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Genes from *Cellvibrio mixtus* Encoding β-1,3 Endoglucanase

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Two genes encoding β-1,3 glucanase activity were cloned from the gram-negative soil bacterium *Cellvibrio mixtus*. The two clones, designated *cwd* (cell wall degradation) and *lam* (laminarin degradation), had distinct endonuclease restriction patterns and encoded enzymes with distinct substrate specificities. The 3.7-kilobase *cwd* insert encoded an enzyme which degraded yeast cell walls as well as the soluble β-1,3 glucan laminarin and the insoluble β-1,3 glucans zymosan and pachyman. The 1.8-kilobase *lam* insert encoded an enzyme which degraded laminarin only. Both enzymes degraded laminarin in an endohydrolytic manner to yield laminarobiose, laminarotriose, and laminarotetraose as major end products. Radiolabeled translation products of the *cwd* and *lam* transcripts were identified.

Recent studies of plant-pathogen interactions have indicated that the induction of the enzymes β-1,3 glucanase and chitinase is an important feature of plant defense against fungal phytopathogens (6). These enzymes are induced in plants by fungal attack (23), and purified preparations of induced β-1,3 glucanase and chitinase have been shown to have antifungal activity in vitro (24). Plant β-1,3 glucanases also release elicitors of phytoalexin accumulation from the cell walls of fungi (18). Consequently, it may be possible to engineer plants genetically for resistance to fungal pathogens by manipulating the expression of chitinase and β-1,3 glucanase genes.

Bacterial chitinases (17, 33) and β-1,3 glucanases (19, 26) have been shown to have some antifungal activity in vitro and consequently represent candidate genes for expression in plant cells. Recently, it was reported that plant chitinases show >50 times more antifungal activity than bacterial chitinases (30). In general, plant chitinases are endohydrolytic, while bacterial chitinases are exohydrolytic, and it has been proposed that the mode of enzymatic chitin hydrolysis accounts for differences in antifungal activity (30). Plant antifungal β-1,3 glucanases also have an endohydrolytic action (23). If bacterial β-1,3 glucanase genes are to be used for genetically engineering resistance to fungal pathogens, it thus appears to be necessary to clone genes whose products are β-1,3 endohydrolases. In this paper, we report the cloning and characterization of two distinct β-1,3 endoglucanase genes from *Cellvibrio mixtus*.

MATERIALS AND METHODS

Chemicals and enzymes. Zymosan A, laminarin (*Laminaria digitata*), lichenan (*Usnea barbata*), carboxymethyl cellulose (sodium salt, low viscosity), *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-cellobioside, and *p*-nitrophenyl-β-D-gentiobioside were supplied by Sigma Chemical Co. Barley β-glucan and pachyman were supplied by Biocon Biochemicals Ltd. Restriction endonucleases and T4 DNA ligase were supplied by Promega Pty. Ltd.

Bacterial strains and plasmids. *C. mixtus* UQM 2294 (4), *Escherichia coli* K38 (14), and *E. coli* JM109 (37) have been described previously. Clones expressing β-1,3 glucanase were identified from a cosmid clone bank of *C. mixtus*, which had previously been constructed in the laboratory of J. M. Pemberton with the cloning vector pHC79ΔBamHI::Tn*5* (28) and the *E. coli* host DH1 Rif^r (22). Plasmids used in this study are listed in Table 1.

Preparation of yeast cell walls. Isolated yeast cell walls were prepared by mechanical disruption of commercially supplied baker's yeast with a Bead Beater cell disrupter (Biospec Products), using 0.5-mm glass beads as recommended by the manufacturer. Yeast lysates were examined by phase-contrast microscopy for >90% disruption. Lysates were centrifuged at 7,000 × *g* in a model B20 centrifuge (International Equipment Co.), and the pellets were washed three times by suspending them in distilled water and centrifuging them at 7,000 × *g*. Washed pellets were dried overnight in a dry-air oven, ground to a fine powder, and stored at −20°C.

Media and screening procedures. Bacteria were grown and maintained on LB agar (27) or McConkey agar (36) containing appropriate antibiotics (ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml). Indicator media consisted of Gilardi's mineral salts agar (13), supplemented with yeast extract (0.05%, wt/vol), and laminarin, lichenan, barley glucan, or carboxymethyl cellulose to a final concentration of 2 mg/ml. Plates were developed with Congo red as described previously (31). For the detection of yeast cell wall degradation, LB agar plates were overlaid with 6 ml of LB agar containing 0.4% (wt/vol) isolated yeast cell walls. *E. coli* cultures were incubated for 24 to 48 h at 35°C, and degradation was indicated by clearing of the medium.

