MPMI Vol. 8, No. 1, 1995, pp. 122–130.

Diversity of Cutinases from Plant Pathogenic Fungi: Different Cutinases Are Expressed during Saprophytic and Pathogenic Stages of *Alternaria brassicicola*

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Received 8 August 1994. Accepted 1 November 1994.

The cutinase gene *CUTAB1* of *Alternaria brassicicola* was disrupted by biolistic transformation of conidia with vector pDABC1, containing flanking regions of the cutinase gene fused to a selectable marker construct. Disruption of the cutinase gene had an impact on saprophytic stages of CUTAB1⁻ mutants. The two isozymes cutinase A_c and B_a, which are predominantly expressed by the wild-type strain during saprophytic growth on polymer cutin, were not expressed by respective mutants, and cutin was no longer utilized as a saprophytic carbon source. This correlation suggests a crucial role of *CUTAB1* expression during saprophytic stages of the pathogen. Disruption of *CUTAB1* had no significant effect on the pathogenicity and tissue specificity of CUTAB1⁻ mutants. Although the two cutinase isozymes expressed by the wild-type strain under saprophytic growth conditions were not produced by CUTAB1⁻ mutants in contact with polymer cutin, low levels of two serine hydrolases with molecular weights of 31 and 19 kDa were specifically induced and expressed. The mixture of these hydrolases exhibited cutinase activity. The same hydrolases were expressed by both the wild-type strain and CUTAB1⁻ mutants during early stages of host infection. In contrast, the gene products of the cutinase gene *CUTAB1* with crucial functions in saprophytic stages were not detected on host surfaces inoculated with the wild-type strain. The results suggest that different cutinases evolved with important functions in either saprophytic or pathogenic stages of the pathogen.

Additional keywords: cuticle, penetration.

Plant cuticles are the first defensive barriers encountered by directly penetrating plant pathogenic fungi. The role of fungal cutinases in cuticle penetration has been studied with a variety of experimental approaches (Kolattukudy 1985; Köller 1991). Cutinase isozymes were first purified from cultures of *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*) saprophytically grown on cutin as sole carbon source (Purdy and Kolattukudy 1975). Several lines of evidence indicated that these and other cutinases purified from saprophytic cultures of several pathogens were also crucially involved in early stages of host infection.

Presence of cutinase at the site of infection was demonstrated (Shayhk *et al.* 1977), and antibodies as well as chemical inhibitors of cutinase prevented fungal infection (Kolattukudy 1985; Köller 1991; Köller *et al.* 1991). The virulence of cutinase-deficient mutants was low but could be restored by addition of exogenous cutinases (Köller *et al.* 1982; Dantzig *et al.* 1986; Dickman and Patil 1986). Addition of a cutinase gene derived from *F. solani* f. sp. *pisi* to the wound parasite *Mycospherella* spp. enabled transformants to infect intact surfaces of papaya fruits (Dickman *et al.* 1989), and evidence was provided that virulence of a cutinase-deficient strain of *F. solani* was increased after addition of the same gene (Kolattukudy *et al.* 1989).

Results contradicting the evidence described above have been presented. In several host-pathogen systems, such as *Colletotrichum lindemuthianum* and bean (Wolkow *et al.* 1983), *Magnaporthe grisea* and rice (Woloshuk *et al.* 1983), and *Colletotrichum lagenarium* and cucumber (Bonnen and Hammerschmidt 1989), chemical inhibitors of cutinase did not prevent fungal infection. The cutinase gene *CUT1* in *M. grisea* was disrupted, but pathogenicity of respective transformants was not altered (Sweigard *et al.* 1992b). However, significant amounts of cutinase not affected by the gene disruption remained to be excreted by the mutants, and the role of this cutinase in infection has not been clarified. Similar to the results reported for *M. grisea*, the disruption of the well-characterized cutinase gene of *F. solani* f. sp. *pisi* (Kolattukudy 1991) had no detectable effect on fungal pathogenicity under the infection condition employed (Stahl and Schäfer 1992). Because respective transformants of *F. solani* had lost their saprophytic competence with polymer cutin as sole carbon source, a role of cutinase in saprophytic growth rather than pathogenicity was suggested (Stahl and Schäfer 1992). In two separate studies, the virulence of gene-disrupted transformants was either found to be significantly reduced (Rogers *et al.* 1994) or entirely unimpaired (Stahl *et al.* 1994).

While the general role of cutinases in plant infection remains controversial, involvement of cutinase in steps other than cuticle penetration was suggested. Evidence was presented that cutinase contributes to the adhesion of spores to

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host surfaces (Deising *et al.* 1992; Pascholati *et al.* 1993). Furthermore, it was hypothesized that enzymatically different cutinases are involved in the tissue specificity of plant pathogenic fungi (Trail and Köller 1990). Two isozymes with different enzymatic properties were purified from *Alternaria brassicicola* (Schw.) Wilts. chosen as a model pathogen lacking tissue specificity (Trail and Köller 1993). A single cutinase gene *CUTAB1* of the pathogen was recently cloned and characterized (Yao and Köller 1994). In the present study, we describe the results of the disruption of *CUTAB1*.

RESULTS

Transformation-mediated gene disruption.

A 495-bp PflMI fragment located in the coding region of cutinase gene *CUTAB1* was replaced by a selectable marker to create the gene disruption vector pDABC1 (Fig. 1). The selectable marker contained the hygromycin B phosphotransferase (*hph*) gene of *Escherichia coli*, conferring resistance to hygromycin B, and the *oliC* promoter and *trpC* terminator from *Aspergillus nidulans* (Hilber *et al.* 1994; Ward 1991). *CUTAB1* sequences flanking the hygromycin resistance construct in pDABC1 were 806 and 244 bp long, respectively.

