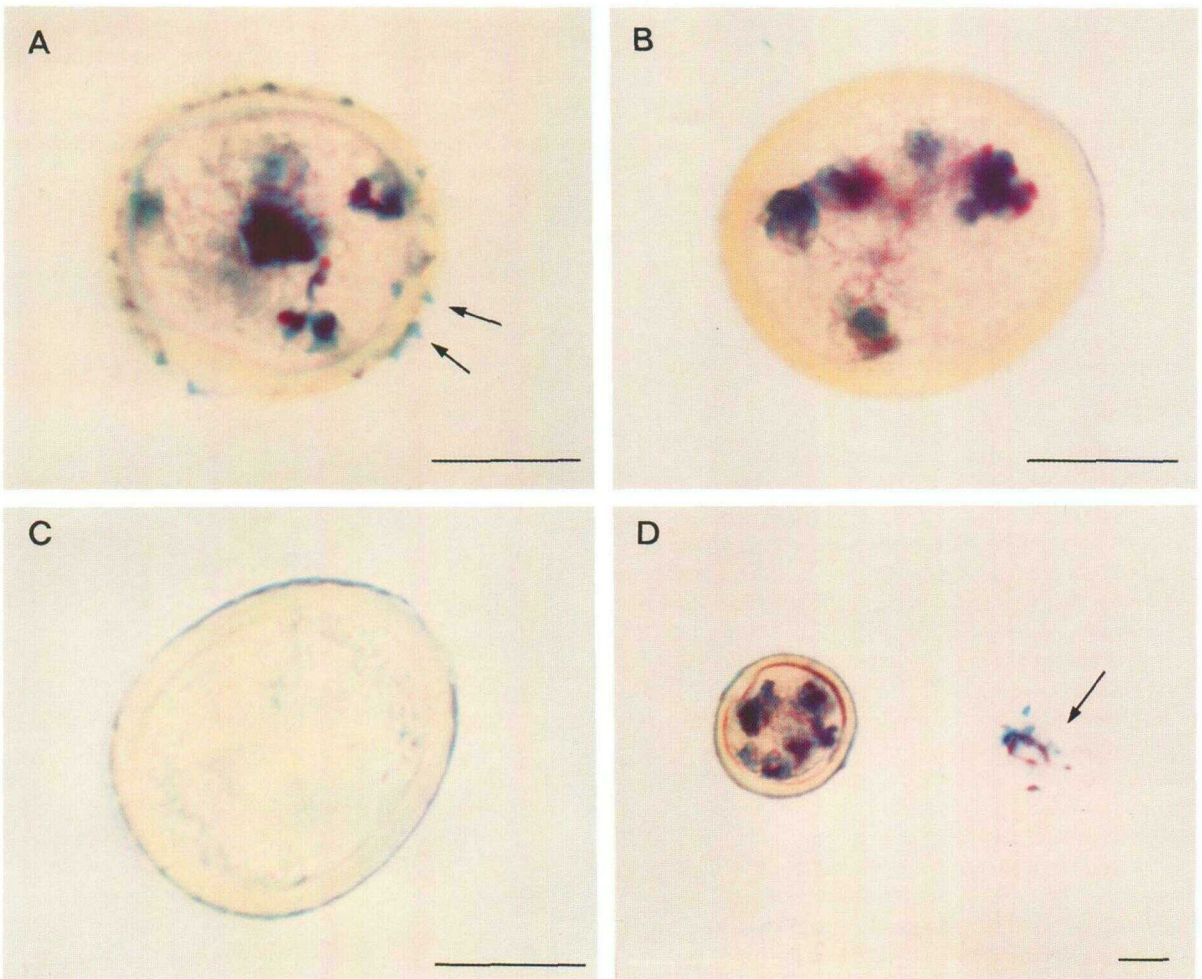

Figure 3. Histochemical Demonstration of Surface-Localized Esterase Activity.

