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Cloning of a Hemolytic Mosquitocidal Delta-endotoxin Gene (*cyt*) of *Bacillus thuringiensis* 73E10-2 (serotype 10) into *Bacillus subtilis* and Characterization of the *cyt* Gene Product

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To illustrate whether a hemolysin in δ-endotoxins of *Bacillus thuringiensis* strain 73E10-2 and subsp. *israelensis* had immunological identity, a *cyt* gene of the strain 73E10-2 which encodes a hemolysin was cloned to *B. subtilis* (transformant 2753). The transformant 2753 containing *cyt* gene produced the hemolysin which lysed sheep erythrocytes after treatment of proteinase K. The hemolysin was proved also to be toxic against mosquito larvae (*Aedes aegypti*). The molecular weight of the hemolysin produced from the transformant 2753 was determined to be about 25 kDa by SDS-PAGE and immunoblot. The hemolysin in δ-endotoxin of subsp. *israelensis* and subsp. *kyushensis* did not react on immunoblot using polyclonal anti-δ-endotoxin of the strain 73E10-2, but 70-140 kDa mosquitocidal toxins in δ-endotoxin of subsp. *kyushuensis* reacted.

Bacillus thuringiensis is a Gram-positive bacterium which produces δ-endotoxin during sporulation (14). Depending upon the subspecies, parasoral inclusions may be composed of one or more δ-endotoxins which are toxic to suceptible hosts such as lepidoptera, diptera, and coleoptera (6). It is well known that *B. thuringiensis* subsp. *israelensis* is highly toxic to diptera. In addition to *israelensis*, a few subspecies such as *morrisoni* PG14 (17), *darmstadiensis* 73E10-2 (16), *kyushuensis* (15), *galleria* (1) and *fukuokaensis* (20) produce diptera active toxins. In these strains, δ-endotoxins have not only cytolytic and hemolytic activity but also mosquitocidal activity. It is interested to note that there is immunologically no relationship between the δ-endotoxins of *israelensis* and those of *darmstadiensis* 73E10-2 (9). Eventhough δ-endotoxins of *darmstadiensis* 73E10-2 and *israelensis* have same mass of mosquitocidal toxin (68 kDa), they are antigenically unrelated each other (9).

Therefore, to illustrate the immunological relationship between the hemolyin of *B. thuringiensis* 73E10-2 and that of *israelensis*, the *cyt* gene of the strain 73E10-2 which encodes a hemolysin was cloned into *B. subtilis* (tansformant 2753) and the characteristics of the toxin produced by transformant 2753 were investigated in this work.

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Key words: *cyt* gene cloning, hemolytic mosquitocidal delta-endotoxin, *Bacillus thuringiensis*

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media and Culture Conditions

The reference strains, *Bacillus thuringiensis* subsp. *darmstadiensis* strain 73E10-2 (73E10-2), *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *kyushuensis* were deposited in this laboratory. Plasmids used were pBR322 for *E. coli* and pCUC19a (*Bacillus/E. coli* shuttle vector; Dr. N. Crickmore, Cambridge University; UK) for expression of the *cyt* gene from the strain 73E10-2 in *B. subtilis*.

The *B. thuringiensis* strains were grown on LB agar (12) at 27°C for 3 days. Spores and parasporal inclusions were harvested by centrifugation at 10,000 rpm for 20 min at 4°C. The sediment was washed three times by centrifugation in 1 M NaCl, and an additional three times with distilled water for purification of δ-endotoxins. The transformants were grown on LB agar, a minimal medium (7) supplemented with casamino acid (0.1%) instead of glucose, or LPB medium (7) for 24 h at 28°C.

