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Identification of an Antifungal Chitinase from a Potential Biocontrol Agent, *Bacillus cereus* 28-9

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***Bacillus cereus* 28-9 is a chitinolytic bacterium isolated from lily plant in Taiwan. This bacterium exhibited biocontrol potential on Botrytis leaf blight of lily as demonstrated by a detached leaf assay and dual culture assay. At least two chitinases (ChiCW and ChiCH) were excreted by *B. cereus* 28-9. The ChiCW-encoding gene was cloned and moderately expressed in *Escherichia coli* DH5α. Near homogenous ChiCW was obtained from the periplasmic fraction of *E. coli* cells harboring *chiCW* by a purification procedure. An *in vitro* assay showed that the purified ChiCW had inhibitory activity on conidial germination of *Botrytis elliptica*, a major fungal pathogen of lily leaf blight.**

Keywords: Antifungal activity, Chitinase, ChiCW, Gene cloning, Protein purification

Introduction

Chitin is an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine (GlcNAc). It is a major constituent of the cell wall of many fungi, insect exoskeletons, and crustacean shells (Bartnicki-Garcia 1969; Sietsma and Wessels, 1979; Gooday 1990; Cohen-Kupiec and Chet 1998). Chitinases (EC 3.2.1.14) are found in a broad range of organisms, including bacteria, fungi, and higher plants, and play different roles in their origin (Flach *et al*., 1992; Graham and Sticklen, 1994; Felse and Panda, 1999). Chitinase-producing microorganisms have been reported as biocontrol agents for different kinds of fungal diseases of plants (Chernin *et al*., 1995; Kobayashi *et al*. 2002; Freeman *et al*., 2004). However, no chitinolytic microorganism has been reported for use in biocontrol of Botrytis leaf and flower blight of lily which causes severe economic loss of cut-flower production in Taiwan (Hsieh and Huang, 1998). Fungicides have been used in high frequency to control this disease, but difficulty in chemical control is increasing due to a rapid development of fungicide resistance by *B. elliptica* (Chastagner and Riley, 1990; Migheli *et al*., 1990). Therefore, biological control involving two or more mechanisms has been chosen as a good alternative for practical use in the control of Botrytis blight (Elad, 1996; Chiou and Wu, 2001).

Bacillus cereus is a large, gram-positive, endospore-forming bacterium that is very common in soils and plants (Brunel *et al*., 1994; Martinez *et al*., 2002). For plant disease control, *B. cereus* UW85, which is capable of producing two antibiotics responsible for disease suppression (Silo-Suh *et al*., 1994), has been proven to be a reliable biocontrol agent of Phytophthora damping off and root rot of soybean (Emmert and Handelsman, 1999). An endophytic *B. cereus* strain 65 producing a chitobiosidase is also found effective against *R. solani* in cotton (Pleban *et al*., 1997). However, the role of chitobiosidase in the antagonism of *B. cereus* strain 65 toward fungal pathogens is not clearly understood.

In this study, a chitinolytic bacterium *B. cereus* 28-9 from lily plant was investigated on its potential biocontrol activity against *B. elliptica*. A chitinase-encoding gene was cloned from *B. cereus* 28-9 and expressed in *Escherichia coli*. This chitinase was purified from a recombinant *E. coli* and used to demonstrate its antifungal activity.

Materials and Methods

Bacterial strains and culture conditions *B. cereus* 28-9 isolated from lily plant in Taiwan showed high chitinase activity on the colloidal chitin plate (1× M9 salts, 0.5% yeast extract, 0.2% colloidal chitin, and 1.5% agar). *E .coli* DH5α (Bethesda Research Laboratories, USA) was used as a host to express foreign chitinase. Bacteria were cultured in Luria-Bertani (LB) medium supplemented with ampicillin at a final concentration of 50 μg/ml if necessary.

Antagonistic activity assay Dual culture assay and leaf disc assay were performed to assess the potential biocontrol activity of

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B. cereus 28-9. *B. elliptica* B061, as a target fungus, was cultured on V-8 agar slant [20% V-8 juice (Campbell Soup Co., Camden, USA), 0.3% $CaCO_3$, 1.8% agar] at 20ºC under near-UV light to induce sporulation (Doss *et al*., 1984). In the dual culture assay, conidial suspension of *B. elliptica* B061 and bacterial suspension of *B. cereus* 28-9 were streaked on the surface of LB agar at a distance of 4 cm. The growth of both was examined daily for the formation of inhibition zone. In the detached leaf assay, the bulbs of oriental lily cv. Star Gazer (*Lilium* oriental hybrid) were grown for four weeks to provide leaf discs. Leaf discs (15 mm diameter) were cut from the middle leaves and placed on water-soaked filter paper in Pyrex petri dish. Bacterial suspension of *B. cereus* 28-9 was atomized on the abaxial surface of lily leaves before, simultaneously with, or after the atomization of conidial suspension of *B. elliptica* B061 (5×10^4 conidia/ml, prepared in sterile distilled water containing 0.05% Tween 20). The disease severity on each leaf disc was recorded as the percentage of necrotic area three days after inoculation. Four leaf discs were used in each treatment. The experiment was repeated three times.

