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Molecular Cloning of *Acinetobacter* sp. WC-17 Gene Encoding Chitinase

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The chitinase gene was cloned from *Acinetobacter* sp. WC-17 for investigating the genetic control and enzymatic properties of bacterial chitinase. A genomic library of *Acinetobacter* sp. WC-17 was prepared in *E.coli* JM109 by using pUC18 as a vector. The chitinase-positive clone containing 3.2kb insert fragment was obtained from 5,000 insert-bearing transformants. The optimum pH and temperature of cloned enzyme were 6.0 and 55℃, respectively. Almost all the chitinase activity of *E.coli* recombinant was localized in the periplasmic fraction, while most of the enzyme activity of *Acinetobacter* sp. WC-17 was found in the extracellular fraction.

Chitin, a major structural component of many agronomically important pests including insect, fungi, and nematodes (1), is a β-1.4 polymer of N-acetylglucosamine and one of the most abundunt biopolymers in nature. All organisms that contain chitin also have chitinases, which are presumably required for morphogenesis of the cell walls and exoskeletons (5). Other organisms that do not contain chitin may produce chitinases to degrade the polymer for food, e.g. soil bacteria that secrete chitinases in respons to chitin in their environment (14). Plants also have been found to have chitinase, often after enzyme synthesis has been induced by microbial infections or other injuries (2). Since plants do not contain chitin in their cell walls, it has been postulated that they produce chitinase to protect themselves from chitin-containing parasites (7, 10). In recent years significant attention has been directed toward the substitution of agrochemicals, criticized for environmental pollution and residual toxicities, by using the antifungal activity of chitinase and the use of chitin in fields such as drug delivery, food additives, and waste treatment. Chitinase-encoding genes have been cloned from plants (9, 20), bacteria (4, 16), yeast (12), and filamentous fungi (15) for the purpose of reinforcing the plant defense system. The gene encoding chitinase of *Serratia liquefaciens* has been cloned as regulon of chitin utilization (11). Recently we have isolated the chitinase-producing bacterial strain, *Acinetobacter* sp. WC-17(18), which has never been reported to produce chitinase, and tested the antifungal activity of the isolated strain against phytopathogenic fungi. Now we are planning to apply the overproduction of chitinase of *Acinetobacter* sp. WC-17 in *E.coli* to various industrial fields and the replacement of agrochemicals. In this report we describe the cloning of the *Acinetobacter* sp. WC-17 gene encoding chitinase and their expression in *E.coli* JM109.

MATERIALS AND METHODS

Chemicals and Enzymes

Restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were purchased from Kosco Biotech (Korea) or Promega Biotech (Madison,Wis, USA). Chitin of crab shell was purchased from Sigma Chemical Co. (St. Louis., Mo, USA). Other chemicals were purchased from Sigma Chemical Co. and were of reagent grade.

Bacterial Strains and Plasmids

Acinetobacter sp. WC-17 isolated from soil was used as a chitinase gene donor. *Escherichia coli* JM109(*rel*1, *end*A1, *Gyr*A96, thi, *hsd*R17, *sup*E44 △(*lac-pro*AB) F' [traD36, *pro*A$^+$, *pro*B$^+$, lacI^q, *lac*ZM15]) was used as a host for all bacterial transformations and plasmid construction, and plasmid pUC18 was used as a cloning vector.

Media and Cultural Conditions

Exponential cultures of *E.coli* JM109 and *Acinetobacter*

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sp. WC-17 were routinely grown in Luria broth medium with ampicillin (50μg/ml) added for the plasmid-containing strain. Chitinase-positive clones were monitored over 14 days and identified by screening Amp^r plasmid-containing strains on chitin overlay plates, prepared by overlaying a 20ml agar base composed of nutrient agar (10mg/ml) plus Bacto-Agar (10mg/ml) with a 10ml layer of the same mixture plus colloidal chitin (10mg/ml) and IPTG (0.1 mM).

Preparation of Chimeric Plasmids

The chromosomal DNA of *Acinetobacter* sp. WC-17 was extracted using the method of Marmur (13). Plasmid DNA was purified using the alkaline lysis method of Ish-Horowitz and Burke (8). To construct a genomic library of *Acinetobacter* sp. WC-17, chromosomal DNA was partially digested with *PstI* and elctrophoresis for purifying 2-10 kilobases using a GENE CLEAN II Kit (Bio101, Lajolla, USA). Cloning vector pUC18 was completely digested with *PstI*, dephosphorylated with CIP, and mixed with partially digested chromosomal DNA of *Acinetobacter* sp. WC-17 and T4 DNA ligase. The reaction was incubated overnight at 12℃. The ligation mixture was used to transform *E.coli* JM109 (6). Other DNA manipulation methods used in this study, are according to description by Sambrook *et al* (17).

