Cloning of two genes from *Bacillus circulans* WL-12 which encode 1,3- β -glucanase activity

MICHAEL J. FISKE,*† KAREN L. TOBEY-FINCHER and ROY L. FUCHS

Monsanto Company, Biological Sciences Department, 700 Chesterfield Village Parkway, St Louis, Missouri 63198, USA

(Received 27 February 1990; revised 25 June 1990; accepted 9 August 1990)

Two genes encoding distinct 1,3- β -glucanases have been cloned from *Bacillus circulans* and expressed in *Escherichia coli*. A cosmid library of *B. circulans* WL-12 DNA was constructed in the broad-host-range cosmid pLAFR1 and screened in *E. coli* for clones which exhibited 1,3- β -glucanase activity. Two 1,3- β -glucanase-positive clones were identified which contained genes encoding two independent 1,3- β -glucanases as shown by biochemical, physical and molecular analyses. The cosmids, designated pMON5401 (27.1 kb insert) and pMON5402 (24.7 kb insert), encoded 68 kDa and 40 kDa 1,3- β -glucanases, respectively. Both 1,3- β -glucanases were purified from their respective *E. coli* strains, characterized biochemically, and were shown to exhibit lytic activity against purified yeast cell wall preparations.

Introduction

Knowledge gained over the past decade has made the engineering and expression of foreign genes in plants a practical method to produce plants tolerant to certain classes of insects (Barry, 1986; Barton *et al.*, 1987; Fischhoff *et al.*, 1988; Vaeck *et al.*, 1987), viruses (Abel *et al.*, 1986; Tumer *et al.*, 1987) and herbicides (De Block *et al.*, 1987; Shah *et al.*, 1986). An analogous approach to generate plants tolerant to fungi suffers from our limited knowledge of specific proteins which have significant antifungal activity. To date, no proteins comparable to the *Bacillus thuringiensis* toxin proteins for insect control have been identified which control fungi. One potential approach for fungal control is to use genes which encode hydrolytic enzymes for specific degradation of fungal cell wall components.

The cell walls of many plant-pathogenic fungi contain 1,3- β -glucans and chitin as essential integral structural components (Kobayashi *et al.*, 1974c; Sietsma & Wessels, 1979; Skujins *et al.*, 1965). Numerous microorganisms produce and secrete the hydrolytic enzymes 1,3- β -glucanase and chitinase, which have been suggested to function synergistically to degrade the cell walls of these pathogens (Chesters & Bull, 1963; Horikoshi & Atsukawa, 1973; Kobayashi *et al.*, 1974c). Plants, likewise, have evolved the ability to induce both 1,3- β -

glucanase and chitinase activities in response to fungal infection (Bell, 1981; Nichols *et al.*, 1980). This suggests that these enzymes may be involved in disease protection (Nitzsche, 1983). It has been reported that although neither the 1,3- β -glucanases nor the chitinases from numerous fungi or bacterial sources function as antifungal agents, the two activities in combination act synergistically to provide effective control against several plant pathogenic fungi, when the enzymes are applied directly to the plant leaf surface (N. Ogasawara & H. Tanaka, Japanese Kokoku patent Sho 53-41025, August 1978).

Our laboratory is interested in determining the feasibility of engineering the genes encoding 1,3- β -glucanase and chitinase into plants to confer resistance to plant-pathogenic fungi. We recently described the cloning and expression in *E. coli* of a gene encoding a *Serratia marcescens* chitinase (Fuchs *et al.*, 1986). Large quantities of the highly purified 57 kDa chitinase encoded by this gene were produced. Studies with 1,3- β -glucanase have been hampered by the limited quantities of protein available for testing antifungal activity. To address this problem, we sought to isolate genes encoding 1,3- β -glucanase from *Bacillus circulans*.

