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Overexpression of Insecticidal Protein Gene of *Bacillus thuringiensis* var. *kurstaki* HD1

Sung-Hei Hwang, Kwan Hee Yoo[1], Eui Sik Moon, Soung Chul Cha, and Hyung Hoan Lee*

Department of Biology & Institute for Genetic Engineering, Konkuk University, Seoul 143-701, Korea
[1]Department of Biology, Sanggi University, Wonju 220-702, Korea

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The Insecticidal protein (ICP) gene from *Bacillus thuringiensis* var. *kurstaki* HD-1 was cloned in pBluescript SK(+) vector and charicterized by overexpression in *Escherichia coli* XL1-blue. Total plasmids in the *B. thuringiensis* were isolated and digested with restriction enzyme *Bam*HI. Then, southern blot was performed with a probe to locate the gene in the fragments. The hybridized 3.8 kb *Nde*I DNA fragment was cloned into the *Sma*I site of pBluescript SK(+) and named pHLN1-80 in forward orientation to the *lacZ* gene promoter and pHLN2-80 in reverse orientation to the *lacZ* gene promoter. Determination of 153 bp nucleotide sequence of 5'-end of the *Nde*I fragment in the pHLN1-80 clone revealed that there are -80 bp region of the ICP gene promoter and +73 bp region of the ICP gene at the 5'end of the ICP gene. In addition, the -80 bp promoter of the ICP gene contained transcription initiation point G at -77 bp point and BtI promoter and Shine-Dalgarno sequence at -14 to -4 bp region. The two clones showed strong insecticidal activity against 3rd the instar *Bombyx mori* larvae. SDS-PAGE analysis revealed that the pHLN2-80 clone clearly produces distinguishable amount (27 times more) of the 130 kDa ICP band and 100 times the insecticidal activity than that of the clone pHLN1-80. These marked differences in production and toxicity due to different orientations of the gene in the vector provide us valuable points for further study on the ICP gene transcription at the molecular level.

Key words: *B. thuringiensis*, δ-endotoxin and gene, overexpression, insecticidal protein gene

Bacillus thuringiensis subsp. *kurstaki* is a gram positive bacterium which produces a bibypyrimidal proteinaceous crystal during sporulation (1). These crystals (insecticidal protein, ICP) are 130 kDa and are toxic to various caterpillars. The *B. thuringiensis* subsp *kurstaki* strains have been isolated and studied by many investigators (5, 14), and numerous reports indicate that the plasmids within the bacteria are involved in the expression of the crystal protein (4, 8, 10, 15, 20, 25). By recombinant DNA techniques, a single gene on certain plasmids from various strains of the *B. thuringiensis* subsp *kurstaki* strains was manipulated and expressed in *E. coli* system (8, 17, 20, 24, 25); however, the overexpression of the inverted genes have not yet been reported. The *B. thuringiensis* strains are important commercial insecticides with low environmental impact on nontarget organisms (5). Further study on the expression of the insecticidal proteins is necessary to develop a better insecticide and to investigate gene regulation. Therefore, we have undertaken the cloning and expression of the *B. thuringiensis* subsp. *kurstaki* HD1 ICP gene coding for this insecticidal protein.

In this paper, we describe the cloning of the ICP gene from the *B. thuringiensis* subsp *kurstaki* HD1 strain (shown toxic to lepidopteran larvae) into the pBluescript SK(+) vector, overexpression of the new recombinant clones in *E. coli* system, and the clones' highly toxic effects.

Materials and Methods

Bacteria and plasmids

B. thuringiensis subsp. *kurstaki* strain HD-1 was obtained from H.D. Dean (Ohio State University, OH). *Escherichia coli* strain XL1-blue bearing a vector pBluescript SK(+) was originally obtained from L. K. Miller (University of Georgia, Athens, GA). *E. coli* strain V517 (16) was obtained from R.M. Faust (U.S. Department of Agriculture) and used as the source of plasmid molecular weight standards.