Subcloning. Plasmid DNA for cloning and restriction analysis was extracted by the method of Birnboim and Doly (3). DNA was digested with restriction endonucleases in accordance with the supplier's specifications, and enzymes were inactivated at 65°C for 1 h before ligation of vector and insert DNA as described by Maniatis et al. (22). Unless otherwise stated, in subcloning experiments pUC12, pUC19, or pPR510 was used as the recipient vector in ligation reactions which were used to transform *E. coli* JM109 by the method of Chung et al. (8). Transformants bearing recombinant clones were detected by their Lac$^-$ phenotype when grown on McConkey agar supplemented with the appropriate antibiotic. Lac$^-$ transformants were screened for their ability to degrade either laminarin or yeast cell walls.

Radiolabeling of plasmid-encoded proteins. The T7 RNA polymerase/promoter system of Tabor and Richardson (34)

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TABLE 1. Plasmids used in this study

Plasmid	Description[a]	Source or reference
pUC12	Ap^r	25
pUC19	Ap^r	37
pPR510	Cm^r	29
pBR322	Ap^r Tc^r	5
pT7-3	Ap^r T7 ϕ10	S. Tabor
pT7-4	Ap^r T7 ϕ10	S. Tabor
pT7-5	Ap^r T7 ϕ10	S. Tabor
pT7-6	Ap^r T7 ϕ10	S. Tabor
pGP1-2	Km^r T7 RNA polymerase	S. Tabor
pJP3204	Ap^r Km^r *cos* Lam^+	This study
pJP3208	Ap^r Lam^+	This study
pJP3210	Ap^r Km^r *cos* Lam^+ Cwd^+	This study
pJP3214	Ap^r Lam^+ Cwd^+	This study

[a] Ap, Ampicillin; Cm, chloramphenicol; Tc, tetracycline; Km, kanamycin; T7 ϕ10, the ϕ10 promoter of bacteriophage T7; *cos*, cohesive termini of bacteriophage lambda; Lam, laminarin degradation; Cwd, degradation of *S. cerevisiae* cell walls.

was used to radiolabel plasmid-encoded proteins exclusively. Inserts encoding β-1,3 glucanse were cloned in the *Eco*RI-*Hin*dIII sites of pT7-3, pT7-4, pT7-5, and pT7-6. Recombinant pT7 plasmids were used to transform the expression host *E. coli* K38 pGP1-2. Proteins were labeled with [^{35}S]methionine and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Gobius and Pemberton (14). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (20).

Enzyme assays. Sonic extracts of recombinant *E. coli* were used as sources of enzyme. LB agar plates were inoculated with *E. coli* and incubated for approximately 8 h at 37°C before the confluent growth was harvested into ice-cold phosphate buffer (0.02 M; pH 7.0). Ten-milliliter volumes were sonicated on ice with a Branson Sonifier 250 (Branson Ultrasonics Corp.). Two 30-s bursts at the maximum setting for the microtip were interrupted by a 30-s interval. Sonic extracts were centrifuged for 10 min at 7,000 × g and 4°C, and the supernatants were filter sterilized through a 2-μm membrane filter (Millipore Corp.) before storage at −70°C.

Reactions for the hydrolysis of carboxymethyl cellulose, laminarin, barley glucan, zymosan, and pachyman were performed by incubation of 200 μl of suitably diluted sonic extract with 400 μl of a 5-mg/ml solution or suspension of substrate in distilled water and 150 μl of phosphate buffer (0.1 M; pH 7.0) at 35°C. Hydrolysis of lichenan was performed by incubating 900 μl of a suitably diluted sonic extract with 400 μl of a 10-mg/ml suspension of lichenan in phosphate buffer (0.02 M; pH 7.0). The lichenan suspension was sonicated previously to disperse aggregated material (90-s burst, maximum setting for the microtip; Branson Sonifier 250). At the end of incubation, insoluble substrates were removed by centrifugation when necessary, and reducing sugars were measured as described previously (7).