The presence of 1,3- β -glucanases has been well documented in a variety of fungi and bacteria (Doi *et al.*, 1973; Manners & Wilson, 1973; Martin *et al.*, 1980; Tanaka & Phaff, 1965; Usui *et al.*, 1985). *B. circulans* WL-12 was selected as the source of the genes encoding 1,3- β -glucanase for these studies for the following

† Present address: Genencor International, 1700 Lexington Avenue, Rochester, New York 14652-3605, USA.

reasons: (1) 1,3- β -glucanases from *B. circulans* have exhibited hydrolysis of fungal cell walls (Horikoshi & Atsukawa, 1973; Kobayashi *et al.*, 1974b); (2) 1,3- β -glucanases from *B. circulans* have been biochemically well characterized (Esteban *et al.*, 1984; Kobayashi *et al.*, 1974a; Rombouts & Phaff, 1976; Tanaka & Phaff, 1965); and (3) the gene(s) encoding 1,3- β -glucanase(s) from *Bacillus* species are likely to be expressed in *E. coli* (Borriess *et al.*, 1985; Cantwell & McConnell, 1983). The molecular cloning and expression in *E. coli* of 1,3/1,4- β -glucanases from both *B. subtilis* (Cantwell & McConnell, 1983; Robson & Chambliss, 1986) and *B. amyloliquefaciens* (Borriess *et al.*, 1985) have been described.

Many micro-organisms, including *B. circulans*, which exhibit 1,3- β -glucanase activity, produce multiple enzyme species (Doi *et al.*, 1973; Kobayashi *et al.*, 1974a; Sietsma & Wessels, 1979; Skujins *et al.*, 1965; Usui *et al.*, 1985; Yamamoto *et al.*, 1983). The isolation of clones which produce unique 1,3- β -glucanases would enable both the circumvention of the problems associated with purifying individual 1,3- β -glucanases from a complex mixture of similar activities and the opportunity to introduce these genes directly into plants for *in vivo* analysis of their efficacies. This paper describes the cloning and expression of the genes encoding two independent 1,3- β -glucanases from *B. circulans* WL-12, and the subsequent purification and characterization of each activity.

Methods

Bacterial strains and culture conditions. *B. circulans* WL-12 was kindly provided by H. J. Phaff through the culture collection of the Department of Food Science and Technology, University of California, Davis, CA, USA. *B. circulans* was grown at 30 °C under constant aeration in medium containing 0.7% Difco yeast nitrogen base, 0.1 M-sodium phosphate (pH 6.8), and either 0.25% yeast cell walls or 0.75% pachyman with or without 0.05% glucose.

E. coli SR193 [C600 *rspL hsdR* Leu⁻ Pro⁻ *supE* (λ C1857 *Bam*58-71 *plac5*)] was obtained from S. Rogers (Monsanto Co., St Louis, MO, USA) and routinely grown at 30 °C to stationary phase in Luria broth (LB) medium (Miller, 1972). Tetracycline (12.5 μ g ml⁻¹) was added for plasmid-containing strains. Plasmid pRmSL26, from which pLAFR1 was derived by *Eco*RI digestion and ligation, was provided by F. M. Ausubel, Harvard University, Boston, MA, USA.

1,3- β -Glucanase-positive clones were identified by screening tetracycline-resistant, cosmid-containing strains on aniline blue-pachyman plates (Mahasneh & Stewart, 1980). Colonies producing active 1,3- β -glucanase developed clear zones on a blue background on this medium due to natural cell lysis and release of intracellular enzyme activity.

Enzyme assay. 1,3- β -Glucanase activity was determined by measuring the reducing power released from the substrate, laminarin, using a modification of the method described by Somogyi (1952). A typical reaction mixture (500 μ l) containing 0.1 M-citrate/phosphate buffer (pH 6.5), laminarin (1 mg ml⁻¹) and enzyme was incubated at 37 °C for 15 min. Specific activities are expressed as μ mol reducing sugar

(glucose) formed min⁻¹ (mg protein)⁻¹. Lytic activity was determined by the addition of an appropriate amount of 1,3- β -glucanase to a dilution of a *Saccharomyces cerevisiae* cell wall suspension in 0.1 M-citrate/phosphate buffer (pH 6.5) to give a final OD₅₅₀ of 1.2 in a final volume of 1.0 ml. The decrease in OD₅₅₀ was monitored over time in reaction mixtures shaken at 100 r.p.m. at 37 °C. Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Preparation of yeast cell walls. Cell walls from *S. cerevisiae* were prepared essentially as described by Okagbue & Lewis (1985).