Disruption of *CUTAB1* in the fungal genome employed the one-step gene disruption strategy (Rothstein 1983). Conidia of *A. brassicicola* were biolistically transformed with plasmid pDABC1. Attempts to transform protoplasts of *A. brassicicola* with other techniques (Yelton *et al.* 1984; Richey *et al.* 1989) were unsuccessful. The biolistic process employs high-velocity microprojectiles to deliver nucleic acids into intact cells (Sanford *et al.* 1993). It has been successfully used to transform diverse microbial species, including several filamentous fungi (Armaleo *et al.* 1990; Lorito *et al.* 1993; Hilber *et al.* 1994; Parker *et al.* 1995). The transformation frequency for *A. brassicicola* conidia was six transformants per microgram of DNA per 10^6 conidia, which is comparable with frequencies obtained with other transformation techniques.

Thirty hygromycin-resistant transformants were selected and analyzed by genomic DNA dot blot hybridization with the 495-bp PflMI fragment of *CUTAB1* as probe. DNA from two transformants, Ab8 and Ab17, lacked hybridization and were subjected to Southern analysis. As shown in Figure 2A, the lack of hybridization between DNA from the two transformants and the PflMI fragment deleted from *CUTAB1* confirmed the CUTAB1$^-$ nature of the mutants. Presence of the hygromycin-resistance construct in the middle of the disrupted cutinase gene was demonstrated by the expected 2.2-kb increase in length of the hybridizing DNA fragment with pDABC1 as a probe (Fig. 2B). Furthermore, plasmid pBluecriept SK–, the vector of pDABC1, did not show any hybridization with DNA from two transformants, indicating that the vector sequence was not integrated into the fungal genome (data not shown).

Total RNA was extracted from mycelium of the wild-type and two CUTAB1$^-$ transformants incubated in the presence of cutin hydrolysate as cutinase inducer (Trail and Köller 1993). Absence of *CUTAB1* mRNA in the two transformants was determined by Northern blot analysis using the 495 bp deleted PflMI fragment as a probe. As expected for CUTAB1$^-$ mutants, the fragment only hybridized with a 1.05-kb fragment from the wild-type strain, but no detectable hybridization signals were observed with mRNA isolated from the two transformants Ab8 and Ab17 (Fig. 3).

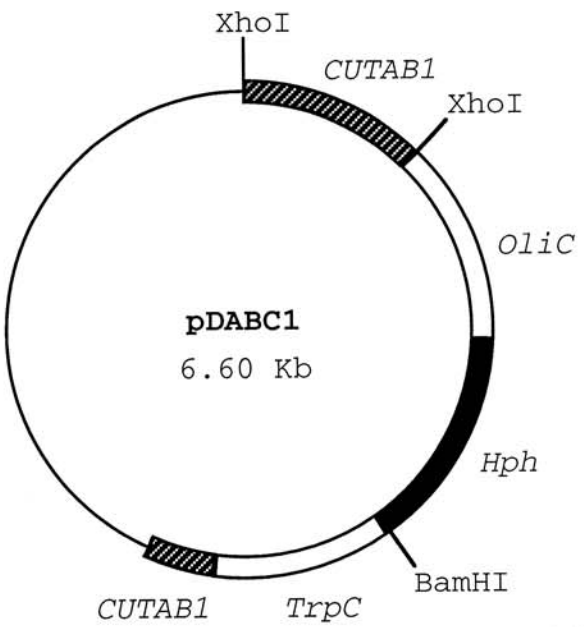

Fig. 1. Cutinase gene-disrupted plasmid pDABC1. An internal 495-bp PflMI fragment of *CUTAB1* was replaced by a selectable marker to generate gene disruption vector pDABC1. The selectable marker contained the hygromycin B phosphotransferase gene *hph* of *E. coli*, and the *oliC* promoter and *trpC* terminator of *A. nidulans*.

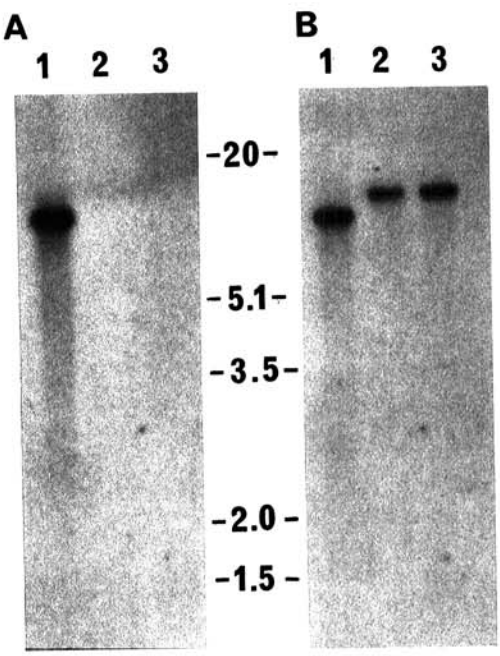

Fig. 2. Southern analysis of the wild-type strain and two CUTAB1$^-$ mutants. Genomic DNA 5 µg isolated from the wild-type strain and mutants was digested with *Bst*XI and subjected to Southern analysis. The probe in A was the deleted 495 bp PflMI fragment of *CUTAB1*, and the probe in B was the transformation vector pDABC1. Lane 1, wild-type strain; lane 2, mutant Ab8; lane 3, mutant Ab17. Molecular weights (middle) are in kb.

Esterase and cutinase production by mutants.