(A) Unwashed, native uredospore. Arrows indicate crystals of indigo blue that are present on the uredospore surface.
(B) Esterase activity of uredospore washed to remove surface-bound esterases. Note the absence of crystals of indigo blue on the spore surface.
(C) Autoclaved uredospore completely lacking indigo blue crystals.
(D) Uredospore removed from the gelatin surface to expose underlying crystals of indigo blue (arrow).
Surface-localized esterase activity was shown using indoxyl acetate as substrate as described in Methods and photographed by bright-field microscopy. Note that in both **(A)** and **(B)** crystals are present in fat bodies within the spores. Bars = 10 μm.

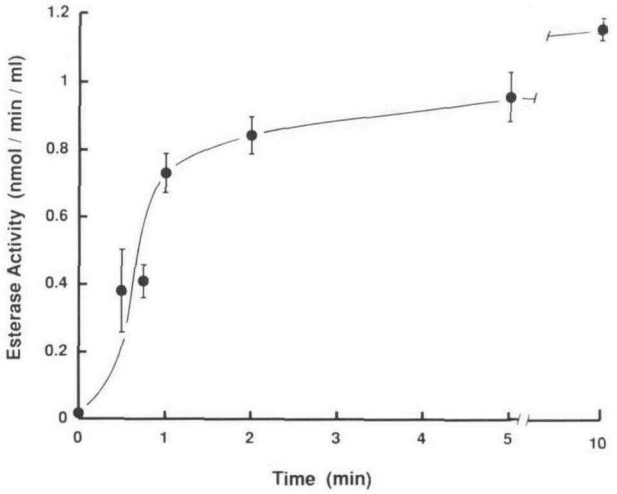

Figure 4. Removal of Esterase Activity from the Surface of Uredospores by Washing.

Enzyme activity in spore washes was determined with *p*-nitrophenyl butyrate as the substrate. Vertical bars represent ± SE.

(Figure 6). However, after 90 min of incubation, the adhesion of washed spores began to increase.

Chromatographic Separation of Esterases and Substrate Specificity

When protein obtained by washing spores was separated by Sephadex G75SF column chromatography, hydrolytic activity, as measured by the *p*-nitrophenyl butyrate assay, eluted in two peaks corresponding to molecular masses of 38 and 18 kD, respectively (data not shown). Cutinase activity coeluted with esterase activity corresponding to a molecular mass of 38 kD. This is consistent with prior observations for the expected molecular weights of fungal hydrolytic enzymes shown to include cutinases (Köller et al., 1982; Kolattukudy, 1985; Köller and Parker, 1989).

Pooled fractions representing peaks corresponding to 38- and 18-kD esterases were assayed for substrate specificity by determining their capacity to cleave tritiated cutin and a variety of fatty acid esters of *p*-nitrophenol. The ability of fractions to cleave the ester substrates decreased as the chain length of the fatty acid component of the ester increased. For both esterase fractions, maximum activity was attained with *p*-nitrophenyl butyrate. Enzyme activity of both preparations with the caproate ester was approximately 25% of the activity with *p*-nitrophenyl butyrate, whereas activities with substrates of greater fatty acid chain length were ⩽5%. Importantly, the cutinase inhibitor DIPF (Kolattukudy, 1984; Trail and Köller, 1990) prevented hydrolysis of both cutin and *p*-nitrophenyl butyrate as did autoclaving the enzyme preparations. The pH optimum of the cutinase was found to be 9.5 (data not shown). These results are consistent with observations of substrate specificities reported for fungal cutinases and serine-esterase preparations (Purdy and Kolattukudy, 1975; Kolattukudy, 1984).

Complementation of Spore Adhesion by Enzymes Separated by Native Polyacrylamide Gel Electrophoresis

Enzymes that had been washed from the surface of uredospores were separated by native polyacrylamide gel electrophoresis. Esterase activity was visualized by the indoxyl acetate assay and revealed the presence of three bands, E1, E2, and E3, as shown in Figure 7. Analysis of gel slices for the ability to hydrolyze tritiated cutin showed that only the esterase designated E1 exhibited cutinase activity (Figure 7).

