J. Gen. Appl. Microbiol., **50**, 177–182 (2004)

Short Communication

PCR cloning and heterologous expression of chitinase gene of endophytic *Streptomyces aureofaciens* CMUAc130

Thongchai Taechowisan,[1] John F. Peberdy,[2] and Saisamorn Lumyong[1,*]

[1] *Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand*
[2] *School of Life and Environmental Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK*

(Received May 12, 2003; Accepted April 15, 2004)

Key Words——chitinase gene; endophytic *Streptomyces*; expression; PCR cloning

Chitin, a β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) polymer, is one of the most abundant and important sources of nutrients and energy in nature. Actinomycetes are well-known decomposers of chitin in soil (Gooday, 1990). Chitinase (EC 3.2.1.14) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) are essential components catalyzing the conversion of insoluble chitin to its monomeric component. These enzymes are found in a wide variety of organisms including bacteria, fungi, insects, plants, and animals. We have investigated the chitinolytic enzyme of the endophyte *Streptomyces aureofaciens* CMUAc130, which can be used for fungal cell wall degradation. The chitinases obtained from *Streptomyces* have been reported for antifungal activity (Tsujibo et al., 2000). In this paper, we describe the PCR cloning and sequencing of *Chi40* from *S. aureofaciens* CMUAc130 and the characterization of its translated products. We also show the activity of *Chi40* in the hydrolysis of insoluble chitin and its antagonism against phytopathogenic fungi.

Chromosomal DNA, of endophytic isolate *S. aureofaciens* CMUAc130, was extracted according to the method of Hopwood et al. (1985). Two oligonucleotide primers, 5′-TTGACCCAGTGGTCCAGACC-3′ (forward primer) and 5′-GTGTGCTGCTCACGCCAG-3′ (reverse primer), designed from the conserved regions among the family 19 chitinase genes of *Streptomyces* sp. were used. A standard PCR was performed in a total volume of 100 µl, containing 100 mM (each) deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 50 pmol of each PCR primer, 1.5 U of *Pfu* DNA polymerase (Promega Co., Madison, WI, USA) and 50 ng of genomic DNA from *S. aureofaciens* CMUAc130. A PCR utilized melting 94°C 5 min, then 35 cycles of 94°C 30 s, 55°C for 1 min, and 72°C extension 1 min, with extension at 72°C for 10 min after the last cycle. The purified PCR products were ligated into the *Sma*I site of pUC18 producing the clone, pChi40_Sau. The nucleotide sequence presented in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB106648.

The complete 1.4-kb sequence of the insert was determined, and the deduced amino acid sequence of Chi40_Sau yielded an open reading frame of 1,242 nucleotides, coding for a protein of 413 amino acids with an estimated molecular mass of 40 kDa (Fig. 1). A database search revealed that the deduced Chi40_Sau protein amino acids sequence was 87, 87, 31, 29, and 28% identical to those of Chit_Strpl from

* Address reprint requests to: Dr. Saisamorn Lumyong, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.
Tel: +66–53–943346 ext 1503 Fax: +66–53–892259
E-mail: scboi009@chiangmai.ac.th