Crude enzyme preparation. *B. circulans* WL-12 culture supernatants were separated from bacterial cells by centrifugation at 20000 g for 20 min and were concentrated in a stirred cell using Amicon YM-10 membranes. The concentrated material was dialysed extensively against assay buffer and stored at -80 °C.

Cells of *E. coli* SR193 and derivatives containing recombinant cosmid clones were suspended in either 50 mM-potassium phosphate buffer (pH 7.2) or 20 mM-ethanolamine (pH 9.0) as indicated in the text. The suspension was disrupted by sonication followed by centrifugation. The extract was then passed through a Sephadex G-25 column, equilibrated in starting buffer, to remove small molecules which might interfere with enzyme assays.

Construction and screening of a *B. circulans* cosmid library. A cosmid library of *B. circulans* WL-12 chromosomal DNA was prepared in pLAFR1 as described by Maniatis *et al.* (1982). *B. circulans* chromosomal DNA was isolated as described by Barry (1986). Cosmid pLAFR1 was isolated by a modification of the procedure of Birnboim & Doly (1979) and purified on NACS-52 resin following the manufacturer's instructions (Bethesda Research Laboratories). *Eco*RI-cleaved pLAFR1 and *B. circulans* DNA were ligated, packaged into λ heads *in vitro*, and used to infect *E. coli* SR193 cells as described by Maniatis *et al.* (1982).

Approximately 1100 tetracycline-resistant colonies were screened for 1,3- β -glucanase activity on aniline blue-pachyman plates at 30 °C. Two independent clones were identified which expressed 1,3- β -glucanase activity in *E. coli*. The plasmids in these clones were designated pMON5401 (27.0 kb of cloned DNA) and pMON5402 (24.7 kb of cloned DNA).

Purification of 1,3- β -glucanase from *E. coli* SR193(pMON5401). The crude extract protein (3.2 g) obtained from *E. coli* SR193 containing pMON5401 was desalted as described above and applied to a DEAE-Sephacrose column (5 $\times$ 35 cm) equilibrated with 50 mM-potassium phosphate (pH 7.2). The column was washed with three bed volumes of starting buffer. 1,3- β -Glucanase activity was eluted with a 7.0 litre linear gradient of NaCl (0-500 mM). Fractions containing 1,3- β -glucanase activity, which eluted at approximately 200 mM-NaCl, were pooled and concentrated in a stirred cell using an Amicon YM-10 membrane. The partially purified concentrate (5 ml) was dialysed against 5 litres of 10 mM-sodium phosphate buffer (pH 7.0) and applied to a hydroxylapatite column (2.5 $\times$ 25 cm) equilibrated with the same buffer. The column was washed with three bed volumes of starting buffer and eluted with a 1.4 litre linear (10-500 mM) sodium phosphate gradient. Fractions containing 1,3- β -glucanase activity eluted at 50-100 mM-sodium phosphate and were pooled, concentrated, and loaded onto an Ultrogel AcA44 column (116 $\times$ 90 cm) equilibrated in 50 mM-potassium phosphate buffer (pH 6.8). The 1,3- β -glucanase-containing fractions were pooled, concentrated, and stored at -20 °C. All the chromatographic steps were carried out at 4 °C.

Purification of 1,3- β -glucanase from *E. coli* SR193(pMON5402). The crude protein extract (1.8 g) derived from *E. coli* SR193 containing pMON5402 was desalted as described above and applied to a DEAE-Sephacrose column (5 $\times$ 30 cm) equilibrated with 20 mM-ethanolamine

(pH 9.0). The column was washed with three bed volumes of starting buffer and 1,3- β -glucanase was eluted with a 6.0 litre linear NaCl gradient (0–125 mM). Fractions containing 1,3- β -glucanase activity, which eluted at approximately 30 mM-NaCl, were pooled, concentrated to 5 ml and dialysed against 5 litres of 10 mM-sodium phosphate buffer (pH 7.0). The dialysate was loaded onto a hydroxylapatite column (1.5 $\times$ 20 cm) equilibrated in the above buffer. The column was washed with three bed volumes of starting buffer and 1,3- β -glucanase activity was eluted with a 300 ml linear (10–300 mM) sodium phosphate gradient. 1,3- β -Glucanase-containing fractions eluted at approximately 100 mM-sodium phosphate and were pooled, concentrated, and stored at -20°C . All fractionation steps were carried out at 4°C .

