Production of Cell Wall-Degrading Enzymes by
Aspergillus nidulans: A Model System for Fungal Pathogenesis of Plants

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The cell wall-degrading enzymes polygalacturonase and pectate lyase have been suggested to be crucial for penetration and colonization of plant tissues by some fungal pathogens. We have found that Aspergillus nidulans (=Emericella nidulans), a saprophytic Ascomycete, produces levels of these enzymes equal to those produced by soft-rotting Erwinia species. Induction of polygalacturonase and pectate lyase in A. nidulans requires substrate and is completely repressed by glucose. Surprisingly, inoculation of excised plant tissues with A. nidulans conidia leads to formation of necrotic, water-soaked lesions within which the organism sporulates. Thus, A. nidulans has phytopathogenic potential. The release of glucose and other sugars from wounded tissues may repress pectolytic enzyme production and limit disease development. Therefore, we tested creA204, a mutation that relieves glucose repression of some A. nidulans carbon utilization enzymes, for its effect on production of pectolytic enzymes. creA204 failed to relieve catabolite repression of polygalacturonase or pectate lyase and had no effect on disease severity.

INTRODUCTION

The cell walls of living plants carry out diverse functions, one of which is the exclusion of pathogens. When plant cells die, their walls provide a source of nutrients for many organisms, including potentially pathogenic microorganisms. The complex chemical composition and physical structure of plant cell walls make them difficult to penetrate and degrade. Various polymers, including cellulose and protein, are embedded in a matrix of highly branched polysaccharides (McNeil et al., 1984; Selvendran and O’Neill, 1987). One of the simplest of these polysaccharides, pectin, is a major component of the middle lamella and acts as an intercellular cement (Talmadge et al., 1973). When presented with purified cell walls in defined culture medium, the first enzymes produced by many microorganisms are pectin-degrading enzymes (Jones et al., 1972; Mankarios and Friend, 1980). Numerous pectin-degrading enzymes have been identified, including endo- and exo-forms of lyases and hydrases (Bateman and Basham, 1976). Depolymerization of pectin has been reported to be a prerequisite for further cell wall breakdown (Bauer et al., 1973, 1977; Keegstra et al., 1973).

Many plant pathogenic fungi produce pectin-degrading enzymes in culture (Cooper, 1983; Keon et al., 1987). In planta, these enzymes may cause tissue maceration and cell death. However, the actual role they play in plant pathogenesis remains to be determined; whether these enzymes are disease determinants or simply modify the course of disease development is unknown. It should be noted that microbial pectin-degrading enzymes may also serve to activate host defense systems; enzymatically released pectin fragments have been proposed to be endogenous elicitors of plant defense responses (Bishop et al., 1981; Lee and West, 1981; Bruce and West, 1982; Davis et al., 1986; Robertsen, 1987).

The mechanisms controlling expression of cell wall-degrading enzymes in eukaryotes are not well understood. In culture, production of pectolytic enzymes by many fungi, including plant pathogens, requires substrate and is repressed by preferred carbon sources such as glucose (Cooper, 1983). Catabolite repression has been proposed to explain the diphasic growth pattern exhibited by some hemibiotrophs and thereby the way in which these pathogens cause disease. For example, few symptoms are visible during initial infection of bean by Colletotrichum lindemuthianum. Subsequent tissue collapse has been correlated with and proposed to result from the production of large amounts of pectolytic enzymes (Wijesundera et al., 1984). On the other hand, with necrotrophs (Botrytis allii and Rhizoctonia solani, for example) production of pectolytic enzymes begins early during and may even precede infection (Cooper, 1983; Marcus et al., 1986). It is thus possible that some phytopathogens circumvent catabolite repression.

Aspergillus nidulans is one of only a few fungi that have
a highly developed molecular genetic system. *A. nidulans* is closely related to many phytopathogens but is not a significant etiological agent itself. However, in this paper, we show that *A. nidulans* has a limited, but significant, phytopathogenic potential. We further demonstrate that it can efficiently utilize polygalacturonic acid as sole carbon source. Polygalacturonase and pectate lyase are produced sequentially during growth on polygalacturonic acid and their production is subject to catabolite repression. Interestingly, the catabolite derepression mutation creA204 (Hynes and Kelly, 1977) does not relieve repression of these enzymes. In the accompanying paper (Dean and Timberlake, 1989), we report isolation, characterization, and inactivation of the single copy *A. nidulans* pectate lyase gene (pelA).