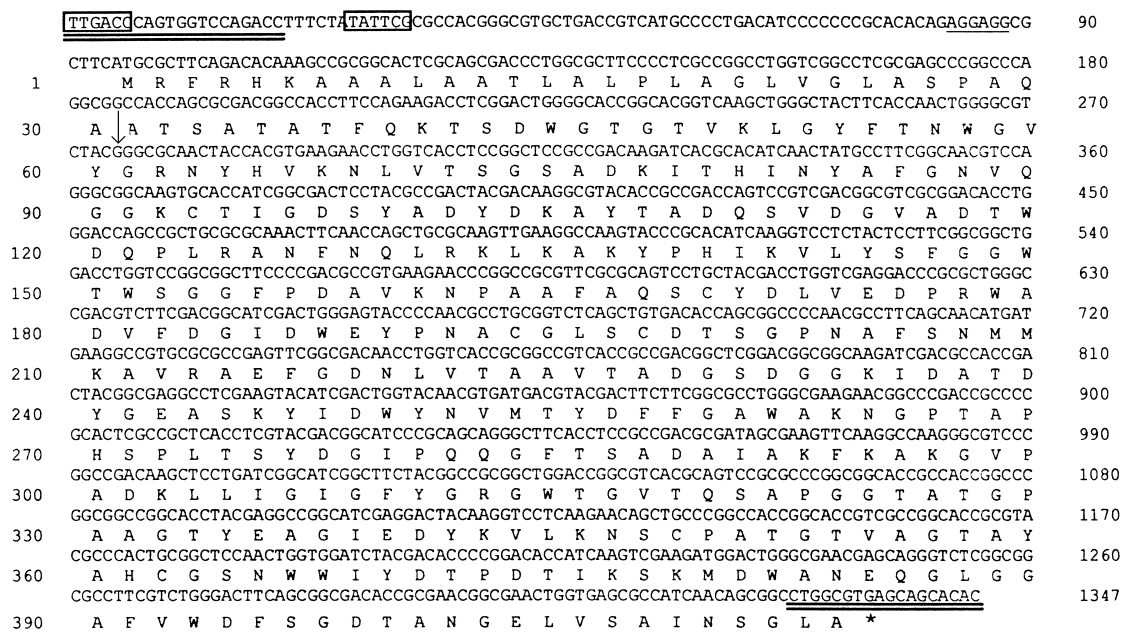

Fig. 1. Nucleotide sequence of the 1.35-kb DNA fragment and the deduced amino acid sequence of Chi40_Sau. The putative ribosome-binding site (AGGAGG) is underlined. The −10 and −35 regions of a possible promoter sequence are boxed. The signal peptide cleavage site is shown with an arrow (↓). The stop codon is indicated by an asterisk (*). The doubly underlined nucleotide sequences denote the conserved regions of *Streptomyces* sp. family 19 chitinase used to design the PCR primers.

S. plicatus ATCC27800 (Robbins et al., 1992), Chia_Strth from *S. thermoviolaceus* OPC-520 (Tsujibo et al., 1993a), Chi1_Bacci from *Bacillus circulans* WL-12 (Watanabe et al., 1993), Chia_Altso from *Alteromonas* sp. strain O-7 (Tsujibo et al., 1993b) and Chia_Serma from *Serratia marcescens* (Jones et al., 1986) (Fig. 2), respectively. These comparisons suggest that the levels of diversity between various chitinases correlate with the evolutionary distances between the bacteria that produce them. The precursor protein of Chi40_Sau had a leader peptide identical to that of Chit_Strli (Miyashita and Fujii, 1993) and Chit_Strpl (Robbins et al., 1992), suggesting that it is also cleaved at the same site. Thus, the mature protein probably starts at Ala31. The type-III homology unit of fibronectin found in *Chi63* and *ChiA* does not exist in the amino acid sequence of Chi40_Sau. The fibronectin-like sequence is also not found in *Chi40* from *S. thermoviolaceus* OPC-520 (Tsujibo et al., 1993a) nor the chitinase gene from *S. erythraeus* (Kamei et al., 1989). We also did not find this fibronectin-like sequence in this gene. These results indicated that the fibronectin type-III sequence was not essential for chitinase activity. The amino acid sequence homology could be found between Chi40_Sau and *Chi40* from *S. thermoviolaceus* OPC-520 except for a short region which may be involved in the heat stability. The *S. aureofaciens* chitinase gene characterized in this paper has high DNA sequence homology (95%) with *Chi40* encoding the chitinase of *S. thermoviolaceus* OPC-520. Identity between the two chitinases at the amino acid level was 87%. Although the amino acid sequence of Chi40_Sau is very similar to that of *Chi40* from *S. thermoviolaceus* OPC-520, the heat stability of the two enzymes is clearly different. It was reported that *Chi40* from *S. thermoviolaceus* OPC-520 is stable when heated in 50 mM Tris-HCl (pH 8.0) at 80°C for 30 min (Tsujibo et al., 1993a). In contrast, Chi40_Sau showed 25% remaining activity after being heated in the same solution at 55°C for 30 min, but was absolutely inactivated when heated at 65°C for 1 min (data not shown). These results indicate a short region of amino acid sequence which differs from *Chi40* of *S. thermoviolaceus* OPC-520 and may be involved in heat stability. One of the regions (amino acid 156–158 of Chi40_Sau) contains an Asp and Glu present in the active site of lysozyme (Metraux et al., 1989). These two portions of Chi40_Sau may therefore constitute the catalytic site of *Chi*, as described in several papers (Kuranda and Robbins, 1991; Tsujibo

