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Characteristics of Hemolysin in Mosquitocidal *Bacillus thuringiensis* strain 21-2

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To describe characteristics of a hemolysin in mosquitocidal *Bacillus thuringiensis* subsp. *gyangiensis* strain 21-2, *Escherichia coli* HB101 was transformed with a gene encoding hemolysin in the strain 21-2. Transformant 47 contained 2.5 kb DNA was selected by ELISA, immunoblot and DNA electrophoresis. Transformant 47-5 was reconstructed after digestion of the 2.5 kb DNA with *Hind* III. Transformant 47-5 contained 1.8 kb DNA and expressed 23 kDa protein which had mosquitocidal activity to *Aedes aegypti*. The 23 kDa protein itself *in vitro* didn't show hemolytic activity on human erythrocytes, but the protein had the activity after proteinase K treatment.

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

Bacillus thuringiensis constitutes a large family of strains found in different habitat and highly specialized as insect pathogens [2, 3]. The main insecticidal factors displayed by these bacilli are δ-endotoxin inclusions synthesized during the sporulation [1]. The δ-endotoxins known so far fall into two categories, Cry and Cyt, that do not share significant sequence homology, although both types of toxins seem to work through pore formation that leads to cell lysis and irreversible damage of the insect mid-gut [19, 20]. *Cyt* genes coding for hemolytic toxins are widely distributed among a range of mosquitocidal *B. thuringiensis* subspecies and constitute another family of highly related genes [10]. The Cry toxins in *B. thuringiensis* subsp. *israelensis* interact synergistically with the Cyt 1A toxin to produce this high level of activity [7, 30]. This synergism was also shown to be important in the relatively low rate of resistance development toward the *israelensis* in *Culex* mosquitoes [9] and can suppress high levels of resistance to Cry 4 and Cry 11 toxins [27, 29]. In addition, Cyt 1A was combined with *B. sphaericus*, an unrelated mosquitocidal bacterium which does not have Cry-type toxins, were synergistic toward highly resistant *Culex quinquefasciatus* [28]. Cyt toxins were implicated as major factors in the lack of resistance to *israelensis* in laboratory-selected *Culex quinquefasciatus* populations [9]. Thus, Cyt proteins may be useful for combating insecticide resistance and for increasing the activity of microbial insecticides.

For screening of Cyt toxins, we isolated a new mosquitocidal *B. thuringiensis* strain 21-2 (H serotype 43) shown in previous paper [18]. In this paper, we described characteristics of Cyt toxin in the strain 21-2 with transformants contained *cyt* gene.

MATERIALS AND METHODS

Bacteria, vectors, media and culture conditions

Bacillus thuringiensis subsp. *gyangiensis* strain 21-2 was kept in this laboratory. Vector pCUC19a (*Bacillus* \ *E. coli* shuttle vector ligated with pC194 (3.3 kb) and pUC19 (2.69 kb) at *Hind* III site) was kindly supplied from Dr. N. Crickmore, Cambridge University. Vector pUC118 was purchased from Wako Co. in Japan. The strain 21-2 was cultured in PYG medium [16] at 28°C for 5 days and transformants were maintained in LB medium [11] at 37°C for 24 hrs.

Purification of δ-endotoxin, formation of anti-δ-endotoxin and protein determination

Delta-endotoxins of the strain 21-2 were purified with discontinuous sucrose gradient centrifugation as described by Cheung and Hammock [4]. The purified δ-endotoxins were observed with a phase contrast microscope, lyophilzed with a freezing-dryer and kept at -20°C. The δ-

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endotoxins were solubilized in carbonate buffer (50 mM Na_2CO_3, 10 mM DTT, 1 mM EDTA, pH10.0) at 37°C for 1 hr as described by Wasano *et al.* [26]. Proteins were determined by Lowry's method [24]. Antiserum against δ-endotoxins (protein 2.0 mg) of the strain 21-2 was raised in a rabbit as described by Cheung and Kim [5]. Purification of immuno-globulin G (IgG) against the δ-endotoxins was performed with Protein A Sepharose 4B (Sigma Co.).

SDS-PAGE and immunoblot

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by Laemmli's method [23] and immunoblot analysis (an immunoblot kit containing peroxidase anti-rabbit-antibody) was performed according to the information provided by Bio-Rad Co.

DNA manipulation and selection of transformants

Plasmids in the strain 21-2 were extracted by alkaline method [25], and the plasmids were digested with *Pst* I (Promega Co.). The digested DNA fragments were ligated with pCUC19a and inserted into *E. coli* HB101. Transfomants were maintained on LB agar contained ampicillin at 37°C overnight. After X-gal treatment for colony selection, the toxin proteins of transformants were detected by enzyme linked immuno-sorbent assay (ELISA) and immunoblot with IgG against δ-endotoxin of the strain 21-2 as described by Kim *et al.* [17]. Transformants produced 22-28 kDa protein on the immunoblot were selected. A inserted DNA fragment and pCUC19a in the selected transformant 47 was confirmed on DNA electrophoresis. Transformant 47-5 was subcloned with pUC118 and the inserted DNA in the transformant 47 after *Hind* III digestion. DNA electrophoresis was carried out on agarose gel with a mini-gel apparatus (Hoefer Scientific Instrument, USA).