Enzymes present in bands E1, E2, and E3 were isolated and applied to autoclaved spores to determine the ability of the individual enzymes to complement spore adhesion. The adhesion of autoclaved spores was reduced to <40% of that of

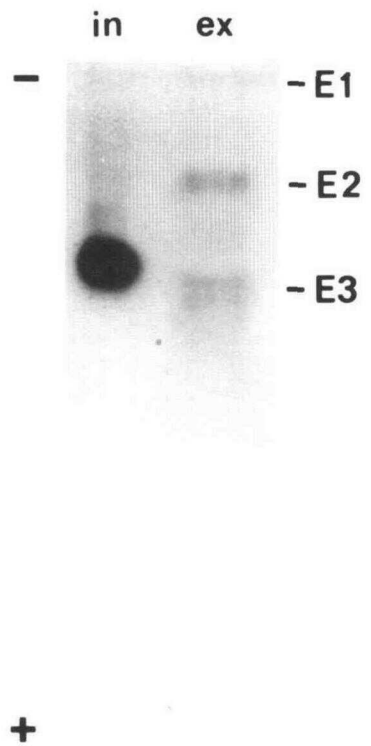

Figure 5. Native Gel Electrophoresis of Intracellular and Extracellular Surface-Localized Proteins from Uredospores of *U. viciae-fabae* Stained for Esterase Activity.

Three different esterases (E1, E2, and E3) were detected in the extracellular surface-localized protein preparation. in, intracellular; ex, extracellular.

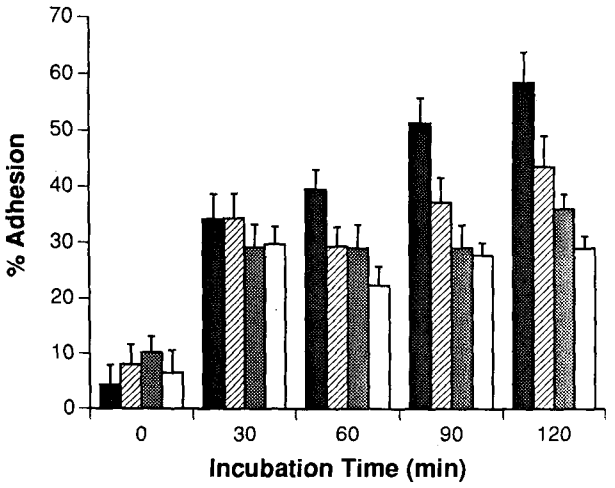

Figure 6. Percentage of Uredospores Adhering to the Cuticle of the Leaf Surface.

Washed (▨), autoclaved (▩), and unwashed (control) spores (■) were dusted onto broad bean leaves. Unwashed spores were also dusted onto leaves that had been pretreated with the esterase-cutinase inhibitor DIPF (□). Adhesion was determined after various times of incubation at 30°C and 100% relative humidity. Vertical bars represent SE.

unautoclaved spores, as shown in Figure 8, bars A and B. Total extracellular material, but not heat-inactivated material, complemented adhesion of autoclaved spores (Figure 8, bars C and D). Treatment of autoclaved spores with the extracellular cutinase isolated by gel electrophoresis (band E1) significantly complemented the level of adhesion, restoring it to >80% of the control (Figure 8, bar E). Treatment of spores with proteins represented by esterase bands E2 and E3 also restored significant levels of adhesion (Figure 8, bars G and I). Importantly, when the esterase inhibitor DIPF was added to the cutinase and esterase enzyme preparations used to treat spores, spore adhesion was reduced to the same level observed for autoclaved spores (Figure 8, bars F, H, and J).