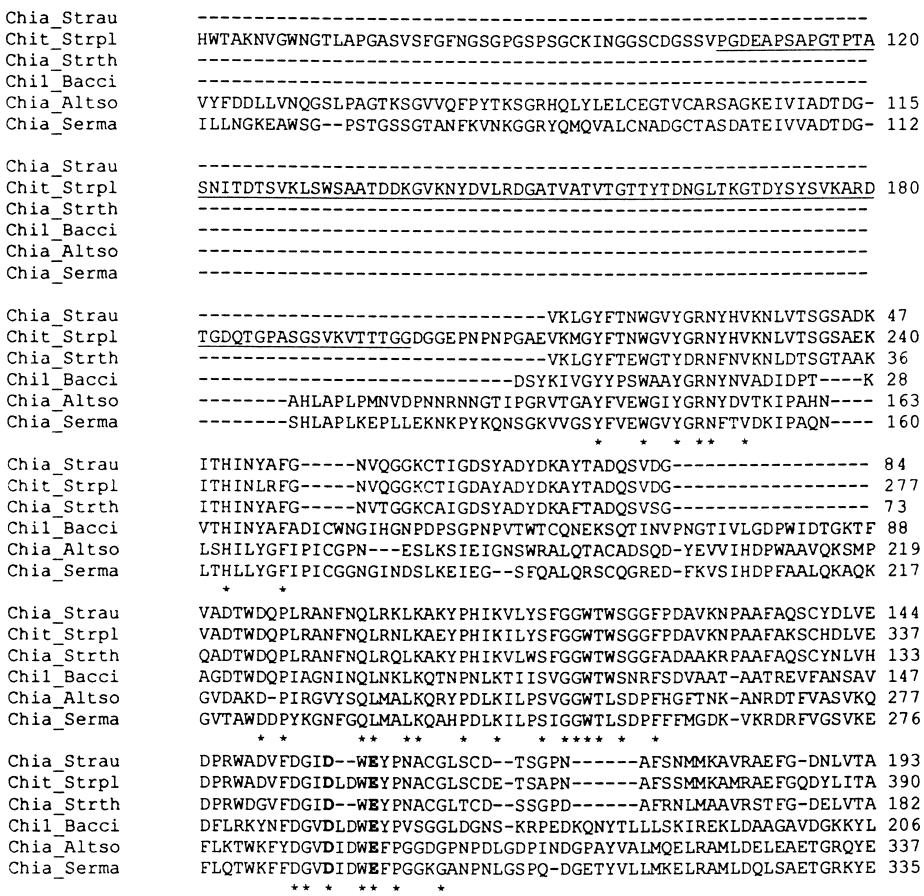

Fig. 2. Alignment of deduced amino acid sequences of some bacterial chitinases.

The amino acid sequences of the chitinases from *S. aureofaciens* (Chia_Strau, deduced from translation of the DNA sequence obtained in this study), *S. plicatus* (Chit_Strpl, from the Swiss-Prot database, accession no. P11220), *S. thermoviolaceus* (Chia_Strth, from the GenBank DNA sequence, accession no. JC2135), *B. circulans* (Chi1_Bacci, from the Swiss-Prot database, accession no. P20533), an *Alteromonas* sp. (Chia_Altso, from the Swiss-Prot database, accession no. P32823) and *S. marcescens* (Chia_Serma, from the Swiss-Prot database, accession no. P07254) were aligned by using the Clustal W 1.82 program (European Bioinformatics Institute, Cambridge, UK). Numbering starts from the first residue of the mature protein. Asterisks indicate identical residues in all proteins shown. The type-III homology unit of fibronectin is underlined. The amino acid Asp156 and Glu158 that are identical in the active site of lysozyme are in boldface.

et al., 1993a; Watanabe et al., 1992).

