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Construction of Shuttle Promoter-probe and Expression Vectors for *Escherichia coli* and *Bacillus subtilis*, and Expression of *B. thuringiensis* subsp. *kurstaki* HD-73 Crystal Protein Gene in the Two Species

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A shuttle promoter-probe vector, pEB203, was derived from pBR322, pPL703 and pUB110. Using the vector, a useful DNA fragment, 319 bp *Eco*RI fragment, having strong promoter activity has been cloned from *Bacillus subtilis* chromosomal DNA. Selection was based on chloramphenicol resistance which is dependent upon the introduction of DNA fragments allowing expression of a chloramphenicol acetyl transferase gene. The nucleotide sequence of the 319 bp fragment has been determined and the putative −35 and −10 region, ribosome binding site, and ATG initiation codon were observed. This promoter was named *EB* promoter and the resultant plasmid which can be used as an expression vector was named pEBP313. The crystal protein gene from *B. thuringiensis* subsp. *kurstaki* HD-73 was cloned downstream from the *EB* promoter without its own promoter. When the resultant plasmid, pBT313, was introduced into *Escherichia coli* and *B.subtilis*, efficient synthesis of crystal protein was observed in both cells, and the *cp* gene expression in *B. subtilis* begins early in the vegetative phase. The cell extracts from both clones were toxic to *Hyphantria cunea* larvae.

Bacillus thuringiensis is a typical soil bacterium characterized by its ability to produce proteinaceous crystalline inclusions during sporulation. *B. thuringiensis* subsp. *kurstaki*, one of the most intensively studied subspecies, produces the crystalline protein up to 30% of the dry weight of the sporulated culture, and the protein is known to be toxic to most lepidopteran insect species (12). The genes coding for the crystal protein are reported to be localized on large plasmids in addition to chromosomal DNA (15, 16, 20, 21), and the *cp* genes seemed to be regulated in a manner similar to that of genes specifying other sporulation-specific polypeptides (37). Kronstad *et al.* (23) identified three *cp* genes, which have been designated as 4.5, 6.6, and 5.3 genes (35) or as *cry A1*, *cry A2*, and *cry A3* (6), respectively, in *B. thuringiensis* subsp. *kurstaki* HD-1 based on the *Hind*-III restriction fragments of total or plasmid DNA. The *cp* genes have been cloned from different strains of subsp. *kurstaki* and the DNA sequences of them have been determined (1, 14, 20, 22, 31, 32).

Wong *et al.* (37) identified two adjacent start sites for transcription of a crystal protein gene, which is corresponded to the 4.5 gene or *cry A1*, using RNAs synthesized during sporulation of *B. thuringiensis*: transcription was initiated from one site (Bt I) early in sporulation and from the other site (Bt II) in the middle of sporulation. In an *Escherichia coli* strain carrying the recombinant plasmid, however, only a single species of gene-specific RNA was detected regardless of the growth phase of the cells. In *E. coli* the unique transcription start site (Ec) seems to be located in the toxin gene between Bt I and Bt II. The expression level of crystal protein in *E. coli*, however, was very low. By the way, Schnepf *et al.* (36) obtained an *E. coli* mutant in which

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Tn*5* had inserted 145 bp upstream from Ec and this mutant produced substantial amounts of crystal protein that accumulate in bipyramidal crystals resembling those found in *B. thuringiensis*. They investigated that the expression of a *B. thuringiensis* crystal protein gene in *E. coli* through the use of fusions of the crystal protein gene promoter to β-galactosidase and catechol oxidase genes, and they described that a region, which was referred to as a transcription-suppressing region, of *B. thuringiensis* DNA located betwen 87 and 258 base pairs upstream from the *E. coli* transcriptional start site, Ec, caused reduced transcription from this promoter (33). On the other hand, Shivakumar *et al.* (34) reported that they cloned two crystal protein genes, 6.6 and 5.3 genes (*or cry A2* and *cry A3*), from subsp. *kurstaki* HD-1 and the genes were expressed efficiently in *E. coli* and in *B. subtilis* to form crystal-like inclusions and well-defined bipyramidal crystals, respectively. The amount of crystal protein produced by the 6.6 gene corresponds to about 10% in *E. coli* and 5% in *B. subtilis* of the total cellular proteins. They reported that the toxin genes were transcribed from its own promoter and independent of sporulation. It is interesting that the 6.6 gene subcloned in their study for expression in *E. coli* has only a 158 bp 5′-flanking region from the *E. coli* transcription initiation site, Ec, and this fact seemed to coincide with the results of Schnepf *et al.* (33).