Preparation of Chitinase Enzyme

To assay chitinase activity, *Acinetobacter* sp. WC-17 and *E. coli* were grown in 200ml LB media with or without ampicillin, and culture supernatants were collected by centrifugation at 12,000rpm for 10min. Proteins in the culture supernatant were precipitated by 80% ammonium sulfate saturation and collected by centrifugation at 1 5,000rpm for 30 min. The protein precipitate was dissolved and dialyzed against a 10mM sodium phosphate buffer (pH6.0). Concentrated enzyme solution was obtained by dissolving protein precipitate in minimum volume.

Physical Mapping

The physical map of plasmid pWCS101 was constructed from single and double digests with various restriction endonucleases by using the manufacturers' recommended conditions. Products were separated on 0.8 to 1.2% agarose gels run at 50 to 100V in Tris-acetate buffer. Gels were stained in 1μg of ethidium bromide per ml and photographed by transmitted UV illumination.

Chitinase Assay

Chitinase activity was determined using the method of Somogyi (19) using a standard curve for N-acetylglucosamine (NAG). The assay mixture consisting of 500 μl of 0.5% (w/v) colloidal chitin in 50mM sodium phosphate buffer, pH6.0 and 500μl of enzyme solution. The enzyme reaction was conducted in shaking water bath at 37°C for 2 h. One unit of chitinase activity was defined as the amount of enzyme required to produce 1 μmole of NAG per min under the described conditions.

Cellular Fractionation

The fractionation of extracellular, periplasmic, and intracellular enzymes was performed using a modified method of Cornelis (3). The culture broth was centrifuged at 7,000 rpm for 10 min at 4℃ and washed twice with an equal volume of original broth containing 0.9% NaCl. The cells harvested at 4℃ were washed in 0.5M potassium phosphate buffer (pH6.5) and suspended in a half volume of the original broth with 25% (w/v) sucrose solution in a potassium phosphate buffer (pH6.5), 1mM ethylenediaminetetraacetic acid (EDTA) at room temperature. After 10 min of constant and gentle shaking, the cells were sedimented by centrifugation. The extracellular fraction was calculated from the sum of the culture broth supernatant, the two washes, and the EDTA treatment supernatant. It was then suspended in the same volume of ice-cold distilled water and shaken for another 10 min. The cells were centrifuged and the resulting supernatant fluid was used as the periplasmic fraction. The cells sonicated in the same buffer were centrifuged and the supernatant was used as the intracellular fraction.

RESULTS AND DISCUSSION

Cloning of An *Acinetobacter* sp. WC-17 Gene Encoding Chitinase Activity

Chromosomal DNA of *Acinetobacter* sp. WC-17 was partially digested with *PstI* and purified into 2-10 Kb sizes with GENE CLEAN II Kit (Bio101, La Jolla, USA). The chromosomal DNA fragments were ligated with the dephosphorylated pUC18 linearized by *PstI* digestion and the ligate was used to transform *E.coli* JM109. The transformants grown on X-gal plates containing 50 μg/ml of ampicillin were transfered onto 1% colloidal chitin overlay plate to screen the chitinase-positive clones. One clone from a partial library of 5,000 insert-bearing transformants was found to be positive for chitinolytic activity on chitin overlay plates. This clone contains about 3.2Kb insert DNA in pUC18 plasmid. As shown in Fig. 1, *Acinetobacter* sp. WC-17 and *E. coli* JM109 (pWCS101) hydrolyzed the colloidal chitin on the agar plate and appeared as a halo zone around the cell while *E.coli* JM109 could not hydrolyze the colloidal chitin. To confirm the presence of the chitinase gene in this insert chromosomal DNA, the chimeric plasmid DNA from the chitinase-positive clone retransformed into *E.coli* JM109. All of the transformants showed chitinase activity by appearing the halo around the cells (data are not shown). These results indicated that the chitinase gene exist in the chimeric plasmid. Thus, we named the

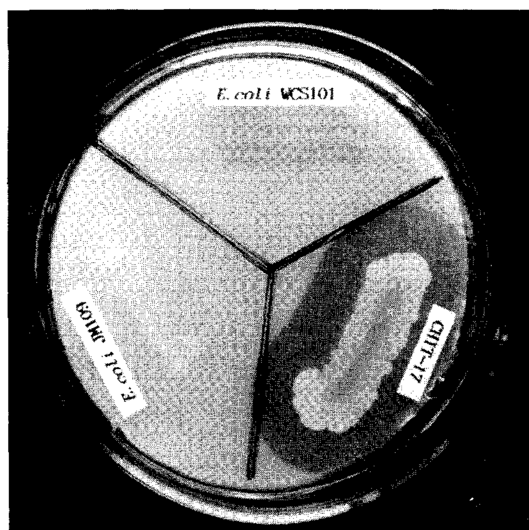

Fig. 1. Chitinolytic activity of recombinant *E.coli* and *Acinetobacter* sp. WC-17
E.coli WCS101; Recombinant *E.coli* harboring pWCS101, CHIT-17; *Acinetobacter* sp. WC-17, *E.coli* JM109; Cloning host