Most of the cloned chitinase enzymes have been found to accumulate in the periplasmic space of the host cells (Robbins et al., 1988; Tsujibo et al., 1993b). In contrast *S. aureofaciens* CMUAc130 chitinase is mostly detected in the culture medium when it was cloned in *E. coli*. This experiment indicated that the signal peptide could be functional in *E. coli*, which has been noted in previous reports by Tsujibo et al. (2000) and Li et al. (2000). The chitinolytic activity found in the culture medium of the transformant JM109/pChi40_Sau was active in the hydrolysis of the colloidal chitin with 1 mM IPTG but very low in the absence of IPTG (Table 1). These data indicate that the chitinase was regulated by the *lac*Z promoter.

To demonstrate directly that the transformed *E. coli* strain produced and secreted the Chi40_Sau chitinase, the immunological detection of secreted proteins of JM109/pChi40_Sau renaturated following separation by SDS-PAGE was examined and compared with that of the original *S. aureofaciens* CMUAc130. The results obtained by Western-blot analysis showed that the specific protein, with an apparent molecular mass of 40 kDa, which is present in the secreted proteins of strain CMUAc130 (Fig. 3, lane 1), corresponding to the 40 kDa chitinase, was detected in the secreted pro-

Table 1. Chitinase activity of *E. coli* JM109 carrying pChi40_Sau in LB after overnight growth at 37°C and then adding 1 mM IPTG 3 h before harvesting.

Source of culture medium	Chitinase activity of heterologous expression (U ml^{-1})[a]	
	1 mM IPTG	No IPTG
E. coli JM109/pChi40_Sau		
Secreted proteins[b]	0.274±0.058	0.079±0.023
Intracellular proteins[c]	0.106±0.072	0.014±0.021
E. coli JM109/pUC18[b]	0.000	0.000

[a] The cultured broth and the sonicated cells were subjected to quantitate chitinase activity. This was quantitated by the method described by Reissig et al. (1955). Chitinase activity was calculated and expressed as unit per ml of the original volume of cell cultures.

[b] The secreted proteins were the total soluble proteins from the cultured broth.

[c] *E. coli* JM109 carrying pChi40_Sau was grown in 100 ml LB broth containing ampicillin (100 μg ml^{-1}) at 37°C overnight. Cell culture was harvested by centrifugation at 8,220×*g* for 10 min at 4°C. The pellets were resuspended in 5 ml of sterile distilled water and sonicated.

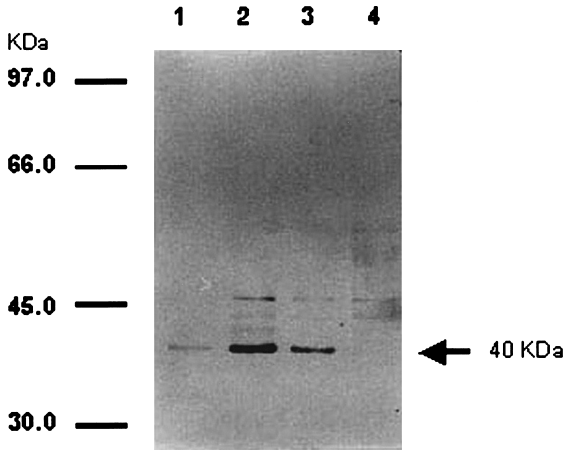

Fig. 3. Western-blot analysis showing the expression of the Chia_Strau gene.

Fifty micrograms of total soluble protein was loaded per lane. Lane: 1, crude chitinase from *S. aureofaciens* CMUAc130; 2, secreted protein from *E. coli* JM109/pChi40_Sau with 1 mM IPTG induction; 3, secreted protein from *E. coli* JM109/pChi40_Sau without IPTG induction; 4, secreted protein from *E. coli* JM109.

teins of JM109/pChi40_Sau with IPTG induction (Fig. 3, lane 2). No bands of immunological reactivity from the secreted proteins of *E. coli* JM109 were found (Fig. 3, lane 4).