Reaction mixtures with *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-cellobioside, and *p*-nitrophenyl-β-D-gentiobioside consisted of 150 μl of a 1-mg/ml solution of substrate in distilled water, 60 μl of phosphate buffer (0.1 M; pH 7.0), and 90 μl of suitably diluted sonic extract. The release of *p*-nitrophenol was measured at 410 nm as described previously (10). Reducing sugars or *p*-nitrophenol was measured at zero time and up to 3 h of incubation for both recombinant *E. coli* sonic extracts and sonic extracts of *E. coli* bearing the cloning vector pUC19, which served as a negative control.

The linearity of each reaction was determined by using a range of dilutions of the appropriate sonic extracts. Protein assays were performed by the method of Lowry et al. (21), using bovine serum albumin as a standard. Specific activities were expressed as micromoles of *p*-nitrophenol or glucose equivalents released per milligram of protein per hour under the assay conditions.

Action patterns of cloned β-1,3 glucanases. The action patterns of β-1,3 glucanase on laminarin were determined by thin-layer chromatography of reaction products over a 3-h period. Four-microliter volumes of a 50-mg/ml solution of laminarin were added to 200-μl volumes of recombinant *E. coli* sonic extract and incubated at 35°C. Proteins were removed from the reaction with two phenol-chloroform extractions followed by a single chloroform extraction. Ten-microliter volumes of the aqueous layer were loaded onto Keiselgel 60 thin-layer chromatography sheets (Merck) and developed for approximately 2 h in ethyl acetate-acetic acid-water (2:1:1, vol/vol/vol, as described previously (15). Oligosaccharides were detected with *p*-anisaldehyde (12). Oligosaccharide standards consisting of glucose, laminarobiose, laminarotriose, and laminarotetraose were the kind gift of B. A. Stone (La Trobe University, Melbourne, Australia) and were prepared as described previously (1). These standards were added to an *E. coli* extract and treated in the same manner as the reactions.

RESULTS

Identification and subcloning of β-1,3 glucanase clones. When a cosmid clone bank of *C. mixtus* was screened, two cosmid clones, pJP3210 (*cwd*) and pJP3204 (*lam*), were identified which directed the degradation of laminarin (Lam^+). A second screening indicated that *E. coli* carrying pJP3210 also degraded isolated cell walls of the yeast *Saccharomyces cerevisiae* (Cwd^+).

Subcloning pJP3204 with *Eco*RI yielded Lam^+ pPR510 clones bearing 3.8-kilobase (kb) inserts. One of these clones was designated pJP3203. Subcloning the pJP3203 insert with a combination of *Eco*RI and *Bam*HI yielded a clone, pJP3206, with a 2.6-kb insert. The insert was further subcloned by ligating the *Sau*3AI partial digestion products of pJP3206 into the *Bam*HI site of pPR510. The smallest Lam^+ clone, pJP3208, had an insert length of 1.8 kb.

The Lam^+ Cwd^+ cosmid clone, pJP3210, was subcloned with *Pst*I, resulting in pPR510 clones containing 18-kb inserts which encoded the degradation of both laminarin and yeast cell walls. One clone, pJP3212, was partially digested with *Sau*3AI and ligated with *Bam*HI-digested pUC12. The smallest Lam^+ Cwd^+ insert was 4 kb in length. The pJP3212 insert was further subcloned by another round of *Sau*3AI partial digestion and ligation with *Bam*HI-digested pPR510. The smallest Lam^+ Cwd^+ subclone, pJP3214, had an insert length of 3.7 kb. At no stage during subcloning of the *cwd* insert were the Lam and Cwd phenotypes separated. Restriction maps of the *cwd* and *lam* inserts are shown in Fig. 1. Deletions from the ends of the *cwd* insert were made by cloning the 3.3-kb *Eco*RI-*Bam*HI and the 3-kb *Hin*dIII-*Cla*I fragments (Fig. 1) in corresponding sites of pBR322, and in both cases both laminarin and yeast cell wall degradation were abolished.

The phenotypes of both the *cwd* and *lam* inserts were expressed when cloned in pUC12 and pUC19, in opposing orientations with respect to the *lacZ* promoter. Thus, it was

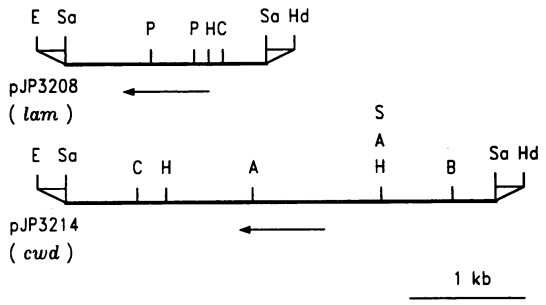

FIG. 1. Restriction maps of subcloned β-1,3 glucanase inserts. Triangles indicate the polylinker region of pUC and pPR510 vectors. Arrows show the direction of transcription of the *lam* and *cwd* genes. A, *Acc*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*cII; Hd, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sa, *Sau*3AI.

concluded that, in both cases, transcription was occurring from a promoter within the insert.