We report here the construction of a recombinant plasmid, pBT313, which can be replicated and produce *B. thuringiensis* crystal protein both in *E. coli* and in *B. subtilis* to a high level. First, a shuttle promoter-probe vector, pEB203, was derived from plasmids pBR322, pPL703 and pUB110, and then a strong promoter, named *EB* promoter, which shows high Cm resistances in the two hosts has been isolated from chromosomal DNA of *B. subtilis* using the vector. By ligation of the *EB* promoter to the 6.6 (or *cry A2*) gene cloned from subsp. *kurstaki* HD-73 without its own promoter, the pBT313 has been constructed. The amount of crystal protein produced by *E. coli* and *B. subtilis* containing the pBT313 was 14% and 13%, respectively, of the total cellular proteins, and the expression began early in the vegetative phase in *B. subtilis*. In this study all of the recombinant plasmids were constructed using *E. coli* as a primary host and then transferred into *B. subtilis* because the generation of recombinant plasmids by direct transformation of ligation mixtures into *B. subtilis* is very inefficient (18).

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Media

Bacterial strains and media used are listed in Table 1. *E. coli* JM83 was used as a host for plasmid construction, promoter cloning and *cp* gene expression. *B. subtilis* strains were used as hosts for promoter screening and *cp* gene expression. The *B. subtilis* plasmid pPL703 obtained from Korean Collection for Type Cultures (KCTC) is promoter cloning vector containing *cat*-86 structural gene cloned from *B. pumilus* NCIB 8600. *E. coli* and *B. subtilis* strains were grown in LB (10 g tryptone, 5 g yeast extract and 10 g NaCl per liter) medium. For solid medium 1.25% agar was added. DMP agar medium was prepared as described by Hardy (19) and used for regeneration of *B. subtilis* protoplast cells. Ampicillin (Ap) and Kanamycin (Km) were added, when necessary, to a final concentration of 50 μg/ml and 10 μg/ml, respectively. For DMP agar medium Km was added to a concentration of 400 μg/ml. Chloramphenicol (Cm) was used at the specifically described concentrations.

Table 1. Relevant properties of bacterial strains and plasmids

Bacterial strains and plasmids	Relevant properties	References
E. coli		
JM83	*ara*, Δ(*lac-pro*), *strA*, *φ80*, *lacZ*ΔM15	7
B. subtilis		
BD170	*thr*-5, *trp*C2	11
BD224	*rec*E4, *thr*-5, *trp*C2	11
RM125	*sp*10(s), *arg*(GH)15, *hsr*M, *Leu*A8, *m*(-)168	38
Plasmids		
pBR322	Ap^r, Tc^r	5
pUB110	Km^r	17
pPL703	Km^r, *cat*-86	2
pMK74	Ap^r, *cp*	28
pEB203	Ap^r, Km^r, Cm^s (*cat*-86)	This study
pEBP313	Ap^r, Km^r, Cm^r (*EB* promoter+cat-86)	This study
pBT203	Ap^r, Km^r, *cp*	This study
pBT313	Ap^r, Km^r, *EB* promoter+*cp*	This study

Transformation

Transformation of *E. coli* was performed as described by Mandel and Higa (26). Transformation of *B. subtilis* protoplast was performed according to the method first reported by Chang and Cohen (8) and described by Hardy (19). Transformations of *B. subtilis* were selected on the DMP plates containing Km (400 μg/ml).