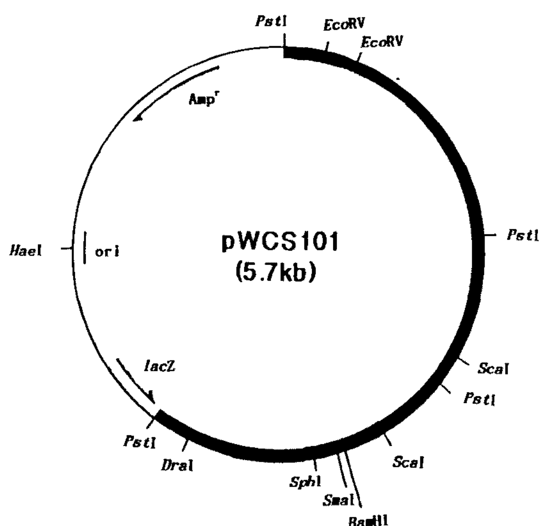

Fig. 2. Restriction endonuclease map of pWCS101
Restriction sites are indicated, and the thick circle represents the cloned fragment.

chimeric plasmid consisting of pUC18 and 3.2Kb chromosomal DNA fragment as pWCS101.

Physical Map of pWCS101

The isolated plasmid pWCS101 was digested with several restriction enzymes and analyzed by agarose gel electrophoresis. A restriction map of plasmid pWCS101 is shown in Fig. 2. No *Hind*III, *Eco*RI, *Sac*I, *Xba*I, and *Kpn*I sites were detected inside the insert. On the contrary, two *Pst*I, *Eco*RV, and *Sca*I sites were found in this fragment. *Sph*I, *Sma*I, *Bam*HI and *Dra*I have single restriction sites.

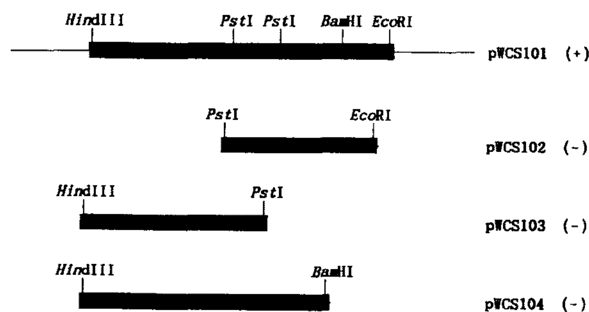

Fig. 3. Localization of the chitinase gene.
The horizontal thick lines represent derivatives of pWCS101. All are hybrid plasmids derived from pUC18 and segments of the cloned fragment. The right side of the figure shows the chitinase activity of *E.coli* JM109 horbouring each plasmid.

Table 1. Localization of the chitinase produced by pWCS 101/*E.coli* JM109 and *Acinetobacter* sp. WC-17

Strains	Relative activity (%)		
	Extracelluar	Periplasmic	Cytosolic
E.coli (pWCS 101)	15.8	76.0	8.2
Acinetobacter sp. WC-17	91.3	6.2	2.5

Subcloning and Deletion Analysis

In order to localize the essential region of the gene coding for chitinase in the 3.2Kb insert fragment, plasmid pWCS101 was digested with *Pst*I and *Bam*HI and religated. Transformants were selected on X-gal plates and chitin overlay agar plates. As shown in Fig. 3, only the original 3.2Kb insert was necessary for chitinase activity of *E.coli* JM109.

Localization of Chitinase in *E.coli* Recombinant and *Acinetobacter* sp. WC-17

To determine the localization of chitinase, *E.coli* recombinant was cultivated in LB broth at 37℃ with appropriate requirements. As shown in Table 1, almost all the chitinase activity of the *E.coli* recombinant carrying pWCS101 was localized in the periplasmic fraction of *E.coli* cell, while most of the enzyme activity of *Acinetobacter* sp. WC-17 was found in the the extracellular space. Thus, most of the expressed chitinase in *E.coli* recombinant was excreted through the inner membrane into the periplasmic space but not through the outer membrane.