Cloning of chitinase-encoding gene from *B. cereus* 28-9 A genomic library was constructed in Lambda ZAPII (Stratagene, La Jolla, USA) using *Eco*RI-digested genomic DNA of *B. cereus* 28-9. The genomic DNA of *B. cereus* 28-9 was isolated and purified using a method described by Keim *et al.* (Keim *et al*., 1997). *Eco*RI partially digested genomic DNA of 4 to 8 kb was size-fractionated by preparative agarose gel electrophoresis and purified from the gel with a Geneclean II kit (Bio-101, La Jolla, USA). The DNA fragments were ligated into *Eco*RI-digested, phosphatased Lambda ZAPII arms. After *in vitro* lambda packaging, *E. coli* XL1-Blue MRF' cells ($OD_{600} = 0.5$) were infected with recombinant phages and grown on NZY plates (0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 1% casein hydrolysate, 1.5% agar) which were overlayed with LB top agar containing 0.2% colloidal chitin. *E. coli* XL1-Blue MRF' cells infected by recombinant phages carrying chitinase genes were screened for the formation of clearer plaque after culturing at 37ºC for several days. Subsequently, DNA inserts in positive phages were excised *in vivo* by rescue of a pBlueScript SK phagemid after superinfection with a helper phage. The nucleotide sequence of DNA insert was determined using an autosequencer ABI-310 (Applied Biosystems, Foster City, USA) and analyzed with the software of NCBI (National Center for Biotechnology) or the Genetics Computer Group (GCG) Wisconsin Package.

Southern blot analysis The genomic DNA of *B. cereus* 28-9 was digested with restriction enzymes. After agarose gel electrophoresis, the DNA fragments were blotted onto a Hybond-N+ nylon membrane (Amersham Biosciences). DNA probe was prepared using a PCR DIG probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany), following the method as described by the manufacturer. The nylon membrane was hybridized with the DIG-labeled DNA probe, following by immunodetection with a DIG luminescent detection kit (Roche Diagnostics GmbH) and high performance chemiluminescence film (Hyperfilm™ ECL™, Amersham Biosciences).

SDS-PAGE and zymogram analysis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli procedure (Laemmli, 1970) using the Mini-Protean II apparatus (Bio-Rad, Herculus, USA). A 10% separating gel containing 0.01% glycol chitin as the substrate of chitinase was used to detect chitinase activity. After electrophoresis, proteins in the gel were renatured in 0.1 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100 at 37ºC. Subsequently, the gel was stained with 0.01% Calcofluor White M2R (Sigma, St. Louis, USA) in 0.5 M Tris-HCl (pH 8.9) and examined for the chitinolytic bands under UV transilluminator (Trudel and Asselin, 1989). In another gel, proteins were stained with Coomassie Brilliant Blue G-250.

Chitinase activity measurements A fluorometric assay was used to determine chitinase activity using 4-methylumbelliferyl-N,N',N''-chitotriose (Sigma, St. Louis, USA) as a substrate. The amount of 4-methylumbelliferone (4-MU) released was measured spectrofluorometrically by using a fluorescence spectrophotometer (F-4500, Hitachi) (excitation 390 nm and emission 450 nm). One unit (U) of chitinase activity was defined as the amount of enzyme required to release one μmol of 4-MU per min at 37ºC. Protein concentration was determined by Bradford's method (Bradford, 1976) using bovine serum albumin as standard.

Expression and purification of chitinase from *E. coli* cells Full-length chitinase-encoding gene was amplified by polymerase chain reaction with a pair of oligonucleotide primers, cwf 5'-GGAATTC GAAAGGAGAAATGGC<u>ATG</u>AGGTC-3' and cwr 5'-GGAATTC <u>CTA</u>GTTTTCGCTAATGAC-3'. The amplified fragment was ligated into pCR2.1-TOPO (Invitrogen, Carlsbad, USA) and transformed into *E. coli* DH5α. Recombinant *E. coli* DH5α harboring chitinase gene was cultured in LB broth supplemented with 50 μg/ml ampicillin on a rotary shaker at 37ºC for 20 h and harvested by centrifugation (8,000×*g*, 10 min). The periplasmic fraction of *E. coli* cells was prepared by an osmotic shock method (Manoil and Beckwith, 1986). The proteins were precipitated by adding solid ammonium sulfate to 70% saturation. The precipitate was dissolved in 25 mM Tris-HCl buffer, pH 8.5 and dialyzed overnight against the same buffer. The dialysate was applied to an anion-exchange (Q Ceramic HyperD) column (Sigma, St. Louis, USA) equilibrated with the same buffer. Proteins were eluted with 0.1 to 0.5 M NaCl gradient in 25 mM Tris-HCl buffer, pH 8.5. Active fractions with chitinase activity were pooled and dialyzed against 100 mM potassium phosphate buffer, pH 6.0. All purification steps were carried out at 4ºC.