DISCUSSION

Previous investigations of cutinases from necrotrophic fungi have addressed the induction of enzyme synthesis (Woloshuk and Kolattukudy, 1986), the mechanism of induction (Podila et al., 1988), and the role of such enzymes in plant–pathogen interactions (Shaykh et al., 1977b; Dickman et al., 1982; Kolattukudy, 1985; Trail and Köller, 1990). Studies involving monospecific antisera to cutinase, the cutinase inhibitor DIPF, and transformation of the papaya wound pathogen *Mycosphaerella* spp with the cutinase gene have demonstrated the importance of this enzyme to fungi that penetrate their hosts directly (Maiti and Kolattukudy, 1979; Dickman et al., 1989). In contrast, other investigations question the importance of cutinase to fungal penetration (Bonnen and Hammerschmidt, 1989; Stahl and Schäfer, 1992; Sweigard et al., 1992). A role for cutinases in diseases caused by obligately parasitic fungi such as the rusts that usually do not penetrate the host cuticle directly has not been investigated. This is partly because isolation of enzymes is frustrated by difficulties of growing the fungi in culture and the relatively small amounts of protein that can be obtained from ungerminated spores or from spore germlings. Thus, the amount of protein available for enzyme studies is limited.

In this investigation, we demonstrated the release of cutinase and esterases from the surface of uredospores of the obligate rust fungus *U. viciae-fabae* and that these enzymes assist the adhesion of spores to the host cuticle. The release of cutinase and esterases from the uredospore surface is similar to the release of these enzymes from the surface of pollen grains (Shaykh et al., 1977a). The localization of such enzymes on the spore or pollen grain surface implies a functional need for enzyme activity at the time of contact with the substratum. In the case of pollen, evidence suggests that such a need may be related to recognition of the stigma surface (Knox et al., 1976; Shaykh et al., 1977a). Similar lines of evidence involving a specific requirement of serine-esterase or cutinase for host recognition do not currently exist for fungal plant pathogens. That surface erosion and cutinase are related to the specificity of the pathogen for a specific host or tissue type (Trail and Köller, 1990) is supported by observations that erosion caused by germlings of *E. graminis* and *E. cichoracearum* may be host specific (Staub et al., 1974).

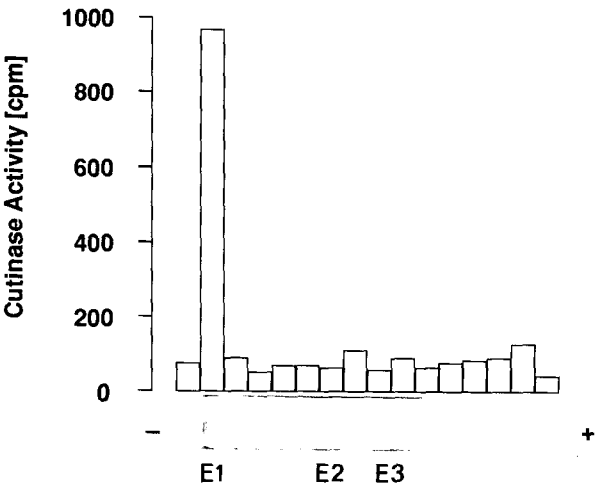

Figure 7. Identification of cutinase activity in extracellular surface-localized esterases. The enzymes (E1, E2, and E3) were separated by native polyacrylamide gel electrophoresis, eluted from the gel, and assayed with ^{3}H-cutin as the substrate.

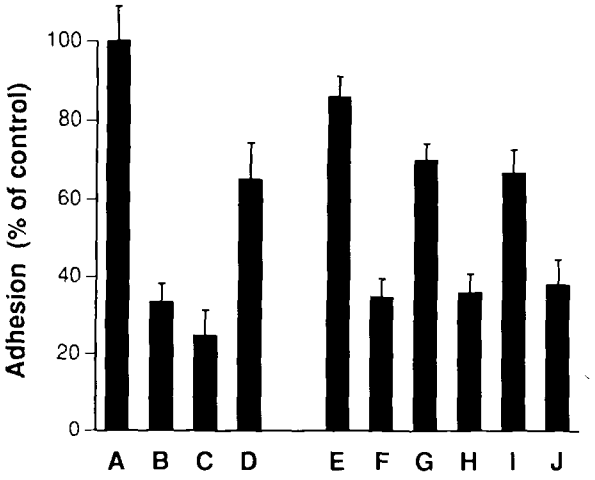

Figure 8. Complementation of Adhesion of Autoclaved Uredospores of *U. viciae-fabae* by the Extracellular Surface-Localized Cutinase and Esterases Isolated from Living Spores.