Enzymes and probe

Enzymes were obtained from Bethesda Research

* To whom correspondence should be addressed.
(Tel) 2-450-3426; (Fax) 2-452-9715
(E-mail) hhlee@kkucc.konkuk.ac.kr

Laboratories. Inc. (Gaithersburg, Md). Calf intestinal alkaline phosphatase was obtained from Boehringer-Mannheim (Mannheim, Germany). All other chemicals were obtained from Sigma (St. Louis, USA), and all enzymes and buffers were used as recommended by the manufacturers. The pHL0.9 plasmid containing 0.85 kb of the *B. thuringiensis* subsp *kurstaki* toxin gene fragment was used as a probe (15).

Purification of plasmids

pBluescript SK(+) plasmid and other plasmid were isolated from *E. coli* by the procedures described by Birnboim and Dolly (2). The plasmid from *B. thuringiensis* var. *kurstaki* HD1 was isolated and purified by the procedures described by Lee and Park (15). A rapid, small-scale plasmid analysis was done using microcentrifugation at 15,000 rpm for 1 min, and then the purified DNAs were examined by 0.8% agarose gel electrophoresis (6, 17).

Purification of insecticidal crystals

Parasporal crystals of *B. thuringiensis* subsp. *kurstaki* HD-1 were separated from spores and cellular debris by NaBr gradient centrifugation (15) and used as antigen for preparation of ICP antiserum from rabbits.

Immunological analysis

Antisera against the solubilized crystal from *B. thuringiensis* subsp. *kurstaki* HD1 were prepared as previously described by Oh *et al.*, (21). Sera were drawn from immunized rabbits, and the immunoglobulin G fraction was purified by DEAE-Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calf.) column chromatography (17). Immunoglobulin G was analyzed by double immunodiffusion precipitation (22) against solubilized crystal from the *kurstaki* HD-1. Western blot was performed with the antiserum as described by Bollag *et al.*, (3).

Polyacrylamide gel electrophoresis

Analysis of the purified insecticidal proteins (ICP) produced in *E. coli* cells was carried out by 7.5% polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots as described by Bollag *et al.*, (3).

Molecular cloning

Total purified plasmid DNA from *B. thuringiensis* subsp *kurstaki* HD1 was digested with *Bam*HI and run on 0.5% agarose gel (3, 19). Then the *Bam*HI fragment containing the ICP gene was detected with a probe (15) by southern blot analysis (25). The hybridized 15 kb *Bam*HI fragment was redigested with 8 different restriction enzymes (*Sal*I, *Hind*III, *Sma*I, *Xho*I, *Nde*I, *Bst*EII, *Hae*II, and *Taq*I) and rehybridized with the probe (15). The hybridized 3.8 kb *Nde*I fragment was cloned into the *Sma*I of pBluescript SK(+) and transformed into *E. coli* XL1-blue (18). The transformed *E. coli* were isolated on the nutrient agar plate containing 50 μg/ml of ampicillin, 2% X-gal (5-bromo-4-chloro-3-indol-β-galactoside) and 100 mM IPTG (isopropyl-β-D-thio-galactopyranoside). The recombinant plasmids were analyzed by *Eco*RI digestion to determine if the ICP gene orientation was either correct or reversed right relation to the *lacZ* gene promoter.

Restriction mapping and sequencing

The DNA fragment containing ICP gene was mapped with restriction enzymes (*Cla*I, *Eco*RI, *Eco*RV, *Dra*I, *Hind*III, *Kpn*I, *Sma*I, and *Nde*I) with the procedure described by Lee *et al.*, (12). The DNA fragment was inserted in pBluescript SK(+) and sequenced by the procedure described by Sanger *et al.*, (23).