Gel electrophoresis. SDS-PAGE analysis was carried out with 12.5% (w/v) acrylamide slab gels by the procedure of Laemmli (1970). Gels were stained for protein using the silver-staining procedure described by Wray *et al.* (1981).

Molecular mass determinations. Subunit molecular masses were estimated by SDS-PAGE analysis using bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa) and trypsinogen (24 kDa) as standards. Molecular masses of native enzymes were estimated by gel filtration on a Superose-12 FPLC column equilibrated in 100 mM-potassium phosphate buffer (pH 6.8); bovine serum albumin (66 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13 kDa) were used as standards.

Isoelectric focusing (IEF). This was performed using an LKB 2117 Multiphor horizontal electrophoresis system with a final acrylamide concentration of 4.8% and a pH gradient of 3.5–9.5. Running conditions consisted of constant power at 25 W, a maximum voltage of 1200 V, and a running temperature of 4°C .

Amino acid sequencing. N-terminal amino acid sequencing was performed using automated Edman degradation chemistry on a gas-phase sequencer (Applied Biosystems model 470A) as described by Hewick *et al.* (1981).

Immunological analysis. Antibodies were prepared in rabbits using purified 68 kDa 1,3- β -glucanase prepared from *E. coli* SR193(pMON5401). Western analyses were performed using the alkaline-phosphatase-conjugated antibody procedure described in the Promega catalogue.

Materials. Protein standards for molecular mass determinations were obtained from Pharmacia. Pachyman was purchased from United States Biochemical Corp. Aniline blue, tetracycline, ampicillin, laminarin, *S. cerevisiae* cells, Sephadex G-25 and DEAE-Sepharose were obtained from Sigma. Hydroxylapatite and electrophoresis reagents were purchased from Bio-Rad. Ultrogel AcA44 and ampholytes were obtained from LKB. Restriction enzymes and DNA ligase were purchased from New England BioLabs and used as recommended by the manufacturers.

Results

Cloning of two 1,3- β -glucanase genes from *B. circulans* WL-12

Approximately 1100 tetracycline-resistant pLAFR1 cosmid *E. coli* clones containing *EcoRI* fragments from partially digested *B. circulans* DNA were screened for 1,3- β -glucanase activity on aniline blue-pachyman plates

Table 1. 1,3- β -Glucanase activity in crude extract preparations

Strain	Plasmid	Sp. act. [$\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$]	Lytic activity*
<i>B. circulans</i> WL-12	None	0.48	1.0
<i>E. coli</i> SR193	None	0.01	0.01
	pMON5401	0.14	0.9
	pMON5402	0.24	1.2

* Lytic activity is expressed as the capacity to degrade yeast cell walls (as measured by the decrease in OD₅₅₀) relative to a crude extracellular enzyme preparation from *B. circulans*, which is designated 1.0.

at 30°C . Three colonies exhibited zones of clearing, due to natural cell lysis, which indicated that a *B. circulans* 1,3- β -glucanase gene(s) was expressed in *E. coli*. Cosmid DNA was isolated from each of the three 1,3- β -glucanase-positive recombinants. *EcoRI* restriction analysis revealed that one of the clones contained *EcoRI* fragments totalling approximately 27.0 kb, whereas the other two clones contained a similar set of *EcoRI* fragments totalling about 24.7 kb. Unique restriction patterns were generated for each of these two classes of clones when digested with five other restriction enzymes (data not shown). No fragments common to both were identified.

The recombinant strains were also examined for their ability to hydrolyse purified yeast cell walls. *E. coli* SR193 containing pMON5401 or pMON5402 was streaked onto an agar medium containing suspended yeast cell walls. Clear zones developed around the colonies of *E. coli* containing either plasmid, indicating that the enzyme(s) produced by each clone does exhibit cell-wall lytic activity (data not shown).