Mycelial growth rates in media containing glucose as carbon source and morphologies of mycelial colonies were not altered by the gene disruption. In contrast, saprophytic growth of the two mutants on polymer cutin as sole carbon source was severely impaired, similar to the results reported for cutinase gene-disrupted mutants of *F. solani* (Stahl and Schäfer 1992). As shown in Figure 4, impaired growth of the mutants on cutin was accompanied by very low levels of esterase activity excreted into culture fluids, when compared with esterase levels accumulating in wild-type cultures. In repeated experiments, residual esterase activities produced by the mutants never exceeded 3% of levels accumulating in wild-type cultures.

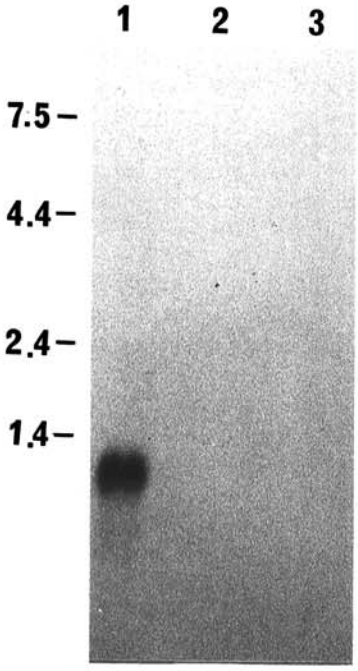

Fig. 3. Northern analysis of the wild-type strain and two CUTAB1$^-$ mutants. Total RNA 5 µg isolated from the wild-type strain and mutants induced with cutin hydrolysate Trail and Köller 1993 was subjected to Northern analysis. The probe was the deleted 495-bp PflMI fragment of *CUTAB1*. Lane 1, wild-type strain; lane 2, mutant Ab8; lane 3, mutant Ab17. Molecular weights (left) are in kb.

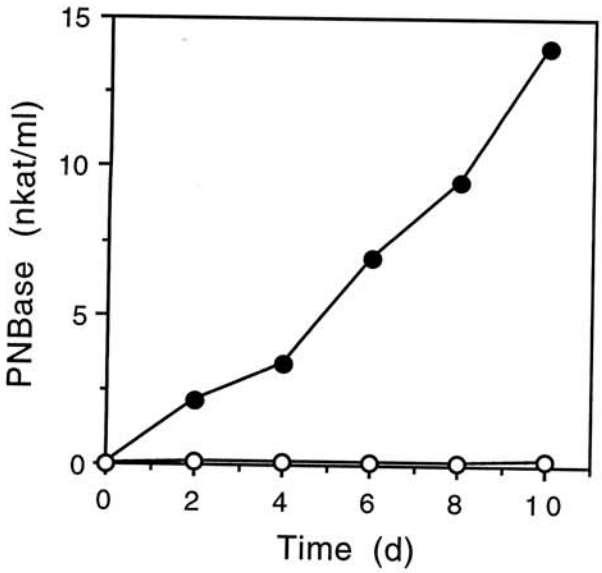

Fig. 4. Esterases excreted by a wild-type strain and a CUTAB1$^-$ mutant. The wild-type strain closed symbols and mutant Ab8 open symbols were grown on cutin as sole carbon source, and esterase activities were assayed with *p*-nitrophenyl butyrate as substrate PNBase. Esterase activities excreted by mutants Ab8 and Ab17 (not shown) were identical.

Extracellular proteins were analyzed with ^{3}H-diisopropylfluorophosphate (DFP) as an active site label for serine hydrolases, including fungal cutinases (Köller *et al.* 1991; Trail and Köller 1993). Equal volumes of extracellular fluids from the wild-type strain and both CUTAB1$^-$ mutants cultured for 4 days in the presence of either cutin or glucose as carbon source were treated with DFP, and labeled esterases were separated by electrophoresis. As shown in Figure 5A, two esterases with molecular weights of 23 and 21 kDa were predominant in wild-type cultures growing on polymer cutin. These two esterases represent the two isozymes cutinase A_c and B_a purified from *A. brassicicola* saprophytically grown on cutin (Trail and Köller 1993). Cutinase A_c (23 kDa) was not present in culture fluids of both CUTAB1$^-$ mutants. Small amounts of a serine hydrolase with a molecular weight very similar to cutinase B_a (21 kDa) were detected in culture fluids derived from both mutants. However, this hydrolase was below detectable levels in some of the repeated experiments and might not resemble cutinase B_a.

Three serine hydrolases with molecular weights of 31, 26, and 19 kDa were consistently secreted from both the wild-type strain and the two mutants (Fig. 5A). The 26-kDa serine

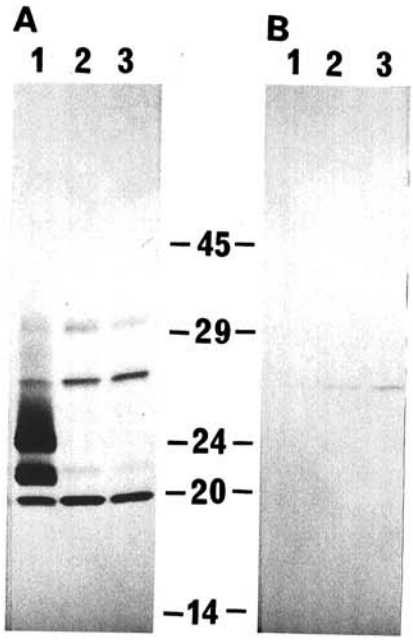

Fig. 5. Serine hydrolases excreted by a wild-type strain and two CUTAB1$^-$ mutants under saprophytic conditions. The wild-type strain lane 1, mutant Ab8 lane 2 and mutant Ab17 lane 3 were grown for 4 days on cutin A or glucose B as carbon source. Equal volumes 2 ml of culture fluids were treated with ^{3}H-DFP and separated by SDS-PAGE. Labeled proteins were identified by fluorography. Molecular weights (middle) are in kDa.

hydrolase was also detected in cultures grown with glucose as carbon source (Fig. 5B) and, thus, was not specifically induced in the presence of cutin. No serine hydrolases were detected in cultures of spores germinating in the absence of any carbon source (data not shown).