Bioassay on *Bombyx mori* larvae

E. coli cells harboring the clones pHLN1-80 or pHLN2-80 in a 50 ml nutrient broth (Difco Laboratories, Detroit, MI.) containing 50 μg/ml of ampicillin were cultured overnight at 37°C, and then the number of cells was calculated. Cells were harvested by centrifugation, washed in 0.85% NaCl solution, suspended in a minimum amount of deionized water containing 1.0 mM EDTA and 1.0 mM dithiothreitol, and sonicated in 30 sec intervals for a total of 2 min at 200 watts. 5.2×108 cells per ml were present in the suspended solution. The sonicated cell lysates were cleared by centrifugation at $5,000\times g$ for 30 min.

Healthy *B. mori* larvae were obtained from Dr. S. P. Lee (National Research Institute of Agricultural Science and Technology, Suwon, Korea). The third instar larvae were used for bioassay. Larvae were starved for 4 h before treatment. Treatment dosages (0.1 ml contained 5.2×10^7 cells) of ICP crude protein extracts were applied uniformly to the surface of a 5×5 cm^2 section of mulberry leaf diet and introduced to 10 larvae per dish. Larvae were maintained under constant light and 40% humidity at room temperature and additional food leaves(untreated) were presented upon consumption of the diet leaves to avoid starvation effects. Mortality was monitored for 18 h after exposure to treatment leaves; lethality was calculated by counting the dead larvae.

Results

Cloning of the ICP gene from *B. thuringiensis* HD1

In the total plasmid fragments of the *B. thurin-*

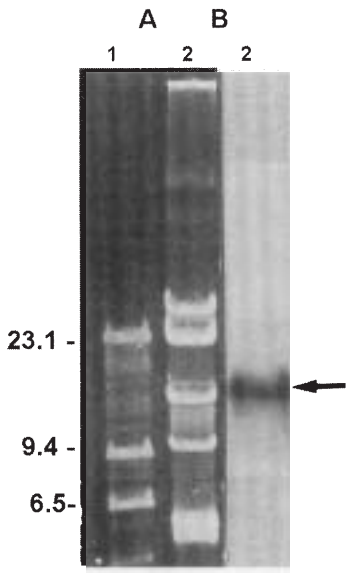

Fig. 1. Detection of the ICP gene among the *Bam*HI-digested plasmid DNA fragments from *B. thuringiensis*. A is the 0.5% agarose gel electrophoresis patterns. Lanes A1: λ DNA digested with *Hind*III; A2, total plasmid DNA fragments from the *B. thuringiensis* digested with *Bam*HI; B2, Southern blot of the gel A. Arrow indicates the 15 kb fragment hybridized with the probe.

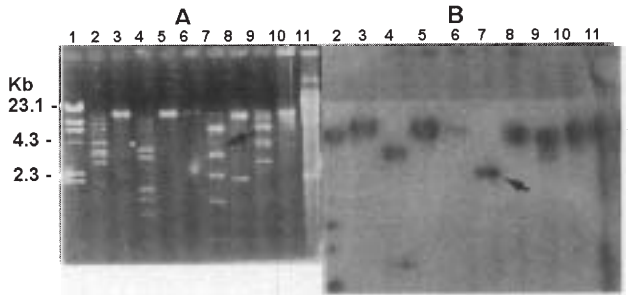

Fig. 2. Detection of ICP gene fragments among the 15 kb *Bam*HI fragments digested with restriction enzymes. The 15 kb *Bam*HI fragment was digested with 9 restriction enzymes, run on the gel, and then hybridized with the probe. Lane A1, λ phage DNA digested with *Hind*III, lanes A2-A10, 15 kb *Bam*HI fragment digested with *Eco*RI, *Sal*I, *Hind*III, *Sma*I, *Xho*I, *Nde*I, *Bst*EII, *Hae*II and *Taq*I respectively; Lane A11, total plasmid DNAs from *B. thuringiensis* digested with *Bam*HI. B is the Southern blot of the gel A.