1,3- β -Glucanase activity in crude enzyme preparations

Direct enzymic assay of 1,3- β -glucanase in crude enzyme preparations confirmed that 1,3- β -glucanase was expressed in *E. coli* SR193 containing pMON5401 or pMON5402 (Table 1). 1,3- β -Glucanase produced from both clones also lysed *S. cerevisiae* cell walls in an *in vitro* assay, with lytic activities similar to the crude mixture of 1,3- β -glucanases released into the medium by *B. circulans* (Table 1).

Purification of 1,3- β -glucanase from *E. coli* SR193 (pMON5401) and (pMON5402)

Crude extract protein from *E. coli* SR193(pMON5401) was passed over a Sephadex G-25 column followed by

Table 2. Purification of 1,3- β -glucanases from *E. coli* SR193(pMON5401) and (pMON5402)

Step	Protein (mg)	Total activity ($\mu\text{mol min}^{-1}$)	Sp. act. [$\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$]	Recovery (%)	Purification (-fold)
(a) <i>E. coli</i> SR193(pMON5401)					
Crude extract	3321	462	0.14	100	1
DEAE-Sepharose	425	398	0.94	87	7
Hydroxylapatite	14	181	12.93	38	92
Ultrogel AcA44	3.5	98	28.00	21	200
(b) <i>E. coli</i> SR193(pMON5402)					
Crude extract	1828	432	0.24	100	1
DEAE-Sepharose	21	159	7.57	37	32
Hydroxylapatite	5	81	16.20	19	68

Table 3. Properties of purified 1,3- β -glucanases

Property	Enzyme purified from <i>E. coli</i> SR193 containing:	
	pMON5401	pMON5402
Mol. mass (kDa)		
SDS-PAGE	68	40
Superose-12	45	15
K_m app. (mg ml $^{-1}$)*	1.25	0.143
pH optimum	6.0-6.5	6.0-6.5
pI	4.6	6.5
Lytic activity†	1.20	0.88

* The K_m data correspond to values obtained from a Lineweaver-Burk plot using laminarin as the substrate.

† Lytic activity is expressed as described in Table 1.

used as growth substrates, and that several enzyme species are likely to act synergistically. To determine whether individual or multiple 1,3- β -glucanases, in combination with chitinase(s), function as antifungal agents, individually purified 1,3- β -glucanases are required.

Two DNA fragments, each containing a unique gene encoding a 1,3- β -glucanase, were isolated from a cosmid library of *B. circulans* WL-12 DNA by screening 1100 tetracycline-resistant pLAFR1 cosmid clones. Expression of the 1,3- β -glucanase genes in *E. coli* was demonstrated by (1) zones of clearing on aniline blue-pachyman plates; (2) zones of clearing on agar plates containing yeast cell walls; (3) direct assay of enzyme activity in crude and purified preparations; and (4) assay of cell-wall lytic activity in both crude and purified preparations using suspensions of purified yeast cell walls. These two genes and their protein products were shown to be distinct based on both genetic and

biochemical evidence. Restriction enzyme patterns generated with six restriction enzymes showed that the two cosmids contained no common fragments. The proteins produced from the two clones differed substantially in size (40 and 68 kDa). Their N-terminal amino acid sequences, determined through fifteen places, showed no homology. Polyclonal antibodies generated against the 68 kDa protein purified from *E. coli* SR193(pMON5401) cross-reacted with the 68 kDa protein purified from SR193(pMON5401) and *B. circulans* crude culture filtrate, but not with the 40 kDa protein purified from SR193(pMON5402). Finally, the purified 40 kDa 1,3- β -glucanase from pMON5402 had a tenfold lower apparent K_m for laminarin than the 68 kDa 1,3- β -glucanase from pMON5401. These data strongly support the unique identity of the cloned genes in plasmids pMON5401 and pMON5402.