Substrate specificities and action patterns of cloned β-1,3 glucanases. The specific enzyme activities of recombinant *E. coli* extracts, with various substrates, are summarized in Table 2. Control extracts from *E. coli* carrying pUC19 did not degrade the substrates tested except laminarin and barley glucan, for which very low background activities were detected. The enzyme activities of extracts from *E. coli* cells carrying the *lam* and *cwd* genes indicated that the *lam* enzyme is specific for the soluble β-1,3 glucan laminarin, while the *cwd* enzyme is specific for laminarin and the insoluble β-1,3 glucans pachyman and zymosan. The β-1,4-linked substrates carboxymethyl cellulose, *p*-nitrophenyl-β-D-cellobioside, and p-nitrophenyl-β-D-glucopyranoside were not hydrolyzed by the *lam* or *cwd* extract. The *lam* and *cwd* extracts also failed to hydrolyze the β-1,6-linked substrate *p*-nitrophenyl-β-D-gentiobioside, and there was no significant hydrolysis of the mixed β-1,3-1,4-linked glucans lichenan and barley glucan.

Figure 2 shows the end products of laminarin digestion with the *cwd* enzyme extract at various intervals. Laminarin was hydrolyzed to oligosaccharides of various unit lengths until, with time, the major end products appeared to be laminarobiose, laminarotriose, and laminarotetraose. Small amounts of glucose were also detected as well as higher-order oligosaccharides. This action pattern is consistent with endohydrolytic cleavage. The *lam* enzyme produces the same pattern of hydrolysis and the same end products from laminarin.

TABLE 2. Comparison of substrate specificities for extracts of *E. coli* carrying cloned *C. mixtus* β-1,3 glucanase genes[a]

Substrate[b]	Activity (μmol of *p*-nitrophenol or glucose equivalents released/mg of protein per h)		
	C. mixtus gene		Control (pUC19)
	lam	*cwd*	
Laminarin	4.8	24.2	0.2
Zymosan	0	4.3	0
Pachyman	0	17.6	0
Barley glucan	0.3	0.2	0.2
Lichenan	0.1	0.1	0
CMC	0	0	0
PNPC	0	0	0
pNPG	0	0	0
PNPGen	0	0	0

[a] *E. coli* carrying pUC19 served as a negative control.
[b] CMC, Carboxymethyl cellulose; PNPC, *p*-nitrophenyl-β-D-cellobioside; PNPG, *p*-nitrophenyl-β-D-glucopyranoside; PNPGen, *p*-nitrophenyl-β-D-gentiobioside.

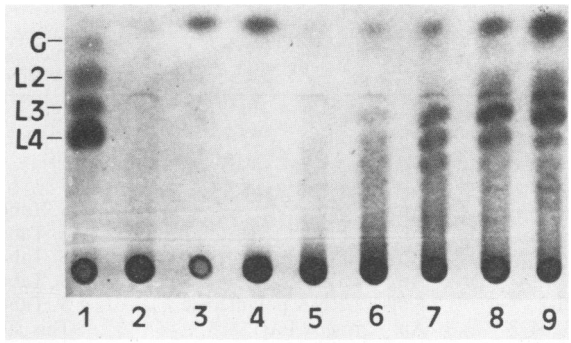

FIG. 2. Thin-layer chromatography of the laminarin hydrolysis products produced by the enzyme *cwd*. Lane 1, Oligosaccharide standards laminarotetraose (L4), laminarotriose (L3), laminarobiose (L2), and glucose (G). Lanes 2 and 3, JM109 pUC19 sonic extracts incubated in the absence of laminarin for 0 and 3 h, respectively. Lane 4, JM109 pUC19 sonic extract incubated with laminarin for 3 h. Lanes 5 to 9, JM109 carrying *cwd* incubated with laminarin for 0, 10, 30, 60, and 180 min, respectively.