giensis subsp. *kurstaki* HD1 digested with *Bam*HI enzyme, a 15 kb *Bam*HI fragment was hybridized with the probe DNA (15) (Fig. 1), redigested with 8 restriction enzymes, and then rehybridized with the probe (Fig. 2). *Sal*I, *Sma*I, and *Xho*I sites were not present in the 15 kb fragment. Four *Eco*RI fragments (9.3 to 1.2 kb), two *Hind*III fragments (7.2 kb, 1.52 kb), one *Nde*I fragment (3.8 kb), one *Best*EII fragment (10.3 kb), one *Hae*II fragment (9.2 kb), and one *Taq*I fragment (10.3 kb) were hybridized with the probe. The hybridized 3.8 kb *Nde*I fragment was cloned into the *Sma*I site of the vector pBluescript SK(+) and named pHLN1-80 recombinant (6.8 kb) for a correct right-orientation to the *lacZ* gene promoter and named pHLN2-80 recombinant (6.8 kb) for reversed-orientation to the promoter (Fig. 3A). Three *Eco*RI sites were present at the 5' end of the ICP gene. So, by the *Eco*RI digestion patterns of the ICP gene, it could be determined whether the gene orientation was correct or reversed. The pHLN1-80 plasmid was cleaved into 3.4, 2.1, and 0.7 kb fragments, and the pHLN2-80 was cleaved into 5.1, 0.7, 0.6, and 0.4 kb fragments by *Eco*RI. These results indicated that the pHLN1-80 contained the correct-oriented *Nde*I fragment and the pHLN2-80 had the reversed *Nde*I fragment. The 3.8 kb *Nde*I fragment in the pHLN1-80 plasmid was mapped with 5 restriction enzymes by single or double digestion(s). The physical map is illustrated in Fig. 3B. The DNA fragment had three sites each for *Eco*RI, *Eco*RV, and *Cla*I and two sites for *Hin*dIII. Fig. 3, lane C is the gene structure map of the 3.8 kb DNA fragment in the clone pHLN1-80.

Nucleotide sequence of the 5'end of the *Nde*I fragment

The 153 bp nucleotide sequence of the 5'-end of the *Nde*I fragment in the pHLN1-80 clone are determined (Fig. 3, lanes C and D). This revealed that there are -80 bp region of part of the ICP gene promoter and a +73 bp region of part of the ICP gene at the 5'end of the ICP gene. In the -80 bp region, the *Sma*I/*Nde*I ligated site sequence (CCC/TATG) at the 5' end and the Shine-Dalgarno sequence (ATGGAGGTAA) at the -14 to -4 bp region were found and underlined. These data indicated that the 3.8 kb fragment sequence was correctly inserted in the pBluescript SK(+) vector. In addition, the -80 bp promoter contained transcription initiation point G at the -77 bp point and BtI promoter region synthesizing RNA type 1 in the *B. thuringiensis* (27). The translation initiation codon ATG is underlined at the +1 bp point in the +73 nucleotide sequence.

The 3.8 kb *Nde*I fragment (Fig. 3, lane C) consisted of a -80 bp promoter of the ICP, the ICP structure gene with the open reading frame of 3,531 bp, the termination codon TAG, and 245 bp of the termination region. The latter three are shown in the data of Schnepf *et al.*, (25).

Evidence for overexpression in *E. coli*

The two *E. coli* clones bearing pHLN1-80 and pHLN2-80 were cultured, and the production of the 130 kDa protein in the cloned cells by the clones was examined using SDS-PAGE analysis (Fig. 4).