Bars represent the adhesion of spores (expressed as a percent of the control) after the following treatments: A, control untreated, viable spores: B, autoclaved spores; C, autoclaved spores treated with a crude preparation of heat inactivated, surface-localized proteins that included both cutinase and esterases; D, autoclaved spores treated with a crude preparation of surface-localized proteins including the active enzymes cutinase and esterase; E, G, and I, autoclaved spores treated with active cutinase (enzyme E1), active esterase (enzyme E2), and active esterase (enzyme E3), respectively; F, H, and J, autoclaved spores treated with active cutinase and esterases as in E, G, and I plus the esterase-cutinase inhibitor DIPF. Vertical lines extending from bars represent SE.

Similar to our results with *U. viciae-fabae*, esterase has been shown to be released from ungerminated conidia of the obligate biotroph *E. graminis*. The physical contact of conidia of this fungus with a substratum causes the immediate release of a mixture of proteins, three of which are esterases (Nicholson et al., 1988). Importantly, a highly concentrated enzyme preparation has been shown to erode the epicuticular layer of the barley leaf cuticle, suggesting the presence of an esterase component, possibly with activity against cutin, that is capable of altering the structure of the native cuticle (Kunoh et al., 1990). The erosion of the barley leaf cuticle by esterases from *E. graminis* supports the concept of a functional need for such enzymes in the infection process of this obligate biotroph.

In the case of *U. viciae-fabae*, cutinase and esterases released from spores appear to be associated with the phenomenon of adhesion. Autoclaved spores and spores that had been washed free of cutinase and esterase adhered to the leaf cuticle in significantly lower numbers (Figure 6). However, when autoclaved spores were amended with cutinase or the esterases washed from native spores, adhesion was restored. Importantly, the addition of the serine-esterase inhibitor DIPF to enzyme-treated spores again resulted in significant reduction of adhesion (Figure 8). Additional evidence that supports our hypothesis that cutinase and esterases from the spore surface are involved in adhesion is presented by Beckett et al. (1990), who showed that ungerminated uredospores of *U. viciae-fabae* adhere to the leaf surface and suggested the involvement of enzymes in adhesion.

Our adhesion assays showed that all spore treatments, even those where spores had been killed or exposed to the serine-esterase inhibitor DIPF, exhibited a limited attachment to the leaf surface. We interpret this as a passive, nonspecific binding of uredospores that occurs on both leaves as well as certain hydrophobic surfaces such as Mylar. Such binding is consistent with current thinking that fungal spores, including rust uredospores, tend to adhere nonspecifically to the cuticle (Beckett et al., 1990) as well as to substrata, such as polystyrene and polyethylene (Young and Kauss, 1984; Nicholson and Epstein, 1991), and to lectins (Mendgen et al., 1985). Nonspecific adhesion was also observed in the present LTSEM investigation, which showed that both living and autoclaved spores adhered to Mylar but not to Teflon films (Figures 2C and 2D). Clearly, further studies will be required to characterize the physical or chemical features of a surface that are important to this type of adhesion.

The adhesion pad formed by living *U. viciae-fabae* spores, which still had active cutinase and esterases associated with their surfaces, tightly adhered to the plant cuticle (Figure 1E). However, the adhesion pad formed on autoclaved spores failed to stick to the leaf surface and remained attached to the spore itself (Figure 2A). We interpret this to indicate that adhesion of the pad to the leaf cuticle depends on the presence of cutinase and esterases associated with the spore surface. Thus, we regard the adhesion of the pad to the leaf surface as an active process that requires the presence of cutinase and esterases on the uredospore surface. This interpretation is consistent with observations that demonstrate that adhesion is often associated with enzymatic modification of the host cuticle (Nicholson and Epstein, 1991). Thus, the results suggest that cutinase and serine-esterases localized on the uredospore surface are involved in the adhesion of spores and initiation of the infection process. The results differ from those obtained with necrotrophic fungi that indicate that serine-esterases of the cutinase class are required only for penetration of the host cuticle. It is apparent that the obligately parasitic fungus used in this investigation utilizes the surface-bound enzymes as a means of promoting adhesion to the leaf.