Cutinase activities of the serine hydrolases excreted by the two CUTAB1$^-$ mutants were analyzed subsequent to a 50-fold concentration of respective culture fluids. This concentration step was necessary to warrant similar amounts of esterase activities employed in comparative cutinase tests. Under these conditions, all samples exhibited cutinase activities. Cutinases released by the mutants were active at a broad range of pH values, with highest activity at pH 6 (data not shown). The comparative analysis presented in Table 1 was, therefore, conducted at this pH.

Both esterase and cutinase activities of the 26-kDa serine hydrolase detected in concentrated extracellular fluids of cultures grown in the presence of glucose as carbon source (Fig. 5B) were below detectable limits (Table 1). In contrast, the serine hydrolases excreted by both CUTAB1$^-$ mutants (Fig. 5A) in the presence of polymer cutin were active as cutinases (Table 1) and, thus, were induced by cutin. Although the volume activities of the samples tested were very similar, specific esterase and cutinase activities excreted by the two mutants were considerably lower than those determined for the wild-type strain, which predominantly excreted the two cutinase isozymes characterized before (Trail and Köller 1993). The analysis was repeated twice with very similar results.

Pathogenicity of CUTAB1$^-$ mutants.

Three days after inoculation of *Brassica oleracea* leaves and stems with conidia of the wild-type strain and the two CUTAB1$^-$ mutants, small black oval lesions were formed on leaf surfaces, and small black linear lesions occurred on stems. The disruption of the cutinase gene *CUTAB1* had no apparent effect on pathogenicity. To compare virulence differences between the wild-type strain and the two CUTAB1$^-$ mutants, conidial densities for infections of leaves and stems were adjusted to quantifiable levels of disease symptoms. Ten times more conidia (10^6 ml^{-1}) were required for quantifiable symptoms on stems. At this spore density, large areas of leaves were diseased, although differences between the wild-type strain and the two mutants were also not apparent under these conditions.

A quantitative virulence analysis is presented in Table 2. Although standard deviations were consistently higher for the mutants, *F*-test analysis showed no significant difference (α = 0.05) between lesion numbers caused by the wild-type strain and the two CUTAB1$^-$ mutants. Furthermore, microscopic analysis of infection sites 48 hr after inoculation showed no difference in the mode of infection. Penetration was almost exclusively direct and not through stomata.

Fungal isolates recovered from 10 lesions on plants inoculated with the two CUTAB1$^-$ mutants remained resistant to hygromycin B, and isolates obtained from plants infected with the wild-type strain were sensitive to the inhibitor. The results demonstrate that disease symptoms were not caused by cross contaminations of conidia. In summary, the disruption of the cutinase gene *CUTAB1* had no apparent impact on either pathogenicity, virulence, or tissue specificity of respective mutants under the infection conditions employed.

Table 1. Esterase and cutinase activities in culture fluids of a wild-type strain of *Alternaria brassicola* and two CUTAB1$^-$ mutants

Strain	Carbon source	Esterase[a] (nkat/mg)	Cutinase[b] (Bq/h/mg)
Wild type	Glucose	< 0.2[c]	< 10[c]
Ab8		< 0.2	< 10
Ab17		< 0.2	< 10
Wild type	Cutin	149	1,585
Ab8		4.4	80
Ab17		3.7	70

[a] Esterase activities were tested with *p*-nitrophenylbutrate as substrate.
[b] Mean of two replicate tests at pH 6.
[c] Detection limits were double background values.

Table. 2. Virulence of a wild-type strain of *Alternaria brassicicola* and two CUTAB1$^-$ mutants and stems of the host *Brassica oleracea*

Strain	Tissue	Number of lesions[a]
Wild type	Leaves[b]	248 (38)[c]
Ab8		229 (69)
Ab17		240 (58)
Wild type	Stems[d]	47 (10)
Ab8		46 (17)
Ab17		50 (17)

[a] Figures are the mean of lesion numbers determined with 12 leaves or stems.
[b] Conidial density for inoculation was 10^5 ml^{-1}.
[c] Standard deviation in parentheses.
[d] Conidial density for inoculation was 10^6 ml^{-1}.

Expression of esterases and cutinases on host surfaces.

To determine if cutinases were expressed by *A. brassicicola* during early stages of host infection, leaves of *B. oleracea* were infected with conidia of either the wild-type strain or the