An unidentified product migrated between the laminarotriose and laminarobiose standards. This was not a hydrolysis product of laminarin since it was found in *E. coli* sonic extracts which did not contain laminarin (Fig. 2, lane 2). A second unidentified product migrated further than the glucose standard and appeared to be the end product of a reaction catalyzed by native *E. coli* enzymes on an endogenous substrate. This product was found in sonic extracts of *E. coli* bearing only the cloning vector pUC19, which were incubated in the absence of laminarin (Fig. 2, lane 3).

Visualization of cloned β-1,3 glucanase proteins. The in vivo T7 transcription-translation system of Tabor and Richardson was used to exclusively radiolabel the *lam* and *cwd* proteins. Schematic representations of the pT7 vector series are shown in Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins was performed and autoradiographs showing the labeled products of the *lam* and

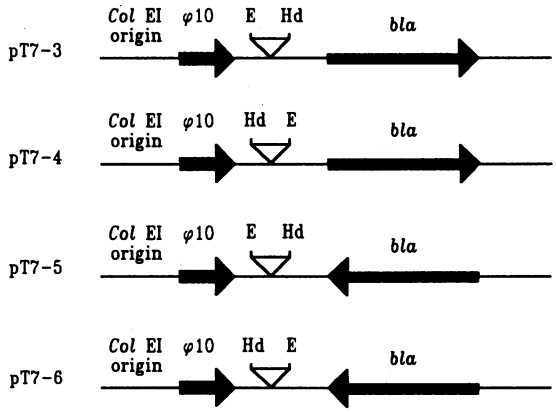

FIG. 3. Schematic representation of the pT7 vector series. Arrows show the direction of transcription of the β-lactamase gene (*bla*) and the direction of transcription from the φ10 promoter. Triangles indicate the polylinker region bounded by *Eco*RI (E) and *Hin*dIII (Hd) sites.

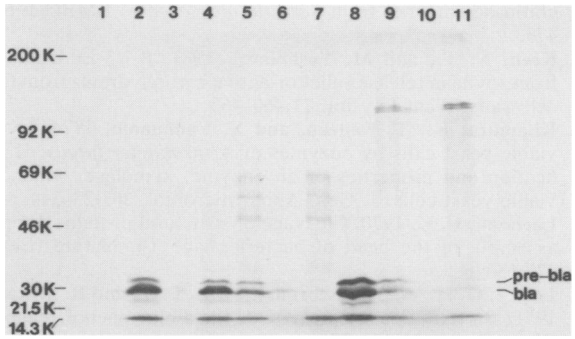

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of radiolabled proteins encoded by pT7-5 (lane 1); pT7-3 (lane 2); pT7-3-*lam*, pT7-4-*lam*, pT7-5-*lam*, and pT7-6-*lam* (lanes 4 to 7, respectively); and pT7-3-*cwd*, pT7-4-*cwd*, pT7-5-*cwd*, and pT7-6-*cwd* (lanes 8 to 11, respectively). Mobilities of protein standards are indicated on the left.

cwd inserts are represented in Fig. 4. When cloned in the *Eco*RI-*Hin*dIII sites of pT7-4 and pT7-6, the *cwd* insert was transcribed from the plasmid-borne ϕ10 promoter and in both cases the insert was in the same orientation with respect to the ϕ10 promoter. When cloned in the *Eco*RI-*Hin*dIII sites of pT7-3 and pT7-5, no insert-encoded proteins were translated and the inserts of these plasmids were in opposite orientation to those of pT7-4-*cwd* and pT7-6-*cwd*. These results showed that the *cwd* gene is transcribed in the direction indicated in Fig. 1. Two insert-encoded proteins were obvious in cells carrying pT7-6-*cwd*, their M_rs being 112,000 and 107,000 (Fig. 4). These proteins plus the mature and immature forms of the pT7-4-encoded β-lactamase were seen in extracts of cells carrying pT7-4-*cwd*. Assuming that the average molecular weight of an amino acid is 110,000, a 112,000-M_r protein would be encoded by a 3-kb gene. This estimated coding region is in agreement with the results of deletion analysis of the *cwd* insert, which indicated a coding region essential for enzyme activity of >2.7 kb.

Insert-encoded proteins were visualized in extracts of *E. coli* carrying pT7-4-*lam* and pT7-6-*lam*, indicating that the *lam* gene is transcribed in the direction shown in Fig. 1. Four radiolabeled proteins of M_rs 66,000, 64,000, 57,000, and 52,000 were seen in cells bearing pT7-6-*lam*. These proteins plus the immature and mature β-lactamases were seen in cells bearing pT7-4-*lam*.