General DNA Manipulation

B. subtilis chromosomal DNA was prepared by the method of Doi (10). Small-scale isolation of plasmid from *E. coli* and *B. subtilis* was performed by the alkaline

lysis method as described by Birnboim and Doly (3), except that the lysozyme treatment was at 37℃ for 20 min for *B. subtilis*. Restriction enzyme digestions, dephosphorylation reactions with calf intestinal alkaline phosphatase (CIP), fill-in of 3'-recessed ends with Klenow fragment, and ligations with T4 DNA ligase were carried out as specified by the suppliers. DNAs were analyzed by electrophoresis on 0.8-1.0% agarose gels. Isolation of DNA fragments from agarose gels was performed using dialysis membrane bags as described by Maniatis *et al.* (27).

Preparation of Cell Extracts for Protein Analysis

E. coli was grown in LB medium containing Ap (50 μg/ml) at 37℃ with shaking. *B. subtilis* was grown in LB medium containing Km (10 μg/ml) at 30℃ with vigorous shaking. Cells were harvested at 10,000×g, washed once in saline solution and resuspended in adequate volume of TE buffer (10 mM Tris-HCl [pH 8.3] and 1 mM EDTA) to make final OD 600= 25. The cells were disrupted by sonication, and the cell extract was mixed with an equal volume of 2x sample buffer (0.1 M Tris-HCl [pH 6.8], 0.2 M dithiothreitol, 4% SDS, 0.2% bromophenol bule and 20% glycerol), boiled for 3 min and cooled. The mixture was analyzed for proteins by SDS-polyacrylamide gel electrophoresis. Cell extracts were also used for insect bioassay.

Detection of Crystal Protein

Electrophoresis on 7.5% polyacrylamide gel was carried out as described by Laemmli (24). Protein bands were stained with 0.1% Coomassie Brilliant Blue R250 (Sigma) in 50% methanol 10% acetic acid. For immunological characterization the proteins were electrophoretically blotted onto a transfer membrane (Immobilon™P, Nihon Millipore Ltd., Tokyo, Japan) and immunostained with the antibody, which was raised against crystal protein in the rabbit, and goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma). The bands were visualized in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$ containing 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as colored substrate (4, 25). The enzymatic reaction was ended after 10 to 30 min with 20 mM Tris-HCl (pH 8.0) and 5 mM EDTA.

Nucleotide Sequencing

Sequencing was performed with double-stranded plasmid DNA using the dideoxy-chain termination method as described by Chen and Seeburg (9), and using 17 bp primers.

Insect Bioassay

To test the toxicity of recombinant *E. coli* and *B. subtilis* strains cell extracts were prepared as described above. The cell extracts was added on an artificial diet, developed in our laboratory, with the ratio of 0.5 ml/4 g of diet. Into each petri dish having the diets ten of third-instar *Hyphantria cunea* larvae were put, and incubated at room temperature for a week. Controls for each test included parental bacterial strain and untreated diet.

Chemicals and Reagents

Restriction enzymes, calf intestinal phosphatase (CIP), T4 DNA ligase and Klenow fragment of *E. coli* DNA polymerase I were purchased from either Bethesda Research Laboratories (Gaithersburg, MD, USA) or Promega Biotec (Madison, WI, USA) and used under conditions described from Amersham International (Amersham, Buckinghamshire, UK). A DNA sequencing kit for sequencing in pUC18 plasmid was purchased Boehringer Mannheim (Mannheim, West Germany), Ampicillin, chloramphenicol and kanamycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