DNA Manipulation and Isolation of Transformations

Total DNA of the strain 73E10-2 was extracted according to Austubel's method (2) and the extracted DNA was partially digested with *Pst*I. The DNA fragments obtained were ligated with pBR322 and transformed into *E. coli* HB101 (18). The transformants were selected with enzyme immuno-assay (ELISA) using anti-

δ-endotoxins of the strain 73E10-2 (21). The inserted toxin protein gene in pBR322 was also ligated into pCUC19a and transformed into *Bacillus subtilis* NA-1. These transformants were also selected by ELISA using alkaline phosphatase conjugated antirabbit-IgG (Bio-Rad Product). DNA was electrophoresed on horizontal 1× TAE [40 mM Tris, 1.14% (v/v) glacial acetic acid, 1 mM EDTA (pH 8.0)] agarose gels at 50 mV for 1 h and visualized with ethidium bromide. The molecular weight marker for DNA (λ DNA; *Hin*dIII digestion) and restriction enzymes were purchased from Boehringer Mannheim Corporation.

Purification of δ-Endotoxin, Formation of Anti-δ-endotoxin and Protein Determination

Spore and parasporal inclusions from the *B. thuringiensis* strains were purified using sucrose and a stepwise gradient as described by Cheung and Hammock (3) and solubilized with 0.1 N NaOH at 37°C for 2 h. Antisera were raised in a rabbit against the solubilized δ-endotoxins (total protein 3.2 mg) of the strain 73E10-2 as described previously (4). Purification of IgG fractions was performed with Protein A Sepharose 4B (Sigma Co.) and protein concentration was determined by Lowry's method (13).

Mosquitocidal Activity

Transformant 2753 was cultured in LB medium containing 25 μg/ml chloramphenicol at 28°C for 24 h, and cultured broth was harvested by centrifugation at 10,000 rpm for 20 mins at 4°C. The supernatant of the transformant 2753 culture was brought to 70% saturation ammonium sulfate and kept at 4°C for 1 h. The precipitation was then solubilized in 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM NaCl and 1 mM EDTA (1/100 vol of cultured broth) after centrifugation, dialyzed against the same buffer at 4°C overnight, and diluted with the same buffer to 4.5 mg/ml protein. Tweenty larvae (the 2nd instars) of *Aedes aegypti* were introduced into a test tube which contained 5 ml-protein suspension. The larvae were kept at 25°C and their mortality was examined.

Manipulation of Hemolysin

Transformant 2753 was cultured in LB medium and the supernatant from the cells of transformant 2753 was collected by centrifugation (10,000 rpm, 20 min, 4°C). Ten ml of the supernatant was mixed with the same volume of 50 mM Na_2CO_3 (pH 10.0) containing 10 mM dithiothreitol and 1 mM EDTA. This solution was treated with proteinase K (final conc., 40 μg/ml) for 1.5 h at 37°C as described by Ishii and Ohba (8). Penylmethylsulfonyl fluoride (PMSF: final conc., 0.2 mM) was added into the solution. The reaction solution was brought to 70% saturation ammonium sulfate and keep at 4°C for 1 h to make precipitation. Precipitation was collected with a centrifuge (4°C, 12,000 rpm, 20 min), solubilized with 1 ml of 20 mM Tris-HCl (pH 8.0) buffer and dialyzed against the same buffer at 4°C overnight.

The hemolytic activity test of the proteinase K-activated hemolysin against sheep erythrocytes was performed according to the method of Yu *et al.* (19). Sheep erythrocytes were purchased from Nippon Bio-Test Lab. Inc. (Tokyo, Japan). These erythrocytes were prepared as 1.5% (v/v) suspensions in 20 mM phosphate buffered saline (PBS, pH 7.4). For assay, 100 μl of erythrocyte suspension was mixed with an equal volume of the activated hemolysin solution in wells of a 96-well microtiter plate (Falcon). After incubation at 27°C, hemolysis of erythrocytes was observed by eyes.