Antifungal assay of ChiCW Purified chitinase from *E. coli* was used for antifungal assay. Conidia of *B. elliptica* B061 was used as a target and the concentration of conidial suspension was adjusted to 10^5 conidia/ml. In each treatment, 4 μl of enzyme solution was mixed with 4 μl of conidial suspension and incubated at room temperature for 12 h. The germination of 100 conidia was examined under a light microscope and the percentage of inhibition was calculated. The experiment was repeated three times.

Nucleotide sequence accession number The nucleotide sequence of *chiCW* has been deposited in the GenBank database under accession number AF416570.

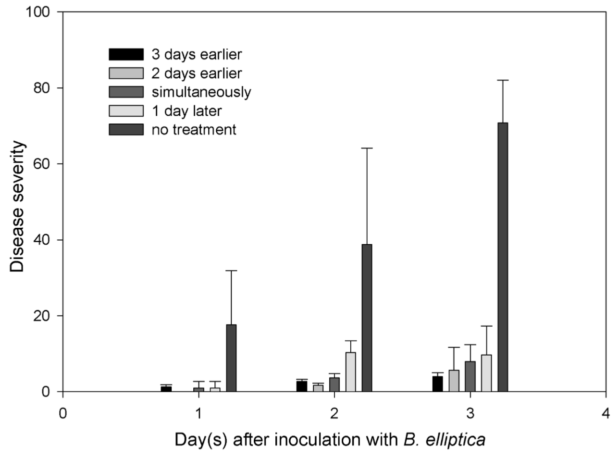

Fig. 1. Biocontrol potential assay on *B. cereus* 28-9 against *B. elliptica. B. cereus* 28-9 at a concentration of 2×10^8 CFU/ml was sprayed onto lily leaf discs at the following time points: 3 days prior to, 2 days prior to, simultaneously with, or 1 day after inoculation with conidial suspension of *B. elliptica* B061. The symptom development on the lily leaf discs were observed three days after inoculation with *B. elliptica*.

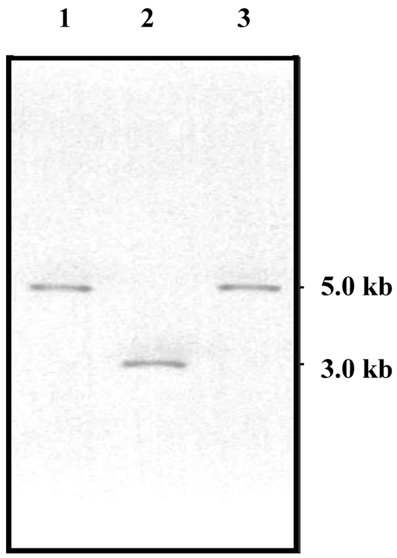

Fig. 2. Southern blot analysis of the genomic DNA of *B. cereus* 28-9. The genomic DNA was digested with *Eco*RI (lane 1), *Eco*RV (lane 2) or *Hin*dIII (lane 3). The DNA probe was generated to target *chiCW* gene.

Results

Biocontrol potential of *B. cereus* 28-9 The antagonistic activity of *B. cereus* 28-9 against fungi was demonstrated by dual culture assay and a detached leaf assay against *B. elliptica* B061. In the detached leaf assay, when *B. cereus* 28-9 was applied at a concentration of 2×10^8 CFU/ml simultaneously to the leaf disc inoculated with conidial suspension, a significant inhibition on the development of lesions was observed. The necrotic lesions appeared browning in a very few cells without undergoing water soaking. Furthermore, the development of lesions was significantly suppressed when *B. cereus* 28-9, also 2×10^8 CFU/ml, was applied two or three days before or one day after inoculation with *B. elliptica* B061 (Fig. 1). Dual culture of *B. cereus* 28-9 and *B. elliptica* B061 showed that *B. cereus* 28-9 inhibited the growth of *B. elliptica* B061 *in vitro* by secreting antifungal compound(s) out of the bacterial cells, as indicated by the formation of inhibition zone.