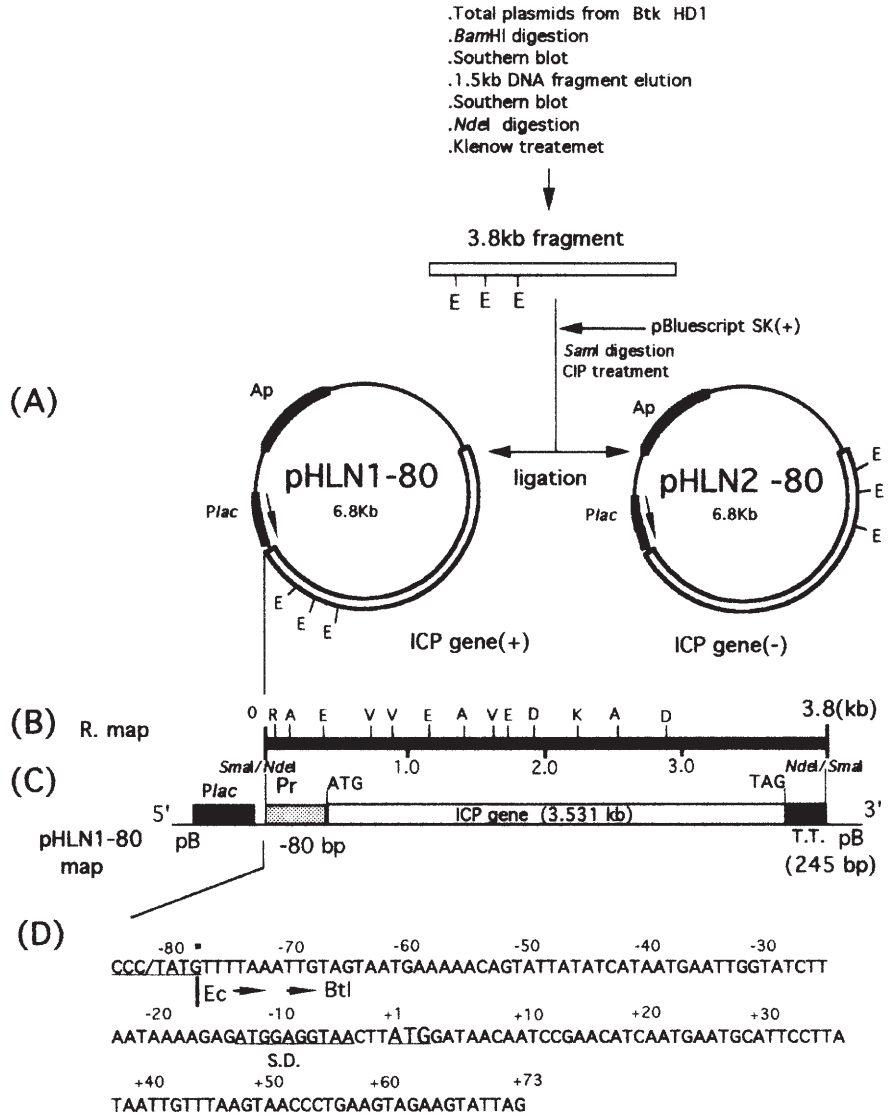

Fig. 3. Schematic diagram for construction of the pHLN1-80 and pHLN2-80 plasmids bearing the ICP gene from *B. thuringiensis* (A). (B), a physical map of the 3.8 kb fragment. (C), gene map and (D), sequence of the 3.8 kb in the pHLN1-80. Abbreviations: R(*Dra*I), E(*Eco*RI), V(*Eco*RV), D(*Hind*III), A(*Cla*I), K(*Kpn*I), S(*Sma*I), and N(*Nde*I). *Plac*, the *lacZ* gene promoter of pBluescript SK(+); -80 pr, -80 bp promoter; Ec and BtI, transcription initiation sites(Wong *et al.*, 1983); ATG, translation initiation codon; TAG, translation termination codon; TT, termination region (Wong *et al.*, 1983); and pB, pBluescript SK(+).