METHODS

Plant and Fungal Material

A uredinial culture of *Uromyces viciae-fabae* was from a single-spore line obtained from naturally infected plants. Uredospores were produced on broad bean (*Vicia faba* cv Con Amore) in growth chambers at 22°C under a 16-hr light/8-hr dark photoperiod (Deising et al., 1991). Spores were harvested 14 days after inoculation and stored at −70°C.

Scanning Electron Microscopy

Spores were distributed evenly on the lower surface of fully expanded broad bean leaves or artificial membranes, either Mylar film (Spex Industries, Metuchen, NJ) or Teflon 50 LP (Du Pont), with a soft brush. Samples were processed for low temperature scanning electron microscopy (LTSEM) either by observing the material in the dry state, by misting the preparations with deionized water or 5 μM diisopropyl fluorophosphate (DIPF) and incubating at 100% relative humidity for 1 hr, or by drying the misted preparations at ambient room conditions for 20 min. Samples were mounted on specimen tables with SH 75-125 embedding medium (Fisher Scientific, Orangeburg, NJ) and plunged into a nitrogen slush and then transferred to the cold stage of a scanning cryo unit (SCU 020; Balzers, Lichtenstein) as described previously (Müller et al., 1991). Frozen samples were then transferred under vacuum to an attached Hitachi S4000 scanning electron microscope, partially freeze dried at −80°C or −95°C, and coated with 8-nm platinum within the scanning cryo unit (−120°C and 2.2×10^{-2} millibars) with a planar magnetron sputter coater. Specimens were examined at −150°C with an accelerating voltage of 4 kV.

Histochemical Assay for Surface Localization of Esterase Activity

The presence of esterase activity on the surface of uredospores was assessed by a method in which indoxyl acetate serves as the substrate for nonspecific carboxylic acid esterases (Barnett and Seligman, 1951). Substrate hydrolysis results in the accumulation of pigmented crystals of indigo blue at the site of hydrolysis. Uredospores of *U. viciae-fabae* were applied to the surface of glass slides coated with a 1-mm layer of gelatin containing indoxyl acetate (17.5% gelatin in 20 mM Tris-HCl, pH 8.0, containing 0.99 M NaCl, 44.6 mM $CaCl_2$, and 3.4 mM indoxyl acetate).

Treatments included viable and autoclaved spores (dispensed in units of 100 mg each). A third treatment included unautoclaved spores that had been washed for 30 min in 1 mL of an aqueous solution of 0.1% Tween 20 followed by five successive washes by repetitive centrifugation (12,000*g*, 1 min) with 1 mL each of distilled water. After washing, spores were collected over filter paper (MN615; Macherey Nagel, Düren, Germany) and allowed to dry at room temperature for 2 hr prior to being distributed onto the gelatin-indoxyl acetate medium. Spores were allowed to settle onto the surface of gelatin-coated slides, and the slides were transferred to a moisture chamber to prevent desiccation. Spores were observed by light microscopy (×100) throughout a period of 2 hr following their contact with the gelatin surface. The experiment was repeated in triplicate.

Spectrophotometric Assay for the Demonstration of Surface Localized Esterase

To study the kinetics of the release of enzyme activity from the spore surface, uredospores in batches of 100 mg were washed by agitation for specified intervals in 1 mL of a 0.1% Tween 20 solution. Separate experiments showed that washing with water alone also resulted in the release of esterase activity from the spore surface but that the pattern of activity release was not consistent across treatments because hydrophobicity prevented the uniform wetting of spores. The spore suspension was washed in a syringe to which a polycarbonate membrane (0.8-μm pore size, 25-mm-diameter; Nucleopore Corp., Pleasanton, CA) was attached. At the end of the washing interval, the wash liquid was collected by filtration to ensure the absence of spores. Microscopic observation demonstrated that the filtration procedure neither damaged uredospores nor reduced their germinability. The experiment was repeated four times.