SDS-PAGE and Immunoblot Assay

Parasporal inclusions from *B. thuringiensis* strains (the strain *israelensis*, *kyushuensis* and 73E10-2) as references were purified by sucrose and a step-wise gradient as described by Cheung and Hammock (3) and solubilized with 0.1 N NaOH at 37°C for 2 h. The supernatant from transformant 2753 was then collected after centrifugation (10,000 rpm, 4°C, 15 min) of the cultured broth. The solution was brought to 70% saturation with ammonium sulfate and dialyzed against 20 mM Tris-HCl (pH 8.0) buffer for SDS-PAGE and immunoblot analysis. The SDS-PAGE (5 mA, 4 h) was carried out according to the method of Laemmli (11) on 15% SDS-polyacrylamide gels and the immunoblot analysis was performed according to information provided by Bio-Rad Co. using an immunoblot kit containing peroxidase antirabbit-antibody.

RESULTS AND DISCUSSION

Transformation of the Toxin Gene and Expression

Total DNA was extraced from the strain 73E10-2, and partially digested with *Pst*I. The digested DNA was ligated with pBR322 and pCUC19a, and transformed into *E. coli* HB101 and *B. subtilis* NA-1, respectively. The plasmid from these transformants were extracted, digested with *Pst*I and electrophoresed on agarose gel. A 3.2 kb foreign DNA from the transformants was observed on agarose gel (Fig. 1), and the transformant 2753 produced much more toxin protein than other transformants. Although the toxin protein from the transformant 2753 was at high level in *B. subtilis*, it is necessary to identify whether the toxin gene expressed was practically producing a main mosquitocidal toxin (68 kDa) or a hemolysin (25 kDa).

Mosquitocidal Test of Transformant 2753

In a preliminary experiment, cells and supernatant from the cultured broth of transformant 2753 were separated by centifugation (10,000 rpm, 15 min, 4°C) for mosquitocidal toxin test. Mosquito larvae was not killed after feeding the cells, but the supernatant was slightly

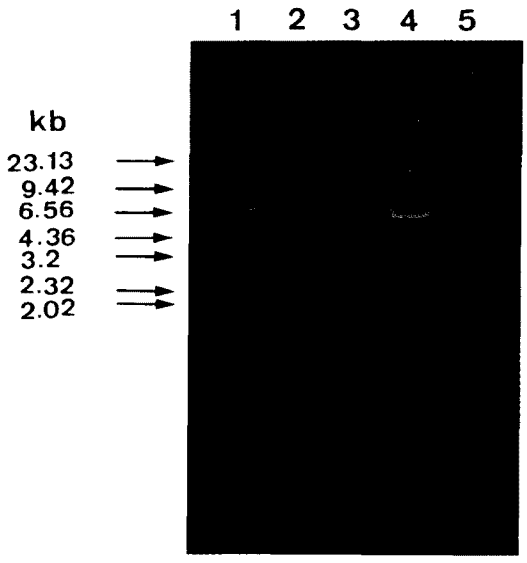

Fig. 1. Inserted toxin protein gene of transformant 27 and 2753 on agarose gel.
Lane 1, Marker (λDNA *Hin*dIII digest); lane 2, pBR322: lane 3, transformant 27; lane 4, pCUC19a; lane 5, Transformant 2753.

toxic to mosquito larvae after concentration (1/10 vol).

Transformant 2753 was the most active toxin protein producer among isolated transformants. Each test tube containing 20 mosquito larvae and 4.5 mg/ml protein of the transformant 2753 was kept at 25°C. Only one fourth of mosquito larvae, 5 among 20 larvae, was dead after incubation (Table 1). Therefore, it was clear that transformant 2753 produced a toxin protein with very low mosquitocidal activity. We thought that there might be 3 possibilities why the the toxin protein from the transformant 2753 had very low activity; i) cloned gene (*cry*IV) from strain 73E10-2 was a fragment of a toxin gene, and the cloned toxin gene therefore expressed a fragment of the mosquitocidal toxin protein, ii) the transformant 2753 produced not only the toxin protein (a product of *cry*IV gene) but also many other proteins (products of chromosomal gene of host), and the toxin protein seemed to have a very low mosquitocidal activity because of contamination of the other proteins, iii) the toxin protein from the transformant 2753 was not a product of *cry*IV gene but a product of *cyt* gene which always has a very low mosquitocidal activity. Three of these strains, *B. thuringiensis* subsp. *israelensis*, subsp. *kyushuensis*, and subsp. *darmstadiensis* 73E10-2, also produce toxins that have cytolytic and hemolytic activity (8, 10). Most of the hemolysins from *B. thuringiensis* showed less mosquitocidal activity than the CryIV protein.