Approximately 130 kDa bands were apparent in lanes B, C, and D on the gel (Fig. 4). Lane B (solubilized crystal band) is a positive control, lane C, the protein band produced by the pHLN1-80, lane D, the band produced by the pHLN2-80, and lane E, the negative control. The original cell lysates of the two clones had the same number of cells (5.2×10^8 cells/ml). Also, the loading volumes of the cloned lysates were the same. Lanes C and D showed different amounts of the 130 kDa band density in the gel. The band from the pHLN2-80 lysate was much dense than that of the pHLN1-80. The pHLN 2-80 lysate was diluted 0, 3, 9, 27, 81, and 243 times, and the pHLN1-80 lysate was diluted 0 and 3 times. The diluted lysates were run on the gel and then confirmed by Western blot (Fig. 5A, lanes 1-8) : Lane 1, the original concentration of pHLN2-80 lysate; lanes 2 to 6, the diluted concentrations of lane 1; lane 7, the original concentration of the pHLN1-80 lysate; lane 8, 3 times diluted solution of the pHLN1-80 lysate. The density of 130 kDa band in lane 8 is similar to the band in lane 5. This indicates that the loaded concentration of lane 5 contained 81 times of that of lane 8. Consequently, the original concentration of pHLN2-80 lysate contained 27 times more ICP than that of the original concentration of pHLN1-80 lysate.

The Western blot analysis (Fig. 5 lane B) of the

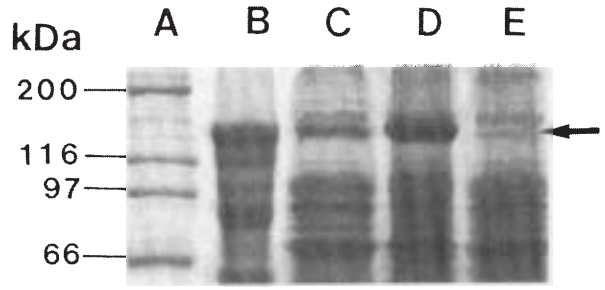

Fig. 4. SDS-PAGE analysis of the total protein from the lysates of clones pHLN1-80 and pHLN2-80. Lane A, molecular weight markers; lane B, solubilized crystal protein; lanes C, D, E: lysates of *E. coli* with pHLN1-80, pHLN2-80, and pBluescript SK(+), respectively. Arrow indicates the 130 kDa protein band.

gel showed results similar to that of the gel analysis (Fig. 5, lane B). A double-diffusion precipitation assay (Ouchterlony) (Fig. 6) was performed using antisera raised against electrophoretically purified and solubilized ICP. This analysis confirmed that an antigenically identical protein was expressed by the clones. The double-diffusion precipitation assay showed that single precipitation bands are without spurs and that the amounts of the protein could be figured out from the densities of the precipitations (Fig. 6). This result was consistent with the results of the Western blot assay and the SDS-PAGE (Fig. 5).

Bioassay evidence on silk worm for overexpression in *E. coli*

Bioassays with *Bombyx mori* larvae were used to demonstrate expression of the ICP gene in the pHLN1-80 clone and the pHLN2-80 clone (Fig. 7). Percent mortalities of the two lysates at equivalent or diluted amounts of cells per treatment are

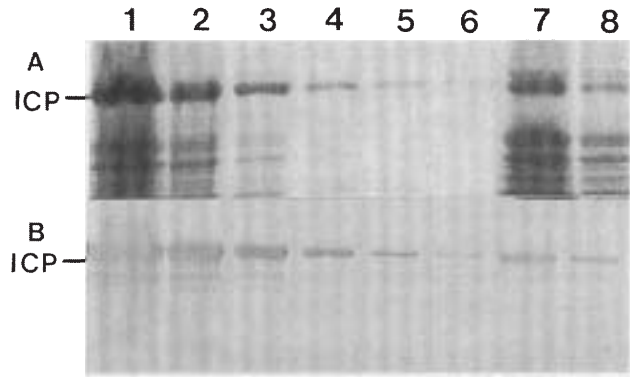

Fig. 5. Comparison of the ICP production in clones pHLN1-80 and pHLN2-80 by SDS-PAGE (A) and Western blot (B) analyses. Lane 1, original lysate of *E. coli* with pHLN2-80; lanes 2-6, samples of 3, 9, 27, 81, and 243 times diluted lysates of the pHLN2-80; lane 7, original lysate of pHLN1-80; lane 8, the 3 times diluted lysate of the pHLN1-80.