Esterase activity released from uredospores was assayed by measuring the hydrolysis of *p*-nitrophenyl butyrate at 400 nm as described previously (Huggins and Lapides, 1947; Kolattukudy et al., 1981). Reaction mixtures consisted of 600 μL of Tris-HCl buffer (0.1 M, pH 8.0), 200 μL of an enzyme preparation, and 200 μL of a stock solution of 37.5 mM *p*-nitrophenyl butyrate in the same buffer. Enzyme assays were also run in the presence of 5 μM DIPF as an inhibitor of serine-esterase activity (Kolattukudy et al., 1981; Köller and Parker, 1989). Assays were run at 30°C.

Column Chromatography

Bulk preparations of proteins released from uredospores by washing were prepared for use in column chromatography. Spores (30 g) were washed in batches of 2.5 g each in 25 mL of 0.1% aqueous Tween 20 for 10 min. After washing, spores were centrifuged (16,500*g*, 2°C, 10 min), and the supernatant was collected and centrifuged again. The final supernatant was filtered through a polycarbonate membrane (0.8-μm pore size; Nucleopore Corp.) and concentrated to 2.0 mL by ultrafiltration using an Amicon 8200 cell and a YM5 filter (62 mm) (Amicon Corp., Danvers, MA). The concentrated filtrate was subjected to Sephadex G75SF chromatography on a column of 84.5 × 2.2 cm. The column was equilibrated with a buffer consisting of 0.1 M Tris-HCl, 0.5 M NaCl, and 0.02% NaN_3 at pH 8.0. Fractions (2.0 mL) were eluted in the same buffer under descending conditions at a flow rate of 15.8 mL/hr at 4°C. Fractions were monitored for absorbance at 280 nm and were assayed for esterase activity with *p*-nitrophenyl butyrate and for cutinase activity as described below.

Esterase-active fractions were pooled and tested for substrate specificity based on chain length of the fatty acid component of the ester. Substrates included the butyrate, caproate, caprylate, caprate, laurate, and palmitate esters of *p*-nitrophenol.

Separation and Isolation of Cutinase and Esterase Activities from Native Polyacrylamide Gels

Native polyacrylamide gel electrophoresis was used to compare esterases localized on the spore surface with esterases present within the spore. Proteins washed from 5 g of spores were brought to 150 μL by ultrafiltration using an Amicon mini-ultrafiltration cell model 3 with a YM5 filter (25 mm) and adjusted to contain 10% glycerol. Intracellular proteins were obtained by homogenization of 500 mg of washed spores in 5 mL of 0.1% Tween 20. The homogenate was clarified by centrifugation (40,000*g*, 4°C, 20 min), and the supernatant was adjusted to contain 10% glycerol. Assay of cytoplasmic malate dehydrogenase (Sigma Technical Bulletin 340-UV) was used as a control for the comparison of enzyme leakage from spores.

Separation of proteins was carried out on a running gel of 10% polyacrylamide with a 4% stacking gel. The buffer was system number 1 as described by Maurer (1971). Gels were run for 14 hr at 4°C and 100 V constant current. Gels were then washed twice for 20 min each in 100 mM Tris-HCl, pH 8.0. Detection of esterase activity was accomplished with a modified indoxyl acetate assay. The indoxyl acetate substrate (35 mg) was dissolved in 1 mL of acetone and added to 49

mL of 100 mM Tris-HCl, pH 8.0. Gels were incubated with the substrate solution by constant agitation at room temperature until bands of desired intensity appeared.

To determine which of the esterase bands separated by the above procedure was a cutinase, the enzymes were extracted from polyacrylamide gels and analyzed for cutinase activity. Gels were cut into 3-mm slices, and the slices were frozen and ground to a fine powder in liquid nitrogen. The powdered gel preparations were then incubated with ^{3}H-cutin for cutinase assays as described below. Reference lanes from the same gels were stained by the indoxyl acetate procedure to identify the position of bands with esterase activity.