The biochemical characteristics listed in Table 3 suggest that the two *B. circulans* 1,3- β -glucanases may correspond to previously reported 1,3- β -glucanases. The 1,3- β -glucanase produced by *E. coli* SR193(pMON5401) possesses lytic activity towards yeast cell walls, has a K_m for laminarin of 1.25 mg ml $^{-1}$ and exhibits a pI of 4.6 (Table 3). Esteban *et al.* (1984) described the isolation of a lytic enzyme, 1,3- β -glucanase IV, with very similar properties. They reported an enzyme with a K_m of 1.23 mg ml $^{-1}$ for laminarin, and an identical pI of 4.6. The molecular masses estimated for the native proteins differ considerably: 68 kDa for the enzyme described in this report versus 21 kDa for that described by Esteban *et al.* (1984). However, as discussed below, this difference in molecular mass may not be accurate. The 1,3- β -glucanase isolated from *E. coli* SR193(pMON5402) proved to be similar to 1,3- β -glucanase I described by Rombouts & Phaff (1976). They purified a cell-wall lytic enzyme with a subunit molecular mass of 40 kDa, a K_m for laminarin of 0.143 mg ml $^{-1}$, and a pH optimum of

Fig. 3. SDS-PAGE analysis of 1,3- β -glucanases and Western blot hybridization with rabbit polyclonal antibodies raised against the 68 kDa glucanase. (a) SDS-PAGE. First lane, molecular mass markers; lane 1, crude culture filtrate from *B. circulans* WL-12; lane 2, purified 68 kDa 1,3- β -glucanase; lane 3, purified 40 kDa 1,3- β -glucanase. (b) Western blot. Lanes 1-3 correspond to lanes 1-3 in (a).

5.5. The enzyme purified from *E. coli* SR193(pMON5402) also lyses yeast cell walls, has an identical subunit molecular mass of 40 kDa, and a similar K_m of 0.105 mg ml⁻¹, but gives a slightly different pH optimum of 6.0-6.5 (Table 3). It is of interest that the molecular mass values obtained for both cloned 1,3- β -glucanases were consistently lower when using gel filtration chromatography as opposed to SDS-PAGE analysis. The reason for this observation is unclear. However, it is possible that 1,3- β -glucanases exhibit a slight affinity for the agarose-based medium used in manufacturing Pharmacia Superose columns, which lowers the apparent molecular mass. Usui *et al.* (1985) observed the same phenomenon in the purification and molecular mass estimation of 1,3- β -glucanases isolated from *Rhizoctonia solani*. The 21 kDa molecular mass reported by Esteban *et al.* (1984) for their 1,3- β -glucanase IV was determined by molecular sieve chromatography and hence may also be an underestimation.

The cloning and expression of genes coding for hydrolytic enzymes which are capable of degrading fungal cell wall components is the first step to the engineering of plants with these activities. With both a chitinase gene from *S. marcescens* and two 1,3- β -glucanase genes from *B. circulans* WL-12 cloned, it will now be possible to test both the *in vitro* effects of the purified enzymes and to insert these genes into plants to

evaluate their *in planta* efficacy against plant-pathogenic fungi.

We thank Bruce Bishop, Ned Siegel, Bob Heeren, David Drahos and Sylvia McPherson for their technical support and suggestions throughout the course of these studies. We also thank Ganish Kishore, Sue MacIntosh and Alan Berry, for critical review of this manuscript and Lori VanDelinder and Dee Rollo for typing this manuscript.