RESULTS AND DISCUSSION

Construction of the Shuttle, Promoter-probe Vector pEB203

For the construction of a shuttle and promoter-probe vector which can be used in both *E. coli* and *B. subtilis*, 2.3 kb *Eco*RI-*Pvu*II fragment of pBR322, 1.5 kb *Eco*RI-*Pvu*II fragment of pPL703 and 2.5 kb *Acc*I-*Nci*I fragment of pUB110 were fused. The 1.5 kb fragment of pPL703 contains *cat*-86 gene cloned from *B. pumilis* NCIB 8600 chromosomal DNA, but lacks promoter sequences that function during the growth-sporulation cycle of *B. subtilis* (13, 30). The strategy for the construction is outlined in Fig. 1. First, the 2.3 kb fragment of pBR322 was ligated with the 1.5 kb fragment of pPL703, and the ligation mixture was used to transform *E. coli* JM83 to Ap^r. From one of the Ap^r transformants a recombinant plasmid, named pRL703, was isolated. Secondly, the 2.5 kb *Acc*I-*Nci*I fragment of pUB110 was filled in using Klenow fragment to make blunt ends, then inserted into *Pvu*II site of the pRL703. The resultant plasmid was named pEB203 (6.3 kb) and the restriction map was shown in Fig. 1. This construction step was also done using JM83 as a host. Vector pEB203 carries Ap^r and Km^r genes, *cat*-86 gene without promoter and the replication origins from pBR322 and pUB110. The pEB203 was able to transform *E. coli* to Ap^r (50 μg/ml) and Km^r (10 μg/ml) and *B. subtilis* to Km^r (10 μg/ml). The pEB203 contains the unique recognition sites for *Eco*RI, *Bam*HI and *Sal*I upstream the *cat*-86 structural gene, and seems to be useful for cloning of DNA fragments having promoter activity in *E. coli* and/or *B. subtilis* using Cm resistance.

Cloning and Analysis of a Promoter from *B. subtilis* Chromosomal DNA

The promoter-probe vector pEB203 was shown to

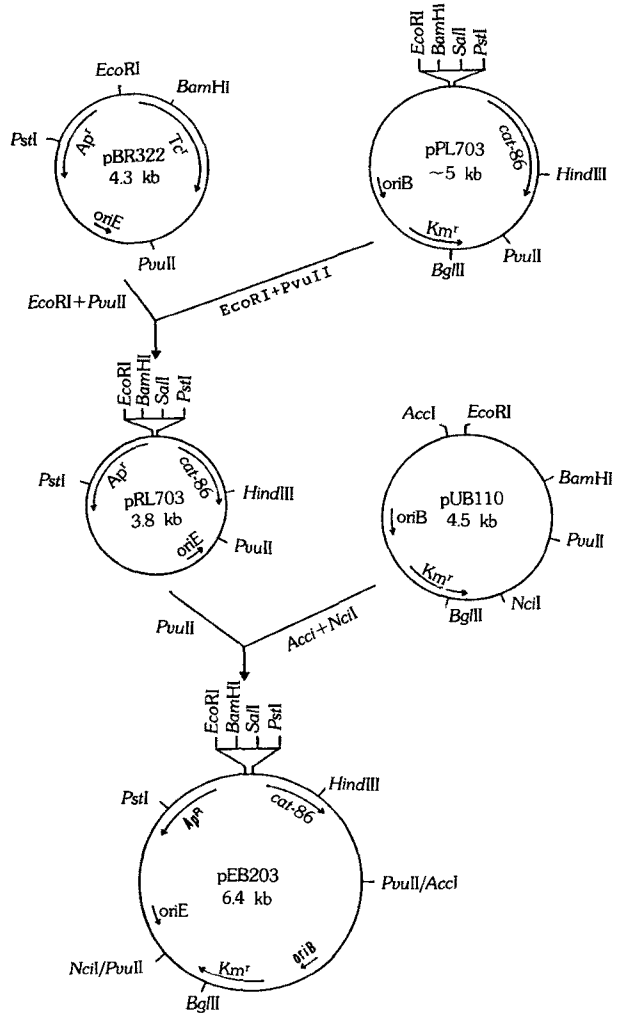

Fig. 1. Construction scheme of the *E. coli-B. subtilis* shuttle, promoter-probe vector, pEB203.
The *cat*-86 represents the chloramphenicol acetyl transferase gene cloned from *B. pumilus* without promoter. Abbreviations: oriE, origin of replication in *E. coli*; oriB, origin of replication in *B. subtilis*; Ap, ampicilin; Km, kanamycin.