Proteinase K treatment

Transformant 47 and 47-5 were maintained in LB [11] broth at 37°C for 24 hrs, and pellets were harvested by micro-centrifuge (Hanil Co. Korea). The pellets were incubated in 50 mM carbonate (pH10.0) containing 10 mM dithiothreitol and 1 mM EDTA at 37°C for 1 hr to solubilize toxin. The solubilized toxin (protein 250 μg/ml) was digested with proteinase K (final conc. 40 μg/ml) at 37°C for 1.5 hrs. After digestion, phenylmethylsulfonyl fluoride (PMSF: final conc., 0.2 mM) was added to the reaction mixture for inactivation of proteinase K as described by Ishii and Ohba [13]. After that, the reaction mixture was dialyzed in 10 mM Tris-HCl buffer (pH8.0) at 4°C.

Table 1. Transformant 47 expressed toxin protein by ELISA.

Strains	Expression of toxin protein
	ELISA (A_{405nm})
E. coli HB101	0.008 ± 0.003*
The strain 21-2	0.452 ± 0.015
Transformant 47	0.082 ± 0.012

*Each value represents the mean SE

Biological activity

Hemolytic activity was determined with human erythrocytes (type O) as described by Yu *et al.* [31]. Mosquitocidal activity was also carried out as described by Chilcott and Ellar [6]; The extracted toxin (1mg/ml) of transformants was suspended into a test tube (Φ1.8 × 10 cm) contained 5 ml of distilled water previously, and 20 larvae of mosquito (*Aedes aegypti* ; the 2nd instars) were introduced into each tube. The larvae were kept at 25°C and their mortality (%) was examined.

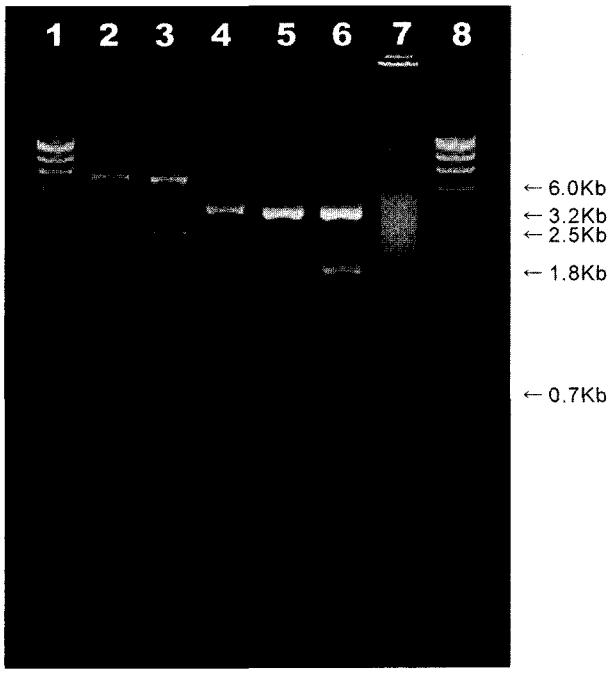

Fig. 1. Inserted DNA in transformants. Lane 1, marker(λDNA *Hind* III digestion); lane 2, pCUC19a; lane 3, transformant 47; lane 4, pUC118; lane 5, transformant 47-3; lane 6, transformant 47-5; lane 7, step ladder DNA marker; lane 8, marker(DNA Hind III digestion).

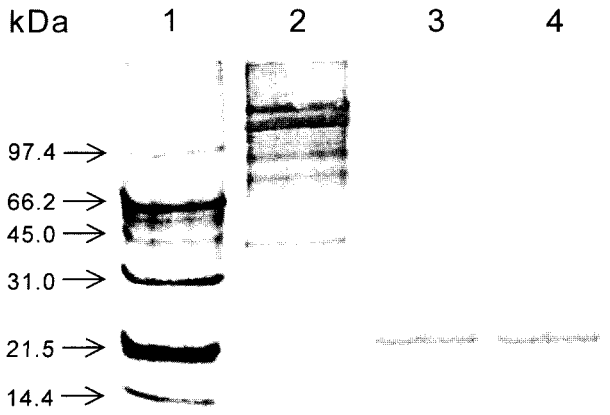

Fig. 2. Toxin proteins of transformants on immunoblot. Lane 1, biotinylated molecular marker; lane 2, delta-endotoxin of strain 21-2; lane 3, transformant 47; lane 4, transformant 47-5.