**RESULTS**

**Growth of *A. nidulans* on Plant Tissues**

We investigated the ability of *A. nidulans* to cause plant disease with an infection assay utilizing detached plant tissues. Tissues were placed on 1% agarose, a medium that does not support growth of the fungus, and inoculated with drops of a conidial suspension. Results from preliminary experiments showed that no detectable infection occurred unless the tissues were wounded at the site of inoculation. Inoculation of wounded sites with heat-killed conidia caused no further tissue damage (Figure 1A). However, when cucumber cotyledons or pea or bean leaves were inoculated with viable conidia, water-soaking and host cell death occurred within a few days. By 5 days, sporulation was evident in the lesions (Figure 1, B to D). Inoculation of the inner surface of orange fruit rinds resulted in rapid fungal growth and extensive sporulation. The tissues were completely colonized within 3 days of inoculation (Figure 1E). When whole orange fruits were inoculated by injecting conidia, the fungus grew into the flesh and by 8 days caused a firm, dark, discolored rot (Figure 1F).

**Utilization of Polygalacturonic Acid as Carbon Source**

Formation of water-soaked lesions on leaves and cotyledons and growth on orange fruit rinds indicated that *A. nidulans* was capable of utilizing pectin as a nutrient source. To test this possibility, we grew the organism in minimal medium containing polygalacturonic acid as sole carbon source. After a lag of ~24 hr, mycelial dry weight increased in a typical sigmoidal fashion, reaching a maximum by ~65 hr (Figure 2). At this time culture filtrates contained two major classes of pectolytic enzymes, polygalacturonase (PG) and pectate lyase (PL). Levels of PG activity produced by *A. nidulans* were similar to those produced by the soft-rotting *Erwinia* species, *E. carotovora* and *E. chrysanthemi* (Table 1). The levels of PL produced by *A. nidulans* and *E. carotovora* were also similar, but were ~10-fold lower than the level produced by *E. chrysanthemi*.

**Production of PG and PL**

The time course of production of extracellular PG and PL by *A. nidulans* grown in minimal medium containing polygalacturonic acid as sole carbon source is shown in Figure 3. PG activity was first detected at 24 hr after inoculation and its appearance coincided with the onset of growth (see Figure 1). Activity in the filtrate continued to increase until it reached a maximum at ~50 hr and then began to decrease. PL activity was first detected at 42 hr and continued to increase until 72 hr. The pH of the culture filtrate increased from 6.8 to 8.6 during the course of the experiment (Figure 1). The optimum pH for PG was ≤4, whereas that for PL was ≥9 (Figure 4). Thus, the times of accumulation of the two enzymes were consistent with their pH optima. PL activity was dependent on Ca²⁺, whereas PG activity was not (Figure 4).

**Catabolite Repression of Pectolytic Enzyme Production**

*A. nidulans* was grown on a variety of carbon sources to investigate the induction and repression of pectolytic enzymes. Table 2 shows that pectolytic enzyme activities were only detected in filtrates from cultures grown with polygalacturonic acid. When glucose was added with polygalacturonic acid, no pectolytic enzyme activities were detected. Thus, pectolytic enzyme production is subject to catabolite repression. Polygalacturonic acid appeared to be a specific inducer of pectolytic enzymes because the enzymes were not produced when *A. nidulans* was grown on other suboptimal carbon sources, including galacturonic acid.

**Effect of the creA204 Mutation on Pectolytic Enzyme Production and Growth on Plants**

One possible explanation for the limited pathogenic ability of *A. nidulans* is that enzymes for cell wall breakdown are subject to carbon catabolite repression in planta. In an attempt to test this hypothesis, we examined the ability of the creA204 mutation to relieve glucose repression of pectolytic enzymes. This mutation partially relieves catabolite repression of a number of enzymes involved in acquisition of suboptimal carbon sources such as ethanol and certain amino acids (Hynes and Kelly, 1977; Kelly and Hynes, 1977). Coisogenic strains RD502 (creA⁰) and RD514 (creA204) were germinated in medium containing glucose and transferred to medium containing either po-
Figure 1. Colonization of Plant Tissues by *A. nidulans*.