References

- ABEL, P. P., NELSON, R. S., HOFFMAN, B. DE N., ROGERS, S. G., FRALEY, R. T. & BEACHY, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738-743.
- BARRY, G. F. (1986). Permanent insertion of foreign genes into the chromosomes of soil bacteria. *Bio/Technology* **4**, 446-449.
- BARTON, K. A., WHITELEY, H. R. & YOUNG, N. S. (1987). *Bacillus thuringiensis* δ -endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. *Plant Physiology* **85**, 1103-1109.
- BELL, A. A. (1981). Biochemical mechanisms of disease resistance. *Annual Review of Plant Physiology* **32**, 21-81.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513-1524.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248-254.
- BORRIS, R., BAUMLEIN, H. & HOFEMEISTER, J. (1985). Expression in *Escherichia coli* of a cloned β -glucanase gene from *Bacillus amyloliquefaciens*. *Applied Microbiology and Biotechnology* **22**, 63-71.
- CANTWELL, B. A. & MCCONNELL, D. J. (1983). Molecular cloning and expression of a *Bacillus subtilis* β -glucanase gene in *Escherichia coli*. *Gene* **23**, 211-219.
- CHESTERS, C. G. C. & BULL, A. T. (1963). The enzymatic degradation of laminarin. 1. The distribution of laminarinase among microorganisms. *Biochemical Journal* **86**, 28-31.
- DE BLOCK, M., BOTTERMAN, J., VANDEWIELE, M., DOCKX, J., THOEN, C., GOSSELE, V., MOVVA, N. R., THOMPSON, C., VAN MONTAGU, M. & LEEMANS, J. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO Journal* **6**, 2513-2518.
- DOI, K., DOI, A. & FUKUI, T. (1973). Purification and properties of lytic β -glucanase from an *Arthrobacter* bacterium. *Agricultural and Biological Chemistry* **37**, 1619-1627.
- ESTEBAN, R., NEBRADA, A. R. & VILLA, T. G. (1984). Synthesis and regulation of *Bacillus circulans* WL-12 1,3- β -D-glucanases. *Journal of General Microbiology* **130**, 2483-2487.
- FISCHHOFF, D. A., BOWDISH, K. S., PERLAK, F. J., MARRONE, P. G., MCCORMICK, S. M., NIEDERMAYER, J. G., DEAN, D. A., KUSANO-KRETZMER, K., MAYER, E. J., ROCHESTER, D. E., ROGERS, S. G. & FRALEY, R. T. (1988). Insect tolerant transgenic tomato plants. *Bio/Technology* **5**, 807-813.
- FUCHS, R. L., MCPHERSON, S. A. & DRAHOS, D. J. (1986). Cloning of a *Serratia marcescens* gene encoding chitinase. *Applied and Environmental Microbiology* **51**, 504-509.
- HEWICK, R. M., HUNKAPILLER, M. W., HOOD, L. E. & DREYER, W. J. (1981). A gas-liquid solid phase peptide and protein sequenator. *Journal of Biological Chemistry* **256**, 7990-7997.
- HORIKOSHI, K. & ATSUKAWA, Y. (1973). β -1,3-Glucanase produced by alkalophilic bacteria *Bacillus* no. K-12-5. *Agricultural and Biological Chemistry* **37**, 1449-1456.
- KOBAYASHI, T., TANAKA, H. & OGASAWARA, N. (1974a). Concerted inductions of multiple β -1,3 glucanases in *Bacillus circulans* WL-12 in response to three different substrates. *Agricultural and Biological Chemistry* **38**, 967-972.
- KOBAYASHI, Y., TANAKA, H. & OGASAWARA, N. (1974b). Multiple β -1,3 glucanases in the lytic enzyme complex of *Bacillus circulans* WL-12. *Agricultural and Biological Chemistry* **38**, 959-965.