Cutinase Assay

The cutinase assay was a modification of the method of Bonnen and Hammerschmidt (1989). Tritiated cucumber cutin (4.5 mg) was washed overnight in two changes of 1 mL each of either Tris-HCl (0.1 M, pH range 8.5 to 9.0) or glycine-NaOH (0.1 M, pH range 9.5 to 10.5) as required for a particular experiment. The cutin was centrifuged and resuspended in 4.5 mL of the required buffer. Cutinase activity was assayed in fractions obtained by column chromatography of spore wash preparations and in proteins separated by gel electrophoresis. Controls included autoclaved enzyme preparations and the addition of 5 μM DIPF to reaction mixtures as an inhibitor of enzyme activity (Köller and Parker, 1989). Reaction mixtures consisted of 250 μL of ^{3}H-cutin, 500 μL of enzyme preparation, and 250 μL of 400 mM glycine-NaOH buffer, pH 9.5. Enzyme reactions were run for 24 hr at 30°C. Reactions were stopped by the addition of 50 μL of 6 N HCl, and the mixture was partitioned twice with 1.5 mL of diethylether. The ether-soluble components were dried and dissolved in 2 mL of Beckman Ready Solv HP scintillation fluor. Radioactivity was counted after 3 hr.

Adhesion Assays

Dry uredospores were dusted onto leaves of broad bean through a copper grid with a 0.8-mm pore size. This allowed the deposition of approximately 160 spores per inoculum site. Inoculum sites were photographed, and the spores were counted. Leaves were incubated at 100% relative humidity and 30°C. After intervals of 0, 30, 60, 90, and 120 min, inoculation sites were washed by delivery of 25 drops of water from a height of 5 cm. Immediately after washing, spores remaining on the leaf surface were counted and data was expressed as a percent of spores originally present. Treatments included spores that had been washed for 60 min with 0.1% Tween 20 solution followed by a thorough water rinse to remove the detergent and spores that had been autoclaved. In an additional treatment, native spores were dusted onto the surface of leaves that had been sprayed with a 4 μM solution of the inhibitor DIPF. The DIPF solution was allowed to dry on the leaf surface prior to the application of spores. Untreated spores served as a control.

To investigate the importance of surface-localized esterases to the process of spore adhesion, spores were autoclaved and dried, supplemented with a crude spore wash and enzyme preparations isolated by native gel electrophoresis, and assayed for the ability to adhere to the cuticle of broad bean leaves. Enzymes were eluted from powdered gel slices by stirring with 500 μL of distilled water for 1.5 hr. The suspensions were centrifuged (14,000*g*, 4°C, 10 min), and the gel pellets were extracted with 500 μL of water. The supernatants were pooled and dialyzed twice against 5 L of distilled water for 10 and 4 hr, respectively. After centrifugation (14,000*g*, 4°C, 10 min), 600 μL of each enzyme supernatant was added to 200 mg of autoclaved dry spores, and the preparations were mixed thoroughly and lyophilized. This corresponded to the addition of 3.59 mg protein of crude spore wash (*p*-nitrophenyl butyrate esterase assay is 0.067 change in A_{400} per min), 0.49 mg protein of the cutinase preparation (*p*-nitrophenyl butyrate esterase assay is 0.038 change in A_{400} per min), 0.074 mg protein of esterase E2 (*p*-nitrophenyl butyrate esterase assay is 0.62 change in A_{400} per min), and 0.023 mg protein of esterase E3 (*p*-nitrophenyl butyrate esterase assay is 0.004 change in A_{400} per min). Protein was measured by the method of Bradford (1976). The lyophilized spore preparations were then applied to the surface of broad bean leaves through copper grids. The number of spores at sites of application were counted, and preparations were incubated for 105 min at 30°C and 100% RH. After incubation, the inoculation sites were washed as described above, and the number of adhering spores was determined. By this procedure, autoclaved spores were treated with enzyme preparations representing a crude spore wash and the cutinase and two distinct esterases isolated from spore surfaces. Treatments also included the addition of the serine-esterase inhibitor DIPF at a concentration of 5 μM prior to freeze drying.

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