ChiCW-encoding gene The chitinase-encoding gene was cloned by functional expression of *Bacillus* chitinase in *E. coli*. Four clones that exhibited chitinolytic activities were selected. A phagemid with a 4.5-kb insert was rescued from the recombinant phage by *in vivo* excision. Nucleotide sequencing analysis revealed an open reading frame (ORF) consisting of 2,022 nucleotides with ATG as a start codon and TAG as a stop codon. This ORF encoded a protein (named as ChiCW precursor) of 674 amino acid residues with a calculated molecular weight of 74.261 and a putative pI of 5.77. The putative Shine-Dalgarno sequence, AGGAGA, was located seven nucleotides upstream of the start codon. The −35 region (TTTACA) and −10 region (TTTAAT) of a putative promoter sequence were found upstream of *chiCW* by computer analysis. Southern blot analysis showed single band signal in *Eco*RI-, *Eco*RV- and *Hin*dIII-digested genomic DNA of *B. cereus* 28-9, indicating that the genome of *B. cereus* 28-9 contained a single copy of *chiCW* (Fig. 2).

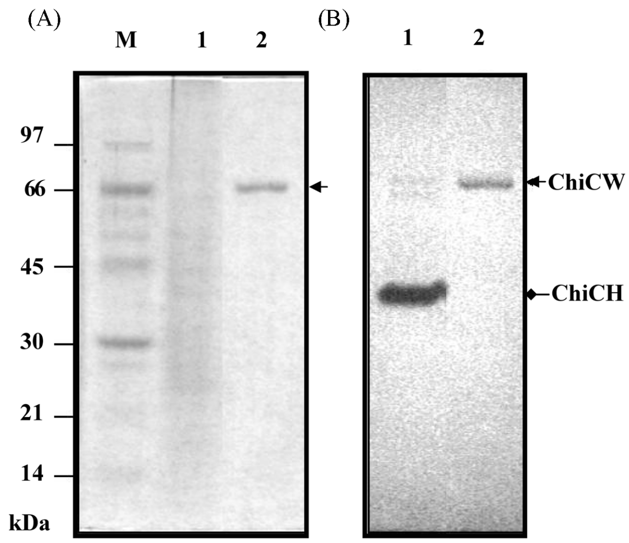

Fig. 3. SDS-PAGE and in-gel activity assay. Proteins from the culture supernatant of *B. cereus* 28-9 (lane 1) and ChiCW purified from the periplasmic fraction of *E. coli* DH5α(pNTU55) (lane 2) were analyzed by SDS-PAGE in a 10% polyacrylamide gel containing 0.01% glycol chitin. One gel was stained with Coomassie Brilliant Blue (A) and another gel was stained with Calcofluor White M2R (B). ChiCW is indicated by the arrow (ca. 70 kDa). M, low molecular weight protein standards (Amersham Biosciences).

Table 1. Purification of ChiCW from periplasmic fraction of *E. coli* DH5α(pNTU55)

Purification step	Total protein (mg)	Total activity (U[a])	Specific activity (U/mg)	Yield (%)	Purification fold
Periplasmic fraction	3.876	0.859	0.222	100.0	1.0
Ammonium sulfate precipitation	0.706	0.212	0.300	24.7	1.4
Anion exchange chromatography	0.158	0.144	0.911	16.8	4.1

[a]1 U of activity = 1 μmol of 4-MU released per min.

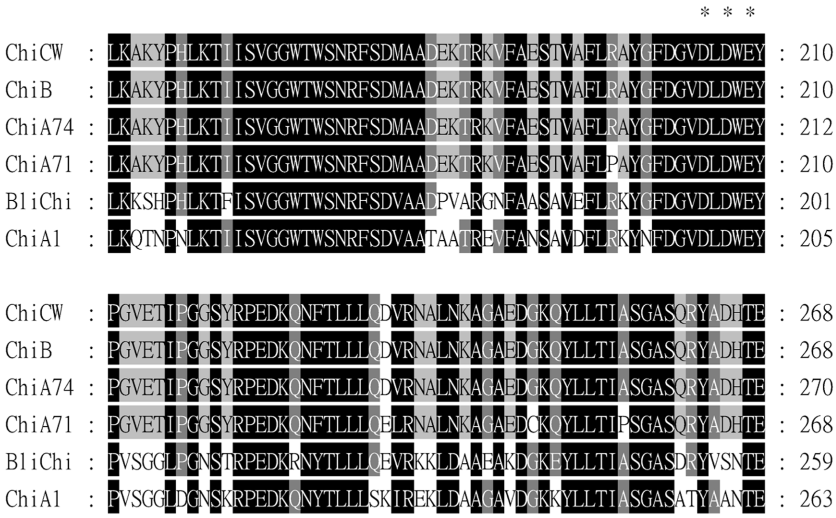

Fig. 4. Alignment of the peptide sequences of the catalytic domains of chitinases from different *Bacillus* spp. ChiCW (this study, accession number AF416570); ChiB, chitinase B of *B. cereus* CH (AB041932); ChiA74, chitinase A74 of *B. thuringiensis* serovar kenyae (AF424979); ChiA71, chitinase A71 of *B. thuringiensis* subsp. *pakistani* (AAB58579); BliChi, chitinase of *B. licheniformis* TP (U71214); ChiA1, chitinase A1 of *B. circulans* WL12 (M57601). The asterisks indicate the essential amino acid residues in the catalytic domains of chitinases.