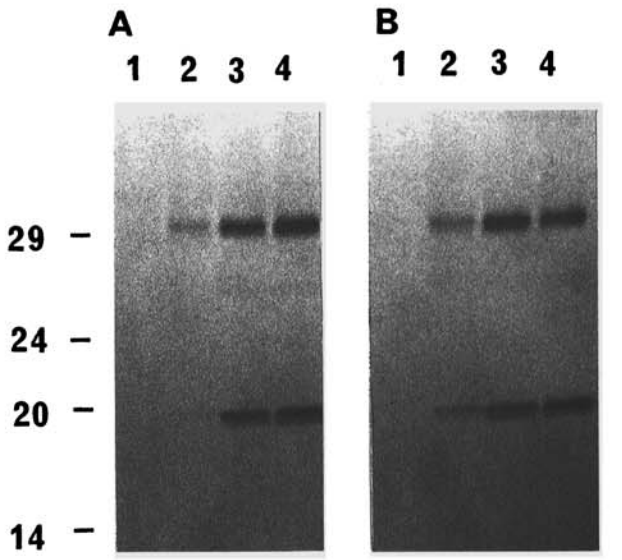

Fig. 6. Serine hydrolases excreted by a wild-type strain and two CUTAB1$^-$ mutants on host surfaces. Enzyme preparations derived from inoculated leaf surfaces 24 **(A)** and 48 hr **(B)** after inoculation and containing 0.1 nkat PNBase were treated with ^{3}H-DFP and separated by SDS-PAGE. Labeled proteins were identified by fluorography. Lane 1, noninoculated control; lane 2, wild-type strain; lane 3, mutant Ab8; lane 4, mutant Ab17. Molecular weights (left) are in kDa

CUTAB1$^-$ mutants. Surfaces of leaves were rinsed 24 or 48 hr after inoculation, at a presymptom stage of disease development. Concentrated samples containing proteins present on leaf surfaces were treated with ^{3}H-DFP, and proteins were separated by electrophoresis. The results are shown in Figure 6. The two cutinases A_c and B_a with molecular weights of 23 and 21 kDa (Trail and Köller 1993), which were strongly expressed during saprophytic growth of the wild-type strain on cutin (Fig. 5A), were not detected on the surface of leaves inoculated with wild-type conidia. Instead, two serine hydrolases with molecular weights of 31 and 19 kDa were excreted by both the wild-type strain and the mutants. These two hydrolases resembled the hydrolases excreted by fungal cultures of the two CUTAB1$^-$ mutants in contact with polymer cutin (Fig. 5A). In repeated experiments, very small amounts of an additional 26-kDa hydrolase, which was also excreted in the presence of glucose (Fig. 5B), were occasionally observed. No serine hydrolases could be recovered from surfaces of noninoculated plants (Fig. 6). Serine hydrolases extracted from mechanically wounded leaves had molecular weights of 90, 60, and 40 kDa (data not shown). The lack of esterases on intact cuticles and the larger sizes of esterases extracted from wounded leaves indicated that the two esterases recovered from inoculated surfaces were of fungal origin.

The absence of cutinase A_c (23 kDa) and B_a (21 kDa) on host surfaces after inoculation with the wild-type strain indicated that the cutinase gene *CUTAB1* was not expressed to detectable levels under these conditions. Poly(A)$^+$ RNA extracted from plants 24, 48, and 72 hr after inoculation with wild-type conidia was, therefore, subjected to Northern analysis, with cutinase cDNA (pCAB23 insert) employed as a probe (Yao and Köller 1994). Transcripts of *CUTAB1* were not detected (data not shown).

The esterase and cutinase nature of the serine hydrolases extracted from surfaces of host leaves 48 hr after inoculation was confirmed in repeated experiments. As shown in Table 3, esterases and cutinases released by both the wild-type and the two CUTAB1$^-$ mutants had very similar specific activities. The lack of serine hydrolases on surfaces of noninoculated leaves (Fig. 6) was accompanied by esterase and cutinase activities below detectable limits. The result also confirmed that cutinases expressed on the host surface were very similar for all strains, regardless of the absence or presence of the intact *CUTAB1* gene. Identical molecular weights of serine hydrolases expressed by CUTAB1$^-$ mutants in contact with polymer cutin (Fig. 5A) and by all strains infecting host leaves (Fig. 6), and similar specific cutinase activities determined for both developmental conditions (Table 1 and Table 3) suggest that these cutinases are identical.

Table 3. Esterase and cutinase activities recovered from leaf surfaces 48 hr after inoculation with a wild-type strain or two CUTAB1$^-$ mutants

Strain	Esterase[a] (nkat/mg)	Cutinase[b] (Bq/h/mg)
None	< 0.5[c]	< 15[c]
Wild type	13	144
Ab8	12	104
Ab17	12	101

[a] Esterase activities were tested with *p*-nitrophenylbutyrate as substrate.
[b] Mean of two replicate tests at pH 6.
[c] Detection limits were double background values.

DISCUSSION

Enzymatic differences between cutinases produced by pathogens infecting either leaf or stem tissue of their hosts led to the hypothesis that cutinases are not only involved in host infection, but also might be one of the determinants of tissue specificity of fungal plant pathogens (Trail and Köller 1990). *A. brassicicola* was chosen as a model system, because the pathogen lacks tissue specificity (Trail and Köller 1990). Two cutinases isozymes with different pH optima of cutin hydrolysis were purified from culture fluids of *A. brassicicola* grown on cutin as sole carbon source (Trail and Köller 1993). To test the hypothesis of cutinase involvement in infection and tissue specificity, the cutinase gene *CUTAB1* was cloned and characterized (Yao and Köller 1994). The disruption of this gene had several effects on the biology of CUTAB1$^-$ mutants.

During saprophytic growth on cutin as sole carbon source, the CUTAB1$^-$ mutants did not excrete cutinase A_c, the most abundant cutinase found in saprophytic cultures of the wild-type strain (Trail and Köller 1993). The disruption of *CUTAB1* also had severe impact on the excretion of cutinase B_a, a second isozyme purified from cultures grown on cutin. Only very small amounts of a hydrolase with a similar molecular weight were detected in mutant cultures, and the enzyme is, most likely, not related to cutinase B_a. This would indicate that the single cutinase gene *CUTAB1* encodes both cutinase isozymes, suggesting a post-transcriptional or post-translational origin of the two cutinase isozymes. A functional relevance of isozyme diversification has yet to be determined.