Production and Properties of Cloned Chitinase of *E.coli* Recombinant

Time course of growth and chitinase production in *E.coli* recombinant were determined as shown in Fig. 4. Chitinase activity in *E.coli* recombinant was detected after the late exponential phase of growth but decreased rapidly after the stationary phase. This might be due to the proteolytic activity of the host. To investigate the

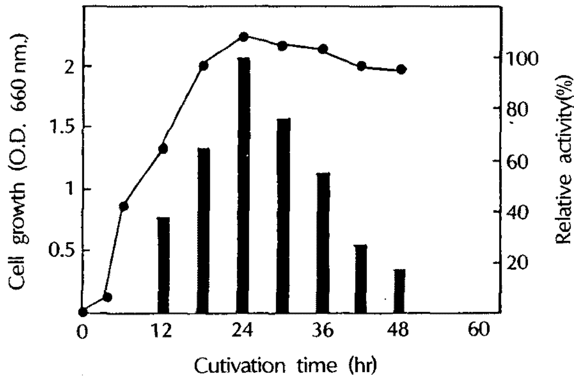

Fig. 4. Growth and chitinase production of recombinant *E.coli* harboring pWCS101 according to culture time.
Recombinant *E.coli* was grown at 37℃ in LB broth containing 0.1% chitin, 50μg/ml ampicillin, and 0.1mM IPTG. Cell growth at 660nm. (●), The bar represents relative activity of cloned chitinase.

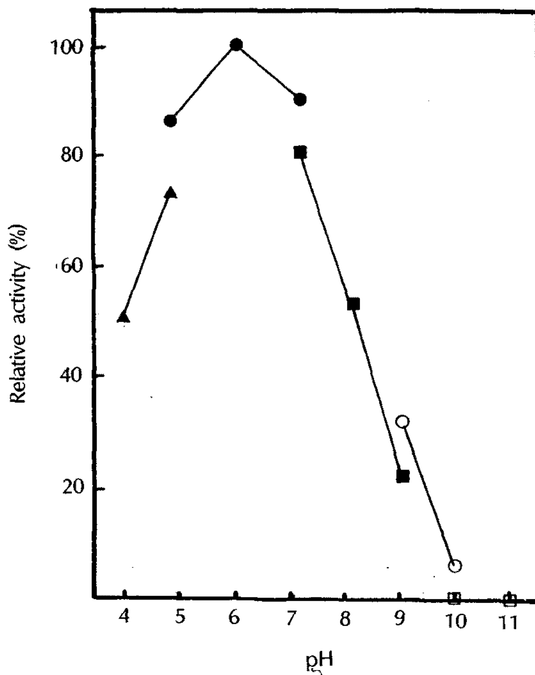

Fig. 5. Optimum pH for the chitinase activity of recombinant *E.coli*
Recombinant *E.coli* was grown at 37℃ in LB broth containing 0.1% chitin, 50μg/ml ampicillin, and 0.1mM IPTG. Na-acetate buffer(▲), Na-phosphate buffer(●), Tris-HCl buffer(■), Boric acid/NaOH(O), and Na-phosphate/NaOH(□).

effect of temperature and pH on cloned chitinase activity, the recombinant *E.coli* harboring pWCS101 was cultivated in LB broth containing appropriate requirements at 37℃, and the chitinase activity in the culture supernatant and total cell extract were measured. The enzyme activity was assayed at 37℃ for 3 h. The optimum pH was 6.0 (Fig. 5), and the optimum temperature for the

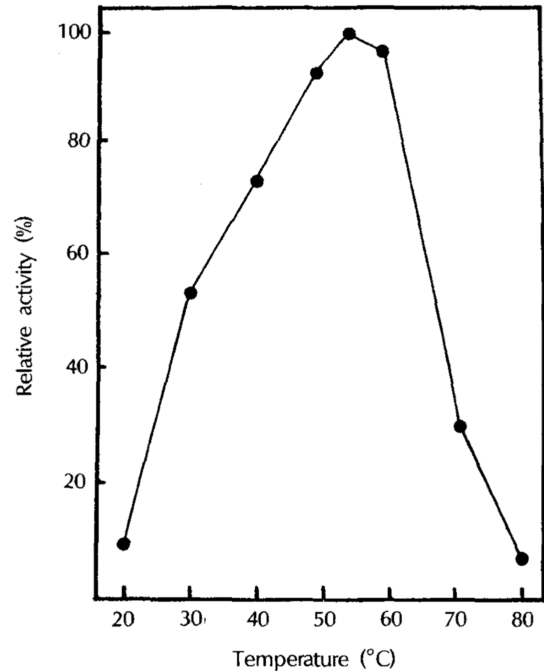

Fig. 6. Optimum temperature for the chitinase activity of recombinant E.coli.
Recombinant *E.coli* was grown at 37°C in LB broth containing 0.1% chitin, 50μg/ml ampicillin, and 0.1mM IPTG.

chitinase activity was 55°C(Fig. 6).

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