Excised leaves, cotyledons, or orange peels were placed in Petri dishes on 1% agarose, inoculated with conidial suspensions, and incubated in an illuminated growth chamber at 30°C. Intact oranges were inoculated by injection. For (A) to (D), the scale bars equal 1 mm. For (E), the scale bar equals 5 mm.

(A) Cucumber cotyledon inoculated with heat-killed conidia.
(B), (C), (D) Respectively, cucumber cotyledon and pea and bean leaves 8 days after inoculation with viable conidia. Water soaking of tissues was evident around lesions containing sporulating colonies.
(E) Sporulation on orange peel 3 days after inoculation. Hyphae from conidia placed in the depression (arrow) colonized the entire surface of the tissue.
(F) Development of firm, dark, discolored rot in whole orange fruit 8 days after injection with viable conidia (right). Heat-killed conidia produced no symptoms (left). Oranges were segmented prior to being photographed.
lygalacturonic acid, polygalacturonic acid plus glucose, or

Figure 2. Growth of A. nidulans on Polygalacturonic Acid.

Cultures were inoculated at 0 hr. Samples were withdrawn at various times and cells were harvested by filtration, dried, and weighed (□). The pH of the culture filtrates was also determined (●).

The reciprocal spore color combinations resulted in lesions that were indistinguishable from one another (data not shown). No differences were observed in lesion size or in the number of viable conidia produced per lesion. Lesions resulting from infections with 1:1 mixtures of yellow-spored and green-spored strains (creA\(^+\) and creA204 and vice versa) produced equal numbers of yellow and green conidia as determined by direct microscopic examination and by dilution platings of conidial suspensions obtained from the lesions.

DISCUSSION

De Bary (1886) postulated an involvement of cell wall-degrading enzymes in plant disease more than 100 years ago. Evidence that these enzymes are essential for penetration and colonization of plant tissues by pathogenic fungi has accumulated but remains circumstantial. For example, extent of tissue damage has been shown to parallel levels of pectolytic enzymes isolated from infected tissues (Harrison, 1983; Barash et al., 1984; Wijesundera et al., 1984; Holz and Knox-Davies, 1985). Isolation of the genes encoding pectolytic enzymes and their subsequent manipulation will allow a direct evaluation of their role in plant disease. For example, it should be possible to inactivate the genes, increase their expression, or alter their regulatory properties. Ideally, coisogenic strains would then be tested for their relative pathogenicity and virulence.

An important problem in this regard is a lack of phytopathogenic fungi that have been intensively studied by using the tools offered by modern genetics and molecular biology. We have demonstrated here that it is possible to study the regulation of pectolytic enzymes in A. nidulans, a saprophyte that is closely related to a number of phytopathogenic fungi. Unlike its phytopathogenic relatives,
however, *A. nidulans* has an extremely well developed genetic system that allows for the most sophisticated types of gene manipulation (reviewed by Timberlake and Marshall, 1988), as is the case with the budding yeast *Saccharomyces cerevisiae*. Unlike yeast, however, *A. nidulans* has demonstrable, although weak, phytopathogenic potential and produces enzymes that degrade plant cell walls.

The results presented in this paper show that *A. nidulans* is able to utilize polygalacturonic acid as sole carbon source and that it produces high levels of PG and PL in culture. *A. nidulans* PG is most active at pH \( \leq 4 \), whereas PL has a pH optimum of \( \geq 9 \). The sequential accumulation of the two enzymes corresponded with an increase in the pH of the growth medium. When the growth medium was maintained at pH 4.5, PG accumulated, whereas PL did not (R. A. Dean and W. E. Timberlake, unpublished results). Thus, the pH of the external medium in part regulates PL production. pH has also been shown to regulate production of other extracellular enzymes in *A. nidulans*, for example, acid and alkaline phosphatases (Caddick et al., 1986). An effect of pH on production of pectolytic enzymes has been observed with other fungi, with PG accumulating preferentially at low pH and PL accumulating at high pH (e.g. Durrands and Cooper, 1988). Arguments concerning the relative roles of PG and PL in plant disease have focused on the pH at which the enzymes are active and the pH of the infected tissues (Cooper, 1983). It is reasonable to assume that production of pectolytic enzymes is regulated by pH in planta as it is in culture.