express no Cm resistance in both *E. coli* and *B. subtilis*. Chromosomal DNA (5 μg) from *B. subtilis* was digested with *Eco*RI, and ligated to *Eco*RI-digested and CIP-treated pEB203 (1μg). *E. coli* JM83 was transformed with the ligation mixture. About 400 transformants were selected on LB plates containing Cm (20 μg/ml), and then toothpicked onto LB plates containing Cm (200~400 μg/ml). Fifty transformants showing growth on the LB Cm (400 μg/ml) plates were inoculated into LB broth medium containing Cm (300~700 μg/ml), and incubated for 14~23 hr at 37℃ with shaking. Through this step 20 transformants showing vigorous growth on LB Cm (300~500 μg/ml) medium and poor growth on LB Cm (600~700 μg/ml) medium were selected. Plasmids were isolated from them and analyzed by agarose gel electrophoresis after digestion with *Eco*RI. Eight plasmids showing high Cm resistances and carrying small inserted fragments (0.3~1.5 kb) were selected, and was used to transform *B. subtilis* RM125. *B. subtilis* transformants carrying each one of the plasmids were screened on DMP plates containing Km (400 μg/ml), and were grown in LB broth medium containing Cm (100~300 μg/ml), respectively. Five transformants showed vigorous growth in LB Cm (100 μg/ml) medium and one of them showed vigorous growth upto Cm 200 μg/ml and poor growth at Cm 300 μg/ml. This transformant was selected finally, and the plasmid isolated from this transformant was named pEBP313. The pEBP313 contained about 0.3 kb *Eco*RI fragment having promoter activity, and nucleotide sequence of the insert (named *EB* promoter) was determined using Sanger's dideoxy chain termination method after subcloning into pUC18. As shown in Fig. 2, the *EB* promoter fragment is 319 bp long between the two *Eco*RI sites. There are putative −35 (TTGAAA) and −10 (TACAAT) regions in the sequence. The spacer between the two regions is 17 bp. Also, putative ribosome binding site and ATG initiation codon were shown in the 3′ distal region, and downstream

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                  20                  40                  60                  80
GAATTCGGCGAAATCGTGGGTGTCTCGATGATTGGCCCGGATGTAACCGAGCTCATCGGGCCAAGCGGCACGGATCATGA

                 100                 120                 140                 160
ATGGTGAGATGACGGCAGATATGGCGGAGCATTTTATCGCCGGCCCATCCGACTTTATCGGAAACATTGCATGAGGCGCT

                 180                 200                 220                 240
GTTAAGCACGATCGGCCTTGCGGTACATGCATAATAAAGGAAAAAGCAGGCGCATGGATATAAGGGCGCCTGCTTTTTTT

                 260                 280                 300          *** 319
ATTGTTGAAAGCGCTTTATTTTTCCCCTACAATAGATGAAAACGGCGTGTAAGGGAGGAGCGATCAAGGAAATGAATTC
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Fig. 2. Nucleotide sequence of the *EB* promoter.
The last digit of each number is aligned with the numbered nucleotide. The putative −35 and −10 regions are underlined, and the Shine-Dalgarno sequence is labeled with a double line. The putative ATG initiation codon is indicated by asterisks.

of the ATG codon was flanked directly by MCS. Therefore, the plasmid pEBP313 seems to be used efficiently as an expression vector or general cloning vector in both *E. coli* and *B. subtilis*.

Subcloning of Crystal Protein Gene into the Expression Vector pEBP313

The *B. thuringiensis* subsp. *kurstaki* HD73 crystal protein gene contained in a 3.7 kb *Bam*HI fragment was isolated from the recombinant plasmid pMK74 (29) after *Bam*HI digestion. The *cp* gene was cloned originally from a 75 kb plasmid of the HD73 using *Bgl*II, and