In addition to the abolished production of cutinases A_c and B_a, disruption of *CUTAB1* had a drastic effect on the saprophytic utilization of polymer cutin as carbon source. Very similar results were reported for cutinase gene-disrupted mutants of *F. solani* (Stahl and Schäfer 1992). However, the gene-disrupted mutants of *A. brassicicola* continued to excrete small amounts of two cutin-induced serine hydrolases with molecular weights of 31 and 19 kDa. These esterases were specifically induced in the presence of cutin and had cutinase activity. Cutinase production not affected by the disruption of one cutinase gene was also reported for *M. grisea* (Swaigard *et al.* 1992b).

The CUTAB1$^-$ mutants of *A. brassicicola* remained pathogenic. Under the infection conditions employed, the CUTAB1$^-$ mutants remained as virulent as the wild-type strain on both leaf and stem tissue of *Brassica oleracea*, without effect on the direct penetration mode. Similar results were originally reported for cutinase gene-disrupted mutants of *M. grisea* (Sweigard *et al.* 1992b) and *F. solani* (Stahl and Schäfer 1992). The impact of gene disruptions on virulence parameters of the *Fusarium* mutants, such as modes of penetration and disease severity under different infection conditions, were studied more recently, and conflicting results have been reported (Rogers *et al.* 1994; Stahl *et al.* 1994).

The two 31- and 19-kDa serine esterases with cutinase activities, which were both induced and expressed by the CUTAB1$^-$ mutants in cultures with cutin as carbon source, were also expressed during early stages of host infection. To the contrary, the two isozymes cutinase A_c and B_a encoded by *CUTAB1*, which accumulate to high levels in cultures of the wild-type strain growing saprophytically on cutin as carbon

source (Trail and Köller 1993), were not detected on leaves inoculated with wild-type conidia. The lack of *CUTAB1* expression under parastic conditions explains why the respective gene disruption had no effect on the pathogenicity of transformants. It also might indicate a different mechanism or a different time course of enzyme induction for the different cutinases.

The two cutinase isozymes expressed to high levels under saprophytic conditions are rapidly induced by cutin monomers (Trail and Köller 1993), very similar to the cutinase of *F. s.* f. sp. *pisi* (Kolattukudy 1991; Kämper *et al.* 1994) and *Venturia inaequalis* (Köller *et al.* 1991). Expression levels, however, are dependent on the dose of cutin monomers (Lin and Kolattukudy 1978; Köller *et al.* 1991). Cuticle penetration by *A. brassicicola* invading leaves of *B. oleracea* is very similar to *A. brassicae* infecting *B. campestris* (Tewari 1985), completed 24 hr after inoculation. This transient exposure of the pathogen to cuticular components might be too short for the liberation of sufficiently high monomer concentrations.

In contrast to the cutinases A_c and B_a encoded by *CUTAB1*, the residual cutinases excreted by the CUTAB1$^-$ mutants were expressed on host surfaces during early stages of cuticle penetration. The lack of cutinase excretion in glucose-grown cultures, the absence of detectable esterase activities released from conidia germinating in the absence of external carbon sources and accumulation of the residual cutinases in the presence of purified cutin strongly suggests that cutinases not affected by the disruption of *CUTAB1* are cutin-induced rather than constitutive. While the induction mechanism remains to be determined, it appears different from the induction of *CUTAB1*.

The unimpaired pathogenicity of CUTAB1$^-$ mutants on both leaves and stems demonstrates that cutinases A_c and B_a are not involved in tissue specificity. The different pH optima of cutin hydrolysis observed for the two isozymes (Trail and Köller 1993), a parameter correlated with tissue specificities of several pathogens (Trail and Köller 1990), was not relevant in the infection of stem or leaf tissue by *A. brassicicola*. The original correlations of cutinase pH optima with tissue specificities were derived from cutinases produced under saprophytic conditions (Trail and Köller 1990). Recent results have indicated a crucial role of these cutinases in saprophytic mobilization of cutin but not necessarily in host invasion, and the functional analysis of cutinase diversity has become more complex. For example, addition of the cutinase gene from *N. haematococca* to *Cochliobolus heterostrophus* mutants containing the pisatin demethylation gene from *N. haematococca* had no impact on the infection of pea roots (Oeser and Yoder 1994). The gene, which is strongly expressed in the presence of cutin as saprophytic carbon source (Stahl and Schäfer 1992), encodes a cutinase with an alkaline pH optimum typical for root and stem pathogens (Trail and Köller 1990). Expression of the cutinase by double mutants of *C. heterostrophus* led to a distinctively darker color of lesions on pea stems (Oeser and Yoder 1994). This different symptomology might indicate that cutinase derived from a stem-base pathogen indeed had impact on the colonization of stem tissue only mildly colonized by the *C. heterostrophus* wild-type strain.

All cutinase genes cloned thus far encode cutinases predominantly expressed during saprophtytic stages of the respective pathogens. Cloning of the first cutinase cDNA from *F. solani* (Soliday *et al.* 1984) and the cloning of the cutinase gene of *C. capsici* (Ettinger *et al.* 1987) was based on immunological properties of cutinases purified from fungal cultures saprophytically grown on cutin as carbon source. Subsequent cloning of cutinase genes from *M. grisea* (Sweigard *et al.* 1992a), *A. brassicicola* (Yao and Köller 1994), and *C. gloeosporioides* (Ettinger *et al.*, 1987) was based on sequence homologies with the original genes. The observations that additional cutinase genes with extensive sequence homologies to the cloned genes did not exist in *M. grisea* (Sweigard *et al.* 1992) and *A. brassicicola* (Yao and Köller 1994), that the disruption of respective genes had no apparent impact on pathogenicity, that the gene product of *CUTAB1* was not expressed on host surfaces inoculated with *A. brassicicola*, and that alternative cutinases were not affected by respective gene disruptions in *M. griseae* (Sweigard *et al.* 1992) and *A. brassicicola* suggests the existence of a not yet characterized class of cutinases.