Expression and purification of ChiCW from recombinant *E. coli* Chitinases secreted by *B. cereus* 28-9 appeared as three chitinolytic bands on the polyacrylamide gel as shown by in-gel activity assay. Two chitinolytic bands corresponded to ChiCH of 37 kDa (Huang and Chen, 2004) and ChiCW of 70 kDa, respectively. A third chitinolytic band appeared at the position of 64 kDa. The recombinant plasmid pNTU55 harboring *chiCW* was constructed to express ChiCW in *E. coli*. ChiCW protein was purified from the periplasmic fraction of *E. coli* DH5α(pNTU55) to near homogeneity as shown by SDS-PAGE analysis and in-gel activity assay. The molecular mass of ChiCW purified from *E. coli* was estimated to be 70 kDa, close to that secreted by *B. cereus* 28-9 (Fig. 3). The degree of purification and yield at individual steps are given in Table 1.

Analysis and comparison of the amino acid sequence of ChiCW with other chitinases According to the conserved domain analysis using programs in NCBI, ChiCW precursor contained a signal peptide at N terminus, consisting of 31 amino acid residues characterized by the hydrophobic, positively charged region and a cleavage site for a specific signal peptidase between Leu-31 and Ala-32 was found (Perlman and Halvorson, 1983). The N-terminal signal peptide was followed by a catalytic domain of chitinase. Based on the homology of amino acid sequences, ChiCW was categorized in family 18 glycosyl hydrolase (Henrissat and Bairoch, 1993) and the sequence of the active site, FLRAYGFDGVDLDWEYPG from Phe-195 to Gly-212, was found (Fig. 4). Three essential conserved amino acid residues (Asp-205, Asp-207 and Glu-209) were found within the active site. These residues have also been found in ChiA1 of *Bacillus circulans* WL-12 (Watanabe *et al*., 1993) and ChiA of *Serratia marcescens* (Perrakis *et al*., 1994). Furthermore, Glu-209 in ChiCW corresponded to Glu-315 of *S. marcescens* ChiA, which has been reported to be involved in chitinase catalysis (Perrakis *et al*., 1994). In addition, these three amino acid residues are fully conserved in chitinases from six *Bacillus* spp. (Fig. 4).

Fibronectin type-III like (FnIII) domain is in the middle region of ChiCW and has been reported to be present in several *Bacillus* chitinases. The amino acid sequence of the FnIII domain of ChiCW revealed a high homology to that of ChiB of *B. cereus* CH, ChiA71 of *B. thuringiensis* subsp. *pakistani* and ChiA74 of *B. thuringiensis* serovar kenyae (Barboza-Corona *et al*., 2003; Mabuchi and Araki, 2001; Thamthiankul *et al*., 2001), but a lower homology to that of *B. licheniformis* TP and ChiA1 of *B. circulans* WL-12

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ChiCWFn3  : ~~~~~~~~~~~~~~~~~~~~~~~~QLNWTASTDNVGVMEYEITAGEEK-WSTTTNSITIKNL : 538
ChiA74Fn3 : ~~~~~~~~~~~~~~~~~~~~~~~~QLNWTASTDNVGVTEYEITAGEEK-WSTTTNSITIKNL : 540
ChiBFn3   : ~~~~~~~~~~~~~~~~~~~~~~~~QLNWTVSTDNVGVTEYEITAGEEK-WSTTTNSITIKNL : 538
ChiA71Fn3 : SQKDTEPPTNVKNIVVTNKNSNSVQLNWTVSTDNVGVTEYEITAGEEK-WSTTTNSITIKNL : 409
BlichiFn3 : ~~~~~~~~SAPVNVRVTGKTATSVSLAWDAASSGTNITEY-VVSFESRSISVKETSAEIGNL : 512
ChiA1Fn3  : ~~~~~~~~SVPGNARSTGVTANSVTLAWNASTDNVGVTGYNVYNGANLATSVTGTTATISGL : 519

ChiCWFn3  : KPNTEYTFSIIAKDAAGN~~~~~~~~~~~~~~~ : 556
ChiA74Fn3 : KPNTEYTFSIIAKDAAGNKSQPTALTVKT~~~~ : 558
ChiBFn3   : KPNTEYKFSVIAKDAAGN~~~~~~~~~~~~~~~ : 556
ChiA71Fn3 : KPNTEYKFSVIAKDAAGIITPTALTVKRSSNAT : 442
BlichiFn3 : NRGTAYSFTVSAKDADG~~~~~~~~~~~~~~~~ : 529
ChiA1Fn3  : TAGTSYTFTIKAKDAAGN~~~~~~~~~~~~~~~ : 537
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Fig. 5. Alignment of the peptide sequences of the Fibronectin type-III like domains of chitinases from different *Bacillus* spp.