- KOBAYASHI, Y., TANAKA, H. & OGASAWARA, N. (1974c). Purification and properties of F-1a, a β -1,3 glucanase which is highly lytic toward cell walls of *Piricularia oryzae* P2. *Agricultural and Biological Chemistry* **38**, 973–978.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MAHASNEH, A. M. & STEWART, D. J. (1980). A medium for detecting β -1,3 glucanase activity in bacteria. *Journal of Applied Bacteriology* **48**, 457–458.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MANNERS, D. J. & WILSON, G. (1973). Studies on β -glucanases: some properties of a bacterial endo- β (1 $\rightarrow$ 3)-glucanase system. *Biochemical Journal* **135**, 11–18.
- MARTIN, D. F., PRIEST, F. G., TODD, C. & GOODFELLOW, M. (1980). Distribution of β -glucanases within the genus *Bacillus*. *Applied and Environmental Microbiology* **40**, 1136–1138.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NICHOLS, E. J., BECKMAN, J. M. & HADWIGER, L. A. (1980). Glycosidic enzyme activity in pea tissue and pea-*Fusarium solani* interactions. *Plant Physiology* **66**, 199–204.
- NITZSCHE, W. (1983). Chitinase as a possible resistance factor for higher plants. *Theoretical and Applied Genetics* **65**, 171–172.
- OKAGBUE, R. N. & LEWIS, M. J. (1985). Influence of mixed culture conditions on yeast-wall hydrolytic activity of *Bacillus circulans* WL-12 and on extractability of astaxanthin from the yeast *Phaffia rhodozyma*. *Journal of Applied Bacteriology* **59**, 243–255.
- ROBSON, L. M. & CHAMBLISS, G. H. (1986). Cloning of the *Bacillus subtilis* DLG β -1,4-glucanase gene and its expression in *E. coli* and *B. subtilis*. *Journal of Bacteriology* **165**, 612–619.
- ROMBOUTS, F. M. & PHAFF, H. J. (1976). Lysis of yeast cell walls: lytic β -(1,3)-glucanases from *Bacillus circulans* WL-12. *European Journal of Biochemistry* **63**, 121–130.
- SHAH, D. M., HORSCH, R. B., KLEE, H. J., KISHORE, G. M., WINTER, J. A., TUMER, N. E., HIRONAKA, C. M., SANDERS, P. R., GASSER, C. S., AYKENT, S., SIEGEL, N. R., ROGERS, S. G. & FRALEY, R. T. (1986). Herbicide tolerance in transgenic plants. *Science* **233**, 478–481.
- SIETSMA, J. A. & WESSELS, J. G. H. (1979). Evidence for covalent linkages between chitin and β -glucan in a fungal wall. *Journal of General Microbiology* **114**, 99–108.
- SKUJINS, J. J., POTGIETER, H. J. & ALEXANDER, M. (1965). Dissolution of fungal cells by a streptomycete chitinase and β -(1 $\rightarrow$ 3)-glucanase. *Archives of Biochemistry and Biophysics* **111**, 358–364.
- SOMOGYI, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry* **195**, 19–23.
- TANAKA, H. & PHAFF, J. H. (1965). Enzymatic hydrolysis of yeast cell walls. I. Isolation of wall-decomposing organisms and separation and purification of lytic enzymes. *Journal of Bacteriology* **89**, 1570–1580.
- TANAKA, H., ITAKURA, K. & TODA, K. (1978). Concerted induction of β -glucanases of *Bacillus circulans* WL-12 in response to various yeast glucans. *Agricultural and Biological Chemistry* **42**, 1631–1636.
- TANAKA, H., KOBAYASHI, Y., ITAKURA, K. & KAMIMIYA, S. (1981). Concerted induction of cell wall lytic enzymes in *Bacillus circulans* WL-12. *Advances in Biotechnology* **4**, 593–598.
- TUMER, N. E., O'CONNELL, K. M., NELSON, R. S., SANDERS, P. R., BEACHY, R. N., FRALEY, R. T. & SHAH, D. M. (1987). Expression of alfalfa mosaic virus coat protein confers cross-protection in transgenic tobacco and tomato plants. *EMBO Journal* **6**, 1181–1188.
- USUI, T., TOTANI, K., TOTSUKA, A. & OGUCHI, M. (1985). Purification of endo-(1 $\rightarrow$ 3)- β -D-glucanases lysing yeast cell walls from *Rhizoctonia solani*. *Biochimica et Biophysica Acta* **840**, 255–263.
- VAECK, M., REYNAERTS, A., HOFTE, H., KANSSENS, S., DE BEUCKELEER, M., DEAN, C., ZABEAU, M., VAN MONTAGU, M. & LEEMANS, J. (1987). Transgenic plants protected from insect attack. *Nature, London* **328**, 33–37.
- WRAY, W., BOULIKAS, T., WRAY, V. P. & HANCOCK, R. (1981). Silver staining proteins in polyacrylamide gels. *Analytical Biochemistry* **118**, 197–203.
- YAMAMOTO, S., MIYAGI, M. & NAGASAKI, S. (1983). Amino acid residues involved in the action of endo-(1 $\rightarrow$ 3)- β -D-glucanase II from *Flavobacterium dormitator* var. *glucanolyticae* FA-5. *Carbohydrate Research* **114**, 137–145.