Regulated production of pectolytic enzymes by *A. nidulans* in culture is similar to that observed with other fungi (Goodenough and Kempton, 1974; Cooper and Wood, 1975; Giltrap and Lewis, 1982). Enzyme induction specifically requires the substrate and is subject to catabolite repression. The monomeric constituent of polygalacturonic acid, galacturonic acid, did not induce enzyme production in *A. nidulans*. On the other hand, galacturonic acid has been demonstrated to be an inducer of pectolytic enzymes in *Verticillium albo-atrum* and *Fusarium oxysporum* (Cooper and Wood, 1975). However, it was only effective when supplied at a low level and a constant rate, most probably to avoid catabolite repression. It is therefore possible that low levels of galacturonic acid can induce pectolytic enzymes in *A. nidulans*.

The levels of pectolytic enzymes produced by *A. nidulans* are substantial, similar to levels produced by soft-rotting *Erwinia* species. Unlike *Erwinia*, however, *A. nidulans*...
Aspergillus nidulans is capable of causing only limited plant disease, even when inoculated onto excised and wounded plant tissues. There are many possible explanations for this weak pathogenic ability. One parameter affecting aggressiveness may be the sensitivity of pectolytic enzyme production by *A. nidulans* to catabolite repression. Catabolite repression has been suggested to underlie disease resistance in some plant-pathogen interactions (Holz and Knox-Davies, 1986a, 1986b). It has also been shown that treatments resulting in increased soluble carbohydrates in plant tissues decrease enzyme production by pathogens and reduce symptom severity (Horton and Keen, 1966; Patil and Dimond, 1968; Weinhold and Bowman, 1974). Genes affecting catabolite repression in the fungus have been identified and cloned in *A. nidulans* (Bailey and Arst, 1975; Hynes and Kelly, 1977). We determined that a mutant allele of one such gene (creA204) has little effect on production of pectolytic enzymes. Mutations at the creA locus have been shown to derepress many enzymes involved in carbon acquisition. However, exceptions, for example quinate dehydrogenase and β-galactosidase, are known (Bailey and Arst, 1975). To alleviate carbon catabolite repression of pectolytic enzymes, we are taking advantage of the *A. nidulans* molecular genetic system. We have cloned the unique gene encoding pectate lyase (pelA) as described in the accompanying paper (Dean and Timberlake, 1989). It is possible to place this and other genes under the control of various regulatory elements and promoters and then to examine the effects of altered levels of expression and regulatory patterns on phenotype (Adams et al., 1988). Evaluation of the importance of PL and PG for pathogenicity and virulence will involve forcing their expression at high levels independent of carbon catabolite repression, pH, and other factors.

### Table 2. Growth and Pectolytic Enzymes Produced by *A. nidulans* on Various Carbon Sources

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Growth b</th>
<th>Pectolytic Enzymes c</th>
</tr>
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<tbody>
<tr>
<td>Polygalacturonic acid</td>
<td>+++ +</td>
<td></td>
</tr>
<tr>
<td>Polygalacturonic acid + glucose</td>
<td>++++ -</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+++ +</td>
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<tr>
<td>Lactose</td>
<td>+++ +</td>
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</tr>
<tr>
<td>Maltose</td>
<td>+++ +</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>+++ +</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>++ -</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>++ -</td>
<td></td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>++ -</td>
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</tr>
</tbody>
</table>

* A. nidulans was grown in liquid culture on minimal medium containing 1% carbon source for 50 hr.

** +++ = good, ++ = moderate, + = little.

* + = polygalacturonase and pectate lyase activities detected in dialyzed culture filtrate, - = neither activity detected.