Antifungal activity was estimated using the hyphal extension-inhibition assay of Roberts and Selitrennikoff (1986). *Fusarium oxysporum*, the causative agent of wilt of wheat was used as the test fungus. The chitinolytic *E. coli* strain carrying the plasmid pChi40_Sau was found to suppress the growth. The original strain CMUAc130 showed stronger suppression of the fungi, whereas almost no suppression was observed with the nontransformed *E. coli* strain.

The effects of crude preparations of the chitinases secreted by the original *S. aureofaciens* CMUAc130 and *E. coli* JM109/pChi40_Sau on spore germination of *F. oxysporum* were studied according to the procedure described by Lorito et al. (1993), using *F. oxysporum* as the test fungus and the crude secreted enzyme preparation from *S. aureofaciens* CMUAc130, *E. coli* JM109/pChi40_Sau, or *E. coli* JM109 as the test solutions. A stock conidial suspension, 10^5 spores ml^{-1} in 30% glycerol, was prepared and kept at −20°C. Equal volumes (20 ml) of spore suspension, 3× potato dextrose broth, and the test solution (about 5 U of chitinase activity) were mixed in a sterile flask. The tested solutions were replaced with sterile water in control samples. Flasks were incubated at 30°C for 24 h. A drop of the mixture from each flask was placed on a microscope slide, and the percentage of conidial germination was determined from the first 100 spores chosen at random.

The rate of spore germination in the control was 80.6%±8.0%. The *F. oxysporum* spore germination assay showed that in the presence of crude preparations of secreted proteins produced by strain CMUAc130 and JM109/pChi40_Sau, the rate of spore germination significantly decreased relative to the control (39.0%±7.5% and 56.6%±5.5%, respectively). When the amount of secreted proteins of the parent strain JM109 equivalent to that in crude preparations of chitinases secreted by strain CMUAc130 and JM109/pChi40_Sau was added to the experimental mixture as a test solution, almost no difference from the control was found (79.0%±4.5%). Morphological changes were observed in microslide-culture of *F. oxysporum* treated with crude chitinase of *E. coli* JM109/pChi40_Sau (5 U ml^{-1}) at 37°C for 12 h in a moist chamber compared with a 5 min-boiled chitinase control under the light microscope (400×). This treatment caused fungal cell wall lysis, as well as inhibition of spore germination and, possibly, conidial damage.

This may be due to enzymatic digestion of the cell wall region rich in chitin. Hyphal width reduction was also observed. Different morphological effects on the fungal cell wall have been reported in studies using *Streptomyces* sp. or other microbial chitinases. These effects were mainly spore germination inhibition, bursting of spore and hyphal tips and germ tube elongation (Gomes et al., 2001). The differences in the effects can be attributed to the different experimental conditions of the tests.

E. coli JM109/pChi40_Sau, expressing the *S. aureofaciens Chi40* gene, acquired the ability to suppress growth of *F. oxysporum* and their spore germination in vitro. The transformant effected less significant fungal suppression in vitro than did the parent strain CMUAc130. This difference can be explained not only by the lower level of chitinolytic activity secreted by the transformant (probably because the *chiA* gene was cloned under the control of the relatively weak *lac* promoter) but also by the fact that the parent strain has been found to produce other chitinolytic enzymes and other antifungal substances.

The results presented in this work confirm the role of chitinases in the antifungal activity of various microbial antagonists (Lorito et al., 1993; Ordentlich et al., 1988; Shapira et al., 1989; Tsujibo et al., 2000). The *S. aureofaciens* CMUAc130 chitinase gene could therefore be used to transform other bacteria or plants and provide them with the ability to control fungal phytopathogens. The application of *S. aureofaciens* CMUAc130 and *E. coli* JM109/pChi40_Sau to soil or plants infested with *F. oxysporum* will be reported elsewhere.

Acknowledgments

This work was supported by the TRF/BIOTEC Special Program for Biodiversity Research and Training grant T_646003 and the graduate school of Chiang Mai University, Chiang Mai, Thailand.