RESULTS AND DISCUSSION

Toxin protein gene and expression

Transformant 47 was selected by ELISA with IgG against δ-endotoxin of the strain 21-2 (Table 1), and contained 2.5 kb DNA (lane 3 in Fig.1). The transformant 47 and 47-5 expressed 23 kDa toxin protein, which was detected on immunoblot (lane 3 & 4 in Fig. 2). However, 23 kDa protein was not observed on lane 2 in Fig. 2. By several attempt to detect the 23 kDa of the strain 21-2, we could finally observe a very faint band, the 23 kDa, on the other immunobot. Unfortunately, the faint band was not enough to photograph. The transformant 47-5 contained 1.8 kb DNA fragment (lane 6 in Fig.1).

Most mosquitocidal *B. thuringiensis* strains contain *cyt* gene encoding cytolytic and hemolytic protein [8, 13, 15, 17, 31]. In the *israelensis,* 28 kDa protein is expressed by *cyt* 1A (former *cyt* A) gene and 25 kDa protein in *kyushuensis* is expressed by *cyt* 2A (former *cyt* B) gene [13, 22]. If the 23 kDa protein of transformant 47-5 is truely a hemolysin, the protein should have not only hemolytic activity but also mosquitocidal activity simultaneously.

Biological activity of transformants

Mosquitocidal and hemolytic activity of transformant 47 and 47-5 were detected with mosquito lavae and human red blood cells (RBC). Both transformants were toxic to mosquito larvae, although mosquitocidal activity of the transformant 47 and 47-5 was lower than that of strain 21-2 (Table 2). In addition, toxin proteins of both transformant 47 and 47-5 had not hemolytic activity *in vitro* but the proteins of the transformants lysed RBC after digestion with proteinase K (Table 3). This phenomena is very similar to Cyt 2A toxin in *kyushuensis*; Delta-endotoxin of *kyushuensis* contains several proteins ranging from 140-14 kDa, and 25 kDa protein itself among them has not a hemolytic activity *in vitro*, but the 25 kDa protein has the activity after proteolytical digestion with proteinase K into 23-22 kDa [13, 21]. However, *cyt* 1A (former *cyt* A) is found in the *israelensis* and the *morrisoni* PG14 and Cyt 1A toxin has hemolytic activity without proteolytic digestion *in vitro* [8, 12]. Juarez-Perez et al. [14] described that none of the Cyt 2 toxins were recognized by antiserum directed against Cyt 1Aa, and the level of sequence identity between *cyt* 1 and *cyt* 2 families is too low. Juarez-Perez et al. [14] also described that none of the Cyt 2B toxin was active after proteinase K processing, and only trypsin treatment revealed the activity of this toxin.

This results showed that strain 21-2 had a *cyt* 2A-like gene. Further work is required to elucidate the role of this Cyt 2 toxin in the strain 21-2.

Consequently, *B. thuringiensis* strain 21-2 contained a *cyt* 2A-like gene and the Cyt toxin had hemolytic activity after proteinase K treatment *in vitro*.

Table 2. Mosquitocidal activity of transformants.

Strain \ Time (h)	Mortality(%)			
	12	24	36	48
E. coli HB101	0	0	0	0
B. thuringiensis strain 21-2	0	100	100	100
Transformant 47	0	65	80	100
Transformant 47-5	5	80	100	100

Table 3. Hemolytic activity of transformants.

Strain	Proteinase K Treatment	Hemolysis (%)	Toxin Protein (μg/ml)
Control	No	0	0
B. thuringiensis strain 21-2	No	0	415
Transformant 47	No	0	415
Transformant 47-5	No	0	415
B. thuringiensis strain 21-2	Yes	100	208
Transformant 47	Yes	100	207
Transformant 47-5	Yes	100	205

국문초록

모기 살충성 *Bacillus thuringiensis* 21-2 균주의 용혈성 내독소 단백질의 특성

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모기 살충성 *Bacillus thuringiensis* subsp. *gyangiensis* 21-2균주의 용혈성 내독소 단백질의 특성을 검토하고자 21-2균주의 용혈성을 가진 유전자를 *Escherichia coli* HB101에 형질전환시켰다. 이들 중에서 형질전환 균주 47은 독소 단백질을 생산하며 2.5 kb DNA을 함유한다는 것을 효소항체법, immunoblot 및 DNA 전기영동법으로 확인하였다. 또한, 형질전환 균주 47-5는 2.5 kb DNA를 다시 *Hind* III로 절단하여 pUC118에 연결시켜서 조제하였다. 그 결과 형질전환 균주 47-5은 1.8kb DNA를 함유하며, 23 kDa의 독소 단백질을 발현하고, 발현된 독소 단백질은 *Aedes aegypti* 모기 유충에 독성을 나타내었다. 또한 23 kDa의 내독소 단백질 그 자체로는 사람의 적혈구를 용해하지 못하였으나, proteinase K로 처리한 후에는 적혈구에 대해 용혈성을 나타내었다.

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