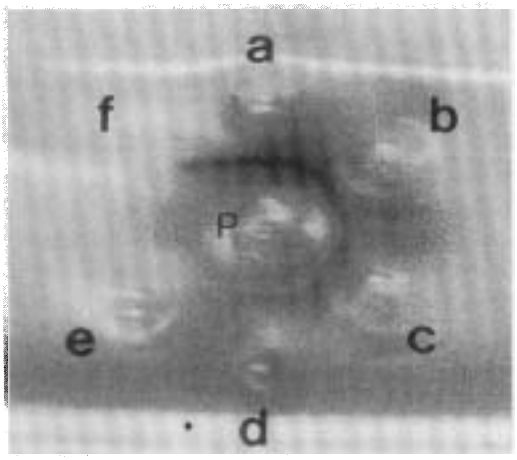

Fig. 6. Immunodiffusion analysis for ICP antigen and antiserum cross-reactions. Well a, concentration of the initial antisera from rabbit, wells b-f; 3, 9, 27, 81, and 243 times diluted antisera, and well P, solubilized crystal antigen (150 μg/15 μl in the well).

shown in Fig. 7. Different mortality curves were observed for each treatment. Neither the cloning host, *E. coli* XL1-blue with pBluescript SK(+) nor the distilled water had any apparent effect. Crude lysates of the above two clones were compared for toxicity to *B. mori* larvae. These lysate samples were spread on the mulberry leaf diet and fed to the third-instar larvae. Then all toxicity symptoms of the larvae were consistently observed. The original lysates contained 5.2×10^8 cells per ml, and 0.1 ml of the lysates was applied to the leaf diets. Comparative insecticidal effects of the two lysates are il-

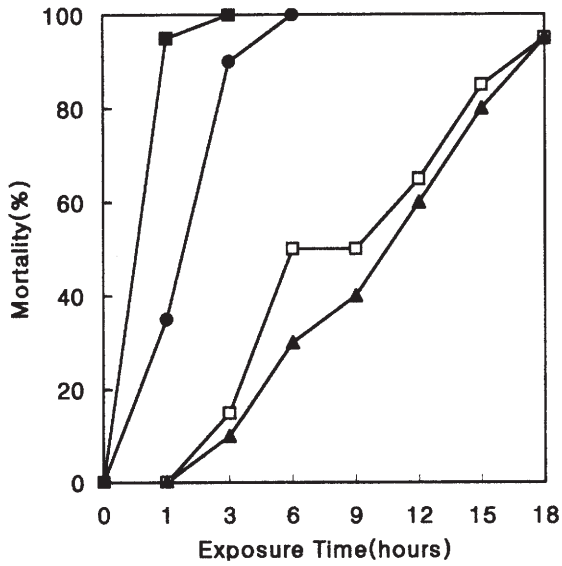

Fig. 7. Comparative insecticidal activity of the pHLN1-80 and pHLN2-80 lysates. Symbols: (-□-Al), lysate of pHLN1-80; (-■-B1), lysate of pHLN2-80; (-●-B10) and (-▲-B100), 10 and 100 times diluted lysates of the B1, respectively.

lustrated in Fig. 7. The pHLN2-80 lysate killed 95% in 1 h and 100% in 3 h. Its one-tenth dilution killed 35% in 1 h, 95% in 3 h, and 100% in 3 h. Its one-hundredth dilution killed 50% in 9 h and about 100% in 18 h. However, the pHLN1-80 lysate killed 50% in 9 h and 100% in 18 h. These results demonstrated that the pHLN2-80 lysate has 100 fold greater toxicity and 100 times greater concentration of the ICP than the lysate of the clone, pHLN1-80.