References

Gomes, R. C., Semedo, L. T. A. S., Soares, R. M. A., Linhares, L. F., Ulhoa, C. J., Alviano, C. S., and Coelho, R. R. R. (2001) Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. *J. Appl. Microbiol.*, **90**, 653–661.

Gooday, G. W. (1990) The ecology of chitin decomposition. *Adv. Microb. Ecol.*, **11**, 387–430.

Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., and Schrempf, H. (1985) Genetic Manipulation of Streptomyces: A Laboratory Manual, John Innes Foundation, Norwich, pp. 79–80.

Jones, J. D. G., Grady, K. L., Suslow, T. V., and Bedbrook, J. R. (1986) Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. *EMBO J.*, **5**, 467–473.

Kamei, K., Yamamura, Y., Hara, S., and Ikenaka, T. (1989) Amino acid sequence of chitinase from *Streptomyces erythraeus*. *J. Biochem.*, **105**, 979–985.

Kuranda, M. J. and Robbins, P. W. (1991) Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **266**, 19758–19767.

Li, H., Plattner, H., Schimz, K. L., Kieb, M., Diekmann, H., and Meens, J. (2000) Cloning, sequencing and heterologous expression of a new chitinase gene, *chi92*, from *Streptomyces olivaceovidis* ATCC11238. *Biotechnol. Lett.*, **22**, 1203–1209.

Lorito, M., Di Pietro, A., Hayes, C. K., Woo, S. L., and Harman, G. E. (1993) Antifungal, synergistic interaction between chitinolytic enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*. *Phytopathology*, **83**, 721–728.

Metraux, J. P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., William, S., Payne, G., Carnes, M., and Ryals, J. (1989) Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. *Proc. Natl. Acad. Sci. USA*, **86**, 896–900.

Miyashita, K. and Fujii, T. (1993) Nucleotide sequence and analysis of a gene (*ChiA*) for a chitinase from *Streptomyces lividans* 66. *Biosci. Biotechnol. Biochem.*, **57**, 1691–1698.

Ordentlich, A., Elad, Y., and Chet, I. (1988) The role of chitinase of *Serratia marcescens* in biocontrol *Sclerotium rolfsii*. *Phytopathology*, **78**, 84–87.

Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955) A modified colorimetric method for the estimation of *N*-acetylamino sugars. *J. Biol. Chem.*, **217**, 959–966.

Robbins, P. W., Albright, C., and Benfield, B. (1988) Cloning and expression of a *Streptomyces plicatus* (Chitinase-63) in *Escherichia coli*. *J. Biol. Chem.*, **263**, 443–447.

Robbins, P. W., Overbye, K., Albright, C., Benfield, B., and Pero, J. (1992) Cloning and high-level expression of chitinase-encoding gene of *Streptomyces plicatus*. *Gene*, **111**, 69–76.

Roberts, W. K. and Selitrennikoff, C. P. (1986) Isolation and partial characterization of two antifungal proteins from barley. *Biochim. Biophys. Acta*, **880**, 161–170.

Shapira, R., Ordentlich, A., Chet, I., and Oppenheim, A. (1989) Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. *Phytopathology*, **79**, 1246–1249.

Tsujibo, H., Endo, H., Minoura, K., Miyamoto, K., and Inamori, Y. (1993a) Cloning and sequence analysis of the gene en-

coding a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520. *Gene*, **134**, 113–117.

Tsujibo, H., Okamoto, T., Hatano, N., Miyamoto, K., Watanabe, T., Mitsutomi, M., and Inamori, Y. (2000) Family 19 chitinases from *Streptomyces thermoviolaceus* OPC-520: Molecular cloning and characterization. *Biosci. Biotechnol. Biochem.*, **64**, 2445–2453.

Tsujibo, H., Orikoshi, H., Tanno, H., Fujimoto, K., Miyamoto, K., Imada, C., Okami, Y., and Inamori, Y. (1993b) Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Alteromonas* sp. strain O-7. *J. Bacteriol.*, **175**, 176–181.

Watanabe, T., Kobori, K., Miyashita, K., Fuji, 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., Ohnishi, K., and Tanaka, H. (1992) Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. *J. Bacteriol.*, **174**, 408–414.