The possibility that specialized functions of fungal cutinases in saprophytic and pathogenic stages have evolved is supported by a recent study with two different subpopulations of *C. gloeosporioides* isolated from citrus (Liyanage *et al.* 1992). Both subpopulations produced cutinases, but only DNA isolated from one subpopulation hybridized with the homologous cutinase gene of *C. gloeosporioides* with strong homologies to the gene of *C. lindemuthianum*. This subpopulation of *C. gloeosporioides* was identified as a competent saprophyte, whereas saprophytic competence of the subpopulation that lacked the respective gene was restricted (Agostini *et al.* 1992).

Although evidence for separate classes of cutinases with functions in either the saprophytic mobilization of polymer cutin or early stages of plant infection is growing, it remains circumstantial until all cutinase genes residing in a given pathogen have been disrupted. Respective work is in progress with the CUTAB1$^-$ mutants of *A. brassicicola*.

MATERIALS AND METHODS

Materials.

A. brassicicola was provided by P. Williams, University of Wisconsin, Madison. Conidia were collected from 5-day-old cultures grown on potato-dextrose agar (PDA). Apple cutin and tritiated grapefruit cutin were prepared according to published procedures (Köller and Parker 1989). Restriction enzymes were purchased from Promega, Madison, WI. Radioisotopes were obtained from Du Pont, Boston, MA. Hybond-N membrane for nucleic acids hybridization was purchased from Amersham, Arlington, IL. All other chemicals were from Sigma Chemical Company, St. Louis, MO, unless otherwise specified.

Generation of a disruption plasmid.

Subclone 19, a deleted derivative of pCUTAB1 (Yao and Köller 1994), contained a 1.55-kb DNA fragment of the cutinase gene from *A. brassicicola* on a modified pBluescript vector. Plasmid pOHT had a 2.65-kb insert of a hygromycin resistance construct, consisting of the *hph* gene of *Escherichia coli*, and the *oliC* promoter and *trpC* terminator from *Aspergillus nidulans* (Ward 1991; Hilber *et al.* 1994). Digestion of subclone 19 with PflMI produced two frag-

ments. The smaller fragment was a 495-bp internal part of the cutinase gene *CUTAB1*, and the larger one contained the pBluescript and flanking cutinase gene sequences. The larger fragments were recovered from low melting agarose gel and blunt ended with T4 DNA polymerase.

Excision of the hygromycin resistance construct was accomplished by double digestion of plasmid pOHT with *Hin*dIII and *Xba*I. The two resulting fragments with similar molecular weights were blunt ended with Klenow fragment and ligated with the larger fragment from subclone 19. Ligated products were used to transform *E. coli* bacterial strain XL1-blue by electroporation according to the protocol provided by the supplier (Bio-Rad, Hercules, CA). The cutinase gene-disrupted plasmids were selected according to hybridization with cutinase cDNA pCAB23 (Yao and Köller 1994) and a *Bam*HI fragment of pOHT containing the *hph* gene, but not with the internal Pf1MI fragment of CUTAB1. Six plasmids were selected and subjected to restriction analysis with *Bam*HI and *Xho*I. A plasmid with identical orientations for both the cutinase gene and hygromycin resistance insert was identified and designated pDABC1 (Fig. 1).

Biolistic transformation of *A. brassicicola* with pDABC1.

Biolistic transformation of conidia of *A. brassicicola* followed the procedures described elsewhere (Smith *et al.* 1992; Hilber *et al.* 1994). In brief, 7 ml of PDA was poured over the wet surface of a sterile Whatman No.4 filter paper (9 cm in diameter) to form a "pagar." An aliquot of 0.2 ml of conidia suspension (1.4×10^7 conidia ml^{-1}) was spread over the pagar, and the surface was dried under sterile condition for 15 min before bombardment.

M10 tungsten particles (Sylvania, GTE Product Corp., Towanda, PA) were used as microprojectiles. DNA of pDABC1 was purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients (Sambrook *et al.* 1989). Particles were coated with plasmid DNA according to the protocol of Sanford *et al.* (1993) with some modifications: For the preparation of aliquots sufficient for six bombardments, 50-µl particle suspension containing approximately 3 mg of tungsten particles in 50% (v/v) sterile glycerol was combined with 5 µl of plasmid DNA (1 mg ml^{-1}), 50 µl of 2.5 M $CaCl_2$, and 20 µl of 0.1 M spermidine. The mixture was shaken for 10 min, and particles were pelleted by brief centrifugation. The pellet was washed in 100 µl of 70% (v/v) and then 100% ethanol. Particles were resuspended in 24 µl of 100% ethanol and mixed by brief sonication in an ultrasonic cleaner bath until the suspension appeared homogeneous. Aliquots of 4-µl particle suspension (approximately 0.8 µg of DNA coated on 0.5-mg particles) were spread over the center of Kapton flying disks.