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ChiCWCBD   : ~TATFSVTSNWGSGYNFSIIIKNNGTTPIKNWKLEFDYSGNLTQVWDSKISSKTNNHYVITN : 640
ChiBCBD    : ~TATFSVTSNWGSGYNFSIIIKNNGTTPIKNWKLEFDYSGNLTQVWDSKISSKTNNHYVITN : 640
ChiA74ChBD : GTATFSVTSNWGSGYNFSIIIKNNGTTPIKNWKLEFDYSGNLTQVWDSKISSKTNNHYVITN : 642
ChiA71CBD  : ~~~~~~~TSNWGSGYNFSIIIKNNGTTPIKKWKLEFDYSGNLTQVWDS~~~~~~~~~~~~~~ : 444
BlichiCBD  : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~YDEWKETNAYTGGERVAFNGKVYEA--------- : 573
ChiA1ChBD  : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AWQVNTAYTAGQLVTYNGKTYKCLQPHTSL-- : 634

ChiCWCBD   : AGWN~~~~~~~~~~~ : 644
ChiBCBD    : AGWN~~~~~~~~~~~ : 644
ChiA74ChBD : AGWNGEIP~~~~~~~ : 646
ChiA71CBD  : ~~~~~~~~~~~~~~~ :
BlichiCBD  : KWWTKGD~~~~~~~~ : 580
ChiA1ChBD  : AGWEPSNVPALWQLQ : 699
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Fig. 6. Alignment of the peptide sequences of the chitin- and cellulose-binding domains of chitinases from different *Bacillus* spp. CBD and ChBD indicate the cellulose-binding domain and chitin-binding domain, respectively.

(Tantimavanich *et al.*, 1998; Watanabe *et al.*, 1990b). However, the conserved amino acid residues could be found in the FnIII domains of ChiCW and chitinases from five other *Bacillus* spp. (Fig. 5).

The C-terminal region of ChiCW was analyzed and specified to be a cellulose-binding domain (CBD). Alignment of the substrate binding domains of these *Bacillus* chitinases indicated that the residues Trp-610 and Tyr-616 of ChiCW were highly conserved among the domains involved in the binding of chitin and/or cellulose substrates (Barboza-Corona *et al*., 2003; Mabuchi and Araki, 2001; Tantimavanich *et al.*, 1998; Thamthiankul *et al*., 2001; Watanabe *et al*., 1994) (Fig. 6).

Inhibition by ChiCW on conidial germination of *B. elliptica* The effect of ChiCW to inhibit conidial germination of *B. elliptica* B061 was demonstrated by an *in vitro* assay using the ChiCW protein purified from *E. coli* harboring *chiCW.* An 84% inhibition on conidial germination of *B. elliptica* B061 was exhibited by the purified ChiCW protein at a dose of 28 μU. The inhibition rate could still be maintained at 20% at a dose of 3.5 μU (Table 2). The conidia of *B. elliptica* B061 incubated with ChiCW frequently became enlarged and the elongation of germination tubes was generally retarded, as observed under a light microscope (Fig. 7).

Discussion

Lily leaf blight caused by the fungal pathogen, *B. elliptica*, is the most severe and destructive disease of the field-grown lilies. It is difficult to control *B. elliptica* by fungicides because this fungus develops resistance to fungicides frequently (Chastagner and Riley, 1990; Migheli *et al*., 1990).

Table 2. Effect of ChiCW on conidial germination of *B. elliptica*[a]

ChiCW activity (μU)	Inhibition rate of conidial germination (%)
28.0	84 ± 1
14.0	78 ± 3
7.0	29 ± 8
3.5	20 ± 2
0.0[b]	0

[a]Each treatment included 4 μl of purified ChiCW from *E. coli* DH5α(pNTU55) and 4 μl of conidial suspension of *B. elliptica* B061 (10^5 conidia/ml). The mixture was placed on slides in a moist petri dish for 12 h before microscopic examination.
[b]Potassium phosphate buffer (100 mM, pH 6.0) was used as a negative control.