DISCUSSION

Our data agree with those of other workers who have shown that bacterial β-1,3 glucanase systems are generally multicomponent in nature. Enzymological studies have shown that *Oerskovia* sp. strain CK, for example, produces three different β-1,3 glucanases with different substrate specificities (26) and *Streptomyces* sp. strain 1228 produces four β-1,3 glucanases (2). Similarly, *C. mixtus* produces at least two distinct β-1,3 glucanases with different substrate specificities, although further study of the *C. mixtus* system might reveal more enzyme components. The biological roles of the *cwd* and *lam* genes in *C. mixtus* are not known. However, *C. mixtus* is a saprophyte isolated from forest floor litter, and presumably a major source of β-1,3 glucan would be from the cell walls of soil fungi.

Only two bacterial β-1,3 glucanase genes, from *Clostridium thermocellum* (32) and *Arthrobacter* sp. strain YCWD3 (11), have been cloned previously. However, the properties of these cloned gene products are quite different from those of the *lam* and *cwd* enzymes of *C. mixtus*. The activities of the *lam* and *cwd* enzymes are strictly limited to the hydrolysis of β-1,3 glucans; hydrolysis of mixed linkage β-1,3-1,4 glucans is negligible. However, the cloned β-1,3 glucanase from *Clostridium thermocellum* effectively hydrolyzes barley glucan and lichenan (32) and probably belongs to the enzyme class EC 3.2.1.6. The cloned glucanase I from *Arthrobacter* sp. strain YCWD 3 also shows different substrate specificity from that of the *lam* and *cwd* enzymes. A clone encoding glucanase I of *Arthrobacter* sp. strain YCWD3 was kindly provided by K. Doi, and sonic extracts of *E. coli* carrying this gene hydrolyze zymosan and pachyman but in our hands have no effect on laminarin (data not shown). These results indicate that bacteria produce very diverse β-1,3 glucanase enzymes.

The results of deletion analysis and in vivo transcription-translation of the *cwd* insert are consistent with the proposal that a single gene encodes a 112-kilodalton (kDa) protein which is proteolytically cleaved to produce a 107-kDa protein. Two tandemly arranged genes encoding proteins of 112 and 107 kDa could not coexist on a DNA insert as short as 3.7 kb. The 107-kDa protein may represent a signal peptidase-processed form of a secreted protein, but this has not been confirmed. In our opinion, a less likely explanation for the presence of the two different proteins is the existence of two overlapping coding sequences of different lengths. Theoretically, the largest protein that could be encoded by the 1.8-kb *lam* insert would be 66 kDa. The presence of 64-, 57-, and 52-kDa proteins in in vivo transcription-translation experiments, as well as of a 66-kDa protein, may be due to proteolytic cleavage of the 66-kDa protein by native *E. coli* proteases or the presence of multiple overlapping coding regions. These questions will be answered by purification of the proteins and comparison of amino acid and DNA sequence data.

The initial aim of this investigation was to clone bacterial genes encoding β-1,3 glucanases so that they could be assessed for antifungal activity in transgenic plants. The genes we have cloned encoded β-1,3 glucanases with substrate specificities and action patterns which resemble those of plant β-1,3 glucanses (15, 23), which are known to be antifungal (24). The 32-kDa β-1,3 endoglucanase purified from germinating barley is specific for β-1,3 glucan (EC 3.2.1.39) and shows a laminarin hydrolysis pattern similar to those of *lam* and *cwd* (15). The β-1,3 glucanase II which was purified from pea pods and has antifungal activity in vitro belongs to the same class of enzymes and liberates di-, tri-, and tetrasaccharides from laminarin but not glucose (23). Current experiments are aimed at the purification of cloned β-1,3 glucanases for a direct comparison of the effects of plant and bacterial enzymes on fungal growth.

Recent work also suggests that plant β-1,3 glucanases may be involved in an early defense mechanism against fungal pathogens, by releasing elicitors of phytoalexin accumulation from the cell walls of invading fungi (18). The *cwd* enzyme of *C. mixtus* partially degrades isolated cell wall preparations of mycelial fungi (data not shown), and we are interested in determining whether the degradation products are active as elicitors. If bacterial enzymes can mimic this postulated function in vivo, constitutive gene expression in transgenic plants may enhance the defense response against fungal pathogens.

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