Hemolytic Activity of Transformant 2753

Transformant 2753 produced a toxin protein which had a very low mosquitocidal activity and was apparently

Table 1. Mosquitocidal activity of transformant 2753.

Strain	Mortality (%)	
	48 h	60 h
B. subtilis NA1	0	0
E. coli HB101	0	0
Transformant 275	5	10
Transformant 2753	15	25

The protein (4.5 mg/ml) extracted from the transformants was suspended into 5 ml of tap water in a test tube (φ1.8×10 cm), and the 20 larvae of mosquito (the 2nd instars of *Aedes aegypti*) was introduced into each test tube for toxicity test. Percentage mortality was calculated after the mosquito larvae was kept at 25°C for 60 h.

Table 2. Hemolytic activity of transformant 2753.

Strain	Protein conc. (μg/ml)			
	265	132	66	33
B. subtilis NA1	–	–	–	–
Transformant 2753				
Ppt	++	++	+	–
Sup	–	–	–	–

Supernatant was harvested from cultured broth of transformant 2753 by centrifugation, and treated with proteinase K (final conc.; 40 μg/ml) for 1.5 h at 37°C. After that, PMSF (final conc.; 0.2 mM) was added into this solution to inactivate proteinase K. The solution was then brought to 70% saturation ammonium sulfate and keep at 4°C for 1 h to make precipitation. The precipitation was collected with a centrifuge, solubilized with 1ml of 20 mM Tris-HCl (pH 8.0) buffer and dialyzed against the same buffer at 4°C overnight. Symbols: ++, 100% hemolysis; +, 50% hemolysis; -, 0% hemolysis; Ppt, Dialyzed solution of precipitation occured by 70% saturation ammonium sulfate; Sup, Dialyzed solution of supernatant occured by 70% saturation ammonium sulfate.

not hemolytic against erythrocytes of sheep (Data not shown). Also, the solubilized crystal proteins of *kyushensis* were apparently not hemolytic, but had a strong hemolytic activity after proteinase K treatment (5, 8). Therefore, the toxin protein from the transformant 2753 was treated with proteinase K and tested for the hemolytic activity using erythrocytes of sheep. In Table 2, we show that proteinase K-activated toxin protein from the transformant 2753 had 50% hemolysis at 66 μg/ml. As compared with 25 μg/ml of *B. thuringiensis* strain PG-14 (19) and 30 μg/ml of *B. thuringiensis* subsp. *israelensis* (8), this hemolysin of transformant 2753 had relatively high hemolytic activity.

It indicates that the transformant 2753 had a *cyt* gene from *B. thuringiensis* subsp. *darmstadiensis* strain 73E102, and the hemolysin of transformant 2753 was activated with proteinase K.

SDS-PAGE and Immunoblot of Hemolysin

Delta-endotoxins were purified from the three reference mosquitocidal strains. The δ-endotoxins of the type strains, *B. thuringiensis* subsp. *israelensis*, subsp. *kyushuensis* and the strain 73E10-2, were analyzed for their protein compositions by SDS-PAGE. As shown in

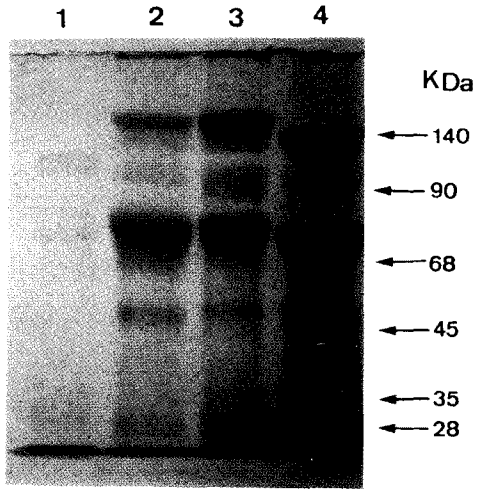

Fig. 2. Toxin protein patterns of transformant 2753 and *B. thuringiensis* strains on SDS-PAGE.