(A) (B)

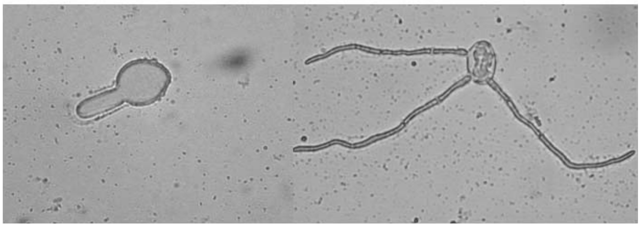

Fig. 7. Effect of ChiCW on conidial germination of *B. elliptica*. Conidia were treated with purified ChiCW from *E. coli* DH5α(pNTU55) (A) or 100 mM potassium phosphate buffer, pH 6.0 (B). The conidium treated with ChiCW became enlarged and did not fully germinate.

Therefore, selection of antagonistic microorganisms to perform biological control is considered an alternative practice. In the study of Chiou and Wu (2001), several antagonistic bacteria were selected and examined for their biocontrol activities. In this study, chitinolytic *B. cereus* 28-9 was selected and its potential biocontrol activity was demonstrated by a detached leaf assay and dual culture assay. According to the studies of Chiou and Wu (2001) and Gould *et al.* (1996), using detached plant tissues for bioassay is a simple, effective and time-saving method. The behavior of antagonists on the detached leaves is positively correlated to that on the whole plants (Chiou and Wu, 2001). Since *B. cereus* 28-9 could inhibit the infection by *B. elliptica* B061 on detached lily leaves and exhibit antifungal activity as demonstrated by dual culture assay, we proposed that *B. cereus* 28-9 could be a biocontrol agent to protect field-grown lilies from *B. elliptica* infection.

In addition, ChiCW purified from the periplasmic fraction of *E. coli* DH5α(pNTU55) effectively inhibited conidial germination of *B. elliptica* B061; thus ChiCW contributing to the antagonistic activity of *B. cereus* 28-9 against *B. elliptica* was presumed. Other factors such as induced plant resistance, antifungal metabolites or a competitive colonization on the leaf surface might also be involved in the antagonism of *B. cereus* 28-9 against *B. elliptica*.

In the zymogram analysis, three chitinases appeared to be secreted by *B. cereus* 28-9. Besides ChiCW (70 kDa) and ChiCH (37 kDa), a third chitinolytic band was observed at position of 64 kDa and was presumed to be a proteolytic modification product of ChiCW. Similar phenomenon of proteolytic modification of chitinases has also been reported in other bacteria (Mabuchi *et al*., 2000; Tantimavanich *et al*., 1998; Thamthiankul *et al*., 2001; Watanabe *et al*., 1990a).

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References

Barboza-Corona, J. E., Nieto-Mazzocco, E., Velázquez-Robledo, R., Salcedo-Hernandez, R., Bautista, M., Jiménez, B. and Ibarra, J. E. (2003) Cloning, sequencing, and expression of the chitinase gene *chiA74* from *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **69**, 1023-1029.

Bartnicki-Garcia, S. (1969) Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annu. Rev. Microbiol.* **22**, 87-108.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Brunel, B., Périssol, C., Fernandez, M., Boeufgras, J. M. and Le Petit, J. (1994) Occurrence of *Bacillus* species on evergreen oak leaves. *FEMS Microbiol. Ecol.* **14**, 331-342.

Chastagner, G. A. and Riley, K. (1990) Occurrence and control of benzimidazole and dicarboximide resistant *Botrytis* spp. on bulb crops in Western Washington and Oregon. *Acta Hort.* **266**, 437-445.

Chernin, L., Ismailov, Z., Haran, S. and Chet, I. (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl. Environ. Microbiol.* **61**, 1720-1726.

Chiou, A. L. and Wu, W. S. (2001) Isolation, identification and evaluation of bacterial antagonists against *Botrytis elliptica* on lily. *J. Phytopathol.* **149**, 319-324.

Cohen-Kupiec, R. and Chet, I. (1998) The molecular biology of chitin digestion. *Curr. Opin. Biotechnol.* **9,** 270-277.

Doss, R. P., Chastagner, G. A. and Riley, K. L. (1984) Techniques for inoculum production and inoculation of lily leaves with *Botrytis elliptica*. *Plant Dis.* **68**, 854-856.

Elad, Y. (1996) Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. *Eur. J. Plant Pathol.* **102**, 719-732.

Emmert, E. A. B. and Handelsman, J. (1999) Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiol. Lett.* **171**, 1-9.

Felse, P. A. and Panda, T. (1999) Regulation and cloning of microbial chitinase genes. *Appl. Microbiol. Biotechnol.* **51**, 141-151.

Flach, J., Pilet, P. E. and Jollès, P. (1992) What's new in chitinase research? *Experientia* **48**, 701-716.

Freeman, S., Minzm O., Kolesnik, I., Barbul, O., Zveibil, A., Maymon, M., Nitzani, Y., Kirshner, B., Rav-David, D., Bilu, A., Dag, A., Shafir, S. and Elad, Y. (2004) *Trichoderma* biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. *Eur. J. Plant Pathol.* **110**, 361-370.