Discussion

The insecticidal protein (ICP) gene of *B. thuringiensis* provides a typical example of sporulation genes specifically expressed in the mother cell cytoplasm. The ICP gene is transcribed in the bacteria from two overlapping promoters (BtI and BtII) which are sequentially used (27). BtI is active at the beginning of sporulation (27). The ICP was encoded by a variety of genes located on various plasmids in *B. thuringiensis* and consisted of a polypeptide(4, 7, 24). Several publications have reported cloning the of ICP genes from *B. thuringiensis* var. *kurstaki* HD-1 (7, 8, 10, 17, 20, 25). These publications support other works demonstrating that ICP genes are borne on plasmids (7, 9). For cloning of the ICP gene, they used about 5~10 kb sized DNA fragments, various restriction enzymes, and pBR322 vector.

The *B. thuringiensis* subsp. *kurstaki* HD-1 contains about 10 plasmid elements (15). The ICP gene was detected on 15 kb *Bam*HI (Fig. 1) and *Nde*I 3.8 kb *Nde*I (Fig. 2) fragments of the bacterial plasmids by the detection of hybridization with probe. The 3.8 kb *Nde*I fragment was cloned into the *Sma*I site of pBluescript SK(+) (because the fragment was only single hybridized band) (Fig. 2). It was named pHLN1-80 (correct-oriented) or pHLN2-80 (reverse-oriented) depending on the gene orientation to the *lacZ* gene promoter (Fig. 3A). The orientation was determined using *Eco*RI digestion shown in the physical map (Fig. 3A).

In the 153 bp nucleotide sequence at the 5'-end of the *Nde*I fragment in the pHLN1-80 clone, there are -80 bp region (contained BtI promoter region) (27) of the ICP gene promoter and +73 bp coding sequence of part of the ICP gene (Fig. 3C and D). Fig. 3D also shows that there are the *Sma*I and *Nde*I ligated site sequence (CCC/TATG) and the translation initiation codon ATG at the +1 bp point in the +73 nucleotide sequences. In addition, the -80 bp promoter of the ICP gene contained transcription initiation point G at the -77 bp point (24, 27) and the Shine-Dalgarno sequence in the -14 to -4 bp region (27). The -80 bp promoter and the +73 bp sequences were identical to those reported by Schnepf *et al.*, (27). Electrophoretical and immunological evidence support the conclusion that the *E. coli* clones produced ICP (albeit not in crystalline form) with molecular weight of 130 kDa (Fig. 5 and 6). Especially, the pHLN2-80 clone produced significantly higher amount of the ICP in *E. coli* XL1-blue than the pHLN1-80 clone (Fig. 4, 5, 6). The bioassay data revealed that the pHLN2-80 lysate caused a 100 fold mortality of the *B. mori* larvae in 1~3 h in comparison with that of the pHLN1-80 lysate (Fig. 7), which supports the above-mentioned results.

The above data reveal four significant points for discussion. First, the simplest interpretation of this result is that all of the ICP-coding capacity resides on the reverse-oriented ICP gene in the plasmid. The *Plac* and the -80 bp promoter (BtI promoter) sequences may have functional contribution in regulating the production of ICP. Second, an *E. coli* clone carrying the ICP gene of the pHLN2-80 produces a noncrystalline active protein in cytoplasm that is equivalent in larvicidal activity on the basis of total milligrams of protein. Third, the transcriptional region of the gene promoters disturbed by the cloned fragment might cause overexpression of the gene. Fourth, mRNA of the ICP gene has a long half-life, and may result in overexpression.

These marked differences in production and toxicity of the ICP in the different orientation of the gene in the vector provide us guidance regarding further studies on regulation of the ICP production in the inverted plasmid.

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