Toxin proteins were analyzed by 15% SDS-PAGE. Lane 1, concentrated solution of cultured supernatant from transformant 2753; lane 2, delta-endotoxin of *B. thuringiensis* strain 73E10-2; lane 3, delta-endotoxin of *B. thuringiensis* subsp. *kyushuensis*; lane 4, delta-endotoxin of *B. thuringiensis* subsp. *israelensis*.

Fig. 2, the major protein profiles of the reference mosquitocidal strains were as follows; i) *B. thuringiensis* subsp. *israelensis*; 27, 68 and 130 kDa (doublet), ii) the strain 73E10-2; 25, 50, 70-80 and 140 kDa, and iii) *B. thuringiensis* subsp. *kyushuensis*; 25, 70-80 (quartet), and 140 kDa. Delta-endotoxin of the strain 73E10-2 had some similarity with the protein profile of the *kyushuensis* but not with the *israelensis*. On the other hand, the protein profile of the transformant 2753 was 25, 70-80 and 100-120 kDa (doublet), but this toxin protein might be contaminated with products of chromosomal gene of host (*B. subtilis* NA1). Because supernatant of cultured broth of the transformant 2753 was precipitated by 70% ammonium sulfate saturated, and dialyzed with 20 mM phosphate buffer (pH 7.0), and electrophoresed. Therefore, it seems to be that 70-80 and 100-120 kDa protein may be products of chromosomal gene of host (*B. subtilis* NA1). Also, the 25 kDa protein band only remained practically after treatment of proteinase K (Data was not shown), because toxin protein is, in general, resistant against proteases.

As shown in Fig. 3, a 25 kDa protein of the transformant 2753 only cross-reacted against polyclonal anti-δ-endotoxin of the strain 73E10-2. Cry gene products such as 70-140 kDa of the strain *kyushuensis* reacted against polyclonal anti-δ-endotoxin of the strain 73E10-2, but the *cyt* gene product, 25 kDa, did not. On the other hand, a 53 kDa protein in δ-endotoxins of the strain *israelensis* only faintly cross-reacted against polyclonal anti-δ-endotoxin of the strain 73E10-2.

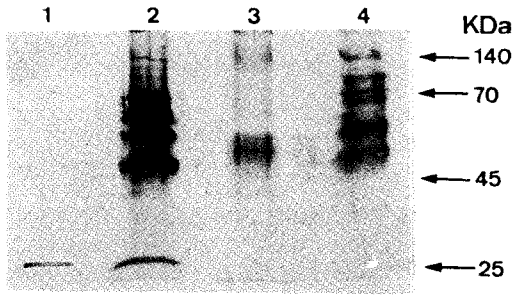

Fig. 3. Immunoblot of toxin proteins of transformant 2753 and *B. thuringiensis* strains against anti-δ-endotoxin of the strain 73E10-2.

Proteins were electrophoretically transferred from an SDS-PAGE gel to nitrocellulose membrane, probed with rabbit antisera against delta-endotoxin of the strain 73E10-2, and incubated with alkaline phosphatase coupled to goat antibody against rabbit IgG. Lane 1, concentrated solution of cultured supernatant from transformant 2753; lane 2, delta-endotoxin of *B. thuringiensis* strain 73E10-2; lane 3, delta-endotoxin of *B. thuringiensis* subsp. *israelensis*; lane 4, delta-endotoxin of *B. thuringiensis* subsp. *kyushuensis*.

This suggests that 25 kDa of the strain 73E10-2 is a different type of hemolysin from that of *kyushuensis* strain at least based on antigenicity, but has the same characteristic such as hemolysin activation by proteinase K.

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