A helium-driven device with flying disks for particle delivery was used for biolistic transformation (Sanford *et al.* 1993). The distance between the helium source and the flying disk was 1 cm, and the distance between the particle launch site and pagar surface was 6 cm. Helium pressure used to accelerate the flying disk was 1,200 psi. The bombarded pagars were transferred immediately to the surface of a plate containing 21 ml of PDA and 133 µg ml^{-1} hygromycin B. The final hygromycin B concentration after full diffusion between both agar layers was 100 µg ml^{-1}, which was sufficient to fully inhibit *A. brassicicola* conidia germination.

After 1 wk of incubation at 22° C, hygromycin B resistant colonies from true transformants appeared. The average number of transformants obtained on each plate was 13. Thirty transformants derivated from single spore cultures were used to screen $CUTAB1^-$ mutants by DNA dot blot hybridization, employing the smaller PflMI fragment of subclone 19 as a probe.

Nucleic acids analysis.

DNA for dot blot analysis was isolated from conidia collected from PDA plates of 5-day-old culture using the procedure of Elder *et al.* (1983). DNA for Southern blot analysis was purified from fungal mycelium according to the method described by Garber and Yoder (1983). Total RNA was extracted using guanidinium thiocyanate followed by centrifugation in cesium chloride solution as described by Sambrook *et al.* (1989). Poly(A)$^+$ RNA was isolated with Promega's polyATract mRNA isolation system (Promega, Madison, WI).

Total or poly(A)$^+$ RNA samples were denatured in 50% (w/v) formamide, 2.2 M formaldehyde, 1× MOPS/EDTA buffer (Sambrook *et al.* 1989) and 10 mg ml^{-1} ethidium bromide at 65° C for 10 min. Gel electrophoresis was performed on 1.2% agarose gels containing 2.2 M formaldehyde in 1× MOPS/EDTA. DNA samples were separated on 1% agarose gels in 1× TBE (Sambrook *et al.* 1989). Gels were blotted onto Hybond-N membranes in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with a vacuum blotter according to the procedures described by the manufacturer (Bio-Rad, Hercules, CA).

All prehybridizations and hybridizations were performed at 65° C in a solution of 5× SSC, 5× Denhardt's (Sambrook *et al.* 1989), 0.1% (w/v) SDS, and 100 mg ml^{-1} denatured salmon sperm DNA. Prehybridization periods ranged from 1 to 24 hr; hybridizations were for 20–24 hr. Membranes were washed at hybridization temperature for 30 min in 1× SSC and 0.1 % SDS twice, then in 0.1× SSC once. All membranes were exposed on Kodak X-OMAT AR film at –70° C for different time periods according to the strength of hybridization signals.

Pathogenicity tests.

Seeds of *Brassica oleracea* 'Roundup' were planted in sterilized soil in plastic pots (15 cm in diameter) and grown in a growth chamber at 22° C with 14 hr light and 10 hr dark periods. Leaves and stems of three plants (4 wk old) were sprayed with 20 ml of 0.1% Tween 20 (w/v) or fungal spore suspensions containing 0.1% Tween 20 (w/v). Conidial densities ranged from 10^5 to 10^6 conidia per milliliter. Following inoculation and drying of surfaces, the plants were moistened with water, wrapped in transparent plastic bags, and incubated in a growth chamber under plant cultivation conditions. Disease symptoms were evaluated 3 days after inoculation.

Enzyme assays and cutinase induction.

Esterase activity was measured spectrophotometrically using *p*-nitrophenyl butyrate (PNB) as substrate, as described previously (Köller and Parker 1989). Cutinase activity was determined using tritiated grapefruit cutin as previously described (Köller *et al.* 1982; Köller and Parker 1989). Cutinase assays were done in duplicates at 30° C for 4 hr. Protein concentrations were determined using the BCA Protein Assay

Reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard.

For enzyme induction under saprophytic growth conditions, 1 ml conidial suspension (1.4×10^7 conidia ml^{-1}) was added to a Roux bottle containing 100 ml of modified Czapek's medium (Trail and Köller 1993) alone, or supplemented with 1 mg ml^{-1} glucose or 5 mg ml^{-1} apple cutin. Cultures were incubated at 22° C and extracellular fluids were obtained by filtration. Equal volumes (2 ml) of extracellular fluids were incubated with 10 µl of ^{3}H-DFP (148 GBq $mmol^{-1}$). Following incubation at room temperature for 2 hr, the proteins were precipated with 7% (w/v) trichloroacetic acid and separated on 14% SDS-polyacrylamide gels (Köller and Parker 1989). Labeled proteins were identified by fluorography (Köller *et al.* 1982).

For enzyme assays, the extracellular fluids derived from mutant cultures were concentrated by a factor of 50 by freeze drying, resuspension of freeze-dried powder in water and centrifugation at 5,000 × *g* for 30 min. Aliquots (0.3 ml) of samples were used to measure cutinase activities and protein contents.

For enzyme induction on host surfaces, leaves of 20 plants (4 wk old) were inoculated with 20 ml conidial suspension (5×10^6 conidia ml^{-1}) in 0.1% Tween 20. Plants sprayed with 0.1% Tween 20 were used as controls. After 24 and 48 hr, leaf surfaces were rinsed with sterile distilled water using a Preval spray gun (Precision Value Co., Yonkers, NY). The samples (approximately 75 ml) were freeze-dried and resuspended in water. Equal portions (0.25 ml) were used for enzyme assays and protein deteminations. Larger portions (2 ml) were used to label serine hydrolases with ^{3}H-DFP, as described above.

ACKNOWLEDGMENTS

We wish to thank F. D. Smith for her technical assistance in the biolistic transformation of *A. brassicicola*, and M. Ward for providing plasmid pOHT. This project was supported by a grant from the National Science Foundation (DCB-9018076).

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