### Methods

#### Strains, Media, and Growth Conditions

*Aspergillus nidulans* FGSC4 (Glasgow wild-type) and FGSC237 (yA2, pbaA1; trpC901) were obtained from the Fungal Genetics Stock Center. Strains RD501 (yA2; creA+), RD502 (yA2; creA+), RD514 (yA2; creA204) and RD518 (yA2; creA204) were meiotic progeny from a cross of FGSC237 and MH664 (biA1; nlaA4; creA204; provided by M. Hynes). Bacterial strains of *Erwinia carotovora* (EC14) and *E. chrysanthemi* (EC16) were provided by D.P. Roberts and N.T. Keen, respectively. Fungal strains were maintained on appropriately supplemented minimal medium (Pon tecorvo et al., 1953; Clutterbuck, 1974; Käfer, 1977; Timberlake and Hamer, 1986).

For enzyme induction studies on different carbon sources and time course experiments with FGSC4, minimal medium cultures containing 1% (w/v) carbon source were inoculated to a density of 1 × 10⁶ conidia/ml and shaken at 37°C. Cultures were harvested by filtration through Whatman No. 1 paper. Filtrates were dried overnight at 55°C and the dry weights of mycelia were measured. The pH of the filtrates was measured prior to dialysis. For time course experiments with creA204 mutants, strains were inoculated at a density of 1 × 10⁵ conidia/ml in minimal medium containing 1% glucose and shaken at 37°C for 14 hr. Cells were harvested by filtration through Mira-Cloth (Behring Diagnostics), washed with minimal medium, divided equally into minimal medium containing either 1% glucose, 1% polygalacturonic acid, or 1% glucose + 1% polygalacturonic acid and incubated as above. Polygalacturonic acid was washed with ethanol prior to use.

The amount of glucose remaining in the culture filtrates was determined by glucose oxidase coupled to the formation of colored quinonemine dye by peroxidase (Sigma Diagnostic Kit No. 315). To compare levels of pectolytic enzymes produced by *A. nidulans* to other organisms, *E. carotovora* and *E. chrysanthemi* cells were grown in M9 minimal medium containing 1% polygalacturonic acid at 30°C with shaking for 24 hr. Cells were pelleted at 10,000g for 10 min. Supernatants were dialyzed and assayed for pectolytic enzyme activities as described above.

#### Inoculation of Plant Tissues

Leaves from pea (*Pisum sativum* cv Alaska) and bean (*Phaseolus vulgaris* cv White half runner) and cotyledons from cucumber (*Cucumis sativus* cv Market) were detached from growth chamber-grown seedlings. The tissues were surface-sterilized with 10% bleach for 5 min, rinsed in sterile distilled water, and placed on 1% agarose in Petri dishes. Tissues were inoculated with 10-μl drops of a suspension containing 1 × 10⁷ conidia/ml and then wounded through the drops with a sterile needle. To determine the effect of the creA204 mutation, plant tissues were inoculated as above with either RD501, RD502, RD514, and RD518 separately or in all pairwise combinations. For the infection of orange, fruits were surface-sterilized with 70% ethanol. Rinds were re-
creA^+ (---) and creA204 (-----) conidia were germinated in minimal medium containing glucose as carbon source, harvested, transferred to medium containing glucose (○), polygalacturonic acid (▲), or polygalacturonic acid + glucose (■), and incubated for 72 hr. At various times, samples were taken and cells were harvested, dried, and weighed (A). Filtrates were assayed for glucose content (B), polygalacturonase activity, units in mg (C), and pectate lyase activity (D).

Figure 5. Growth and Enzyme Production by creA^+ and creA204 Strains.

Reaction mixtures were incubated for 1 hr to 3 hr at 37°C. Na+ acetate and Tris-HCl buffers were adjusted to various pH values for determination of enzyme activities versus pH. Polygalacturonase activity was determined by measuring the formation of reducing groups by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952). A thiobarbituric acid assay (Preiss and Ashwell, 1963) was used to detect the chromogenic products of pectate lyase at 550 nm and that of polygalacturonase at 509 nm (Ayres et al., 1966).

Pectolytic Enzyme Assays

Polygalacturonase activity was assayed in 50 mM Na+ acetate buffer, pH 4.5, containing 0.5% (w/v) Na+ polygalacturonic acid. Pectate lyase activity was assayed in 50 mM Tris-HCl buffer, pH 8.8, containing 0.5% Na+ polygalacturonic acid and 0.5 mM CaCl2.

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