Gooday, G.. W. (1990) The ecology of chitin degradation; in *Advances in Microbial Ecology,* Marshall, K. C. (ed.), pp. 387-430, Plenum Press, New York, USA.

Gould, A. B., Kobayashi, D. Y. and Bergen, M. S. (1996) Identification of bacteria for biological control of *Botrytis cinerea* on petunia using a petal disk assay. *Plant Dis.* **80**, 1029-1033.

Graham, L. S. and Sticklen, M. B. (1994) Plant chitinases. *Can. J. Bot.* **72**, 1057-1083.

Henrissat, B. and Bairoch A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **293**, 781-788.

Hsieh, T. F. and Huang, J. W. (1998) Factors affecting disease development of Botrytis leaf blight of lily caused by *Botrytis elliptica*. *Plant Pathol. Bull*. **40**, 227-240.

Huang, C. J. and Chen, C. Y. (2004) Gene cloning and biochemical characterization of chitinase CH from *Bacillus* *cereus* 28-9. *Ann. Microbiol.* **54**, 289-297.

Keim, P., Kalif, A., Schupp, J., Hill, K., Travis, S. E., Richmond, K., Adair, D. M., Hugh-Jones, M., Kuske, C. R. and Jackson, P. (1997) Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J. Bacteriol.* **179**, 818-824.

Kobayashi, D. Y., Reedy, R. M., Bick, J. A. and Oudemans, P. V. (2002) Characterization of a chitinase gene from *Stenotrophomonas maltophilia* strain 34S1 and its involvement in biological control. *Appl. Environ. Microbiol.* **68**, 1047-1054.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Mabuchi, N. and Araki, Y. (2001) Cloning and sequencing of two genes encoding chitinases A and B from *Bacillus cereus* CH. *Can. J. Microbiol.* **47**, 895-902.

Mabuchi, N., Hashizume, I. and Araki, Y. (2000) Characterization of chitinases excreted by *Bacillus cereus* CH. *Can. J. Microbiol.* **46**, 370-375.

Manoil, C. and Beckwith, J. (1986) A genetic approach to analyzing membrane protein topology. *Science* **233**, 1403-1408.

Martinez, C., Michaud, M., Belanger, R. R. and Tweddell, R. J. (2002) Identification of soils suppressive against *Helminthosporium solani*, the causal agent of potato silver scurf. *Soil Biol. Biochem.* **34**, 1861-1868.

Migheli, Q., Aloi, C. and Gullino, M. L. (1990) Resistance of *Botrytis elliptica* to fungicides. *Acta Hort.* **266**, 429-436.

Perlman, D. and Halvorson, H. O. (1983) A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J. Mol. Biol.* **167**, 391-409.

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S. and Vorgias, C. E. (1994) Crystal structure of a bacterial chitinase at 2.3Å resolution. *Structure* **2**, 1169-1180.

Pleban, S., Chernin, L. and Chet, I. (1997) Chitinolytic activity of an endophytic strain of *Bacillus cereus*. *Lett. Appl. Microbiol.* **25**, 284-288.

Sietsma, J. H. and Wessels, J. G. H. (1979) Evidence for covalent linkages between chitin and β-glucan in a fungal wall. *J. Gen. Microbiol.* **114**, 99-108.

Silo-Suh, L. A., Lethbridge, B. J., Raffel, S. J., He, H., Clardy, J. and Handelsman, J. (1994) Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Appl. Environ. Microbiol.* **60**, 2023-2030.

Tantimavanich, S., Pantuwatana, S., Bhumiratana, A. and Panbangred, W. (1998) Multiple chitinase enzymes from a single gene of *Bacillus licheniformis* TP-1. *J. Ferment. Bioeng.* **85**, 259-265.

Thamthiankul, S., Suan-Ngay, S., Tantimavanich, S. and Panbangred, W. (2001) Chitinase from *Bacillus thuringiensis* subsp. *pakistani*. *Appl. Microbiol. Biotechnol.* **56**, 395-401.

Trudel, J. and Asselin, A. (1989) Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem.* **178**, 362-366.

Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S. and Tanaka, H. (1994) The roles of the C-terminal domain and type III domains of chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. *J. Bacteriol.* **176**, 4465-4472.

Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M. and Tanaka, H. (1993) Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* **268**, 18567-18572.

Watanabe, T., Oyanagi, W., Suzuki, K. and Tanaka, H. (1990a) Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *J. Bacteriol.* **172**, 4017-4022.

Watanabe, T., Suzuki, K., Oyanagi, W., Ohnishi, K. and Tanaka, H. (1990b) Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectin. *J. Biol. Chem.* **265**, 15659-15665.