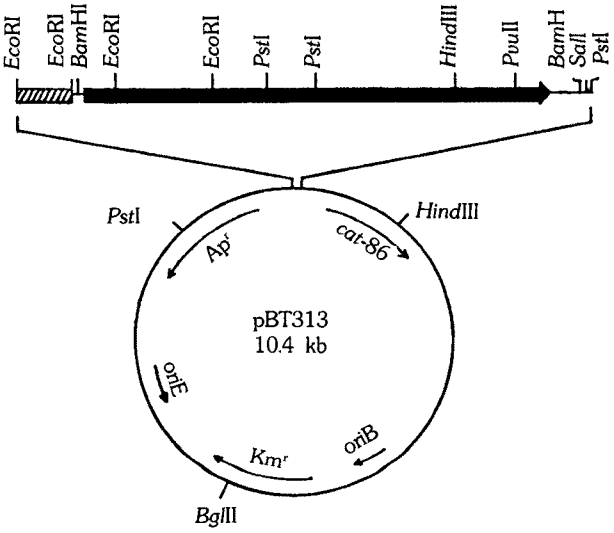

Fig. 3. Restriction map of pBT313.
The hatched line represents the *B. subtilis* chromosomal DNA fragment having promoter activity. The large arrow represents the *cp* gene from subsp. *kurstaki* HD-13.

was subcloned into a 3.7 kb fragment using *Nde*I (28). However, in this process the most part of the promoter region (to just before the *E. coli* transcriptional start site, Ec) was deleted, and the pMK74 carrying the *cp* gene showed very poor synthesis of crystal protein in *E. coli* (unpublished data). The 3.7 kb *Bam*HI fragment was inserted into the same site of pEBP313 and pEB203 in the same orientation as the *cat*-86 gene (Fig. 3). The resultant plasmids were designated as pBT313 and pBT 203, respectively. In the pBT313 the *cp* gene is located on downstream from the 0.3 kb *Eco*RI fragment having promoter activity.

Expression of the Crystal Protein Gene in *E. coli* and *B. subtilis*

The recombinant plasmids constructed using *E. coli* as a host were isolated and introduced into *B. subtilis* strains by the protoplast transformation method. *E. coli* and *B. subtilis* strains containing pBT313 or pBT203 were grown overnight in LB medium containing appropriate antibiotic at 37℃ and 30℃, respectively. The protein extracts were prepared from the cells as described in Materials and Methods, and then analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4A the *cp* gene cloned in pBT313 was expressed to a high level in both *E. coli* and *B. subtilis*, so that the thick protein bands were observed in the region of 135 kDa, but the *cp* gene of pBT203 which has no *EB* promoter shows very low expression. A western blot of a similar gel is shown in Fig. 4B. The amount of crystal protein produced by pBT313 corresponds to about 14% in *E. coli* and 13% in *B. subtilis* of the total cellular proteins. This expression level in *B. subtilis* thought to be very high in comparison with the results of Shivakumar *et*

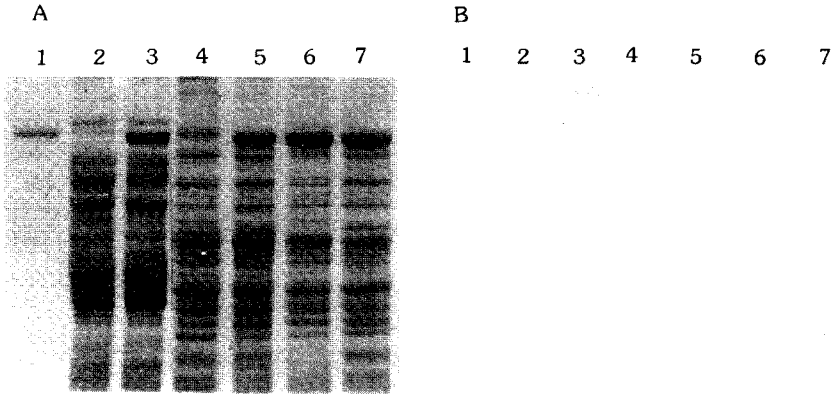

Fig. 4. Expression of HD-73 *cp* gene in *E. coli* and *B. subtilis* strains.
SDS-polyacrylamide gel electrophoresis pattern of total cell extracts of *E. coli* and *B. subtilis* transformants (A) and corresponding protein immunoblot (B) are shown. The cell extracts were prepared from overnight grown cultures, and analyzed using 0.1% SDS-7.5% PAGE. Lanes: 1, purified crystal protein from HD-73; 2, *E. coli* JM83(pBT203); 3, *E. coli* JM83 (pBT313); 4, *B. subtilis* BD170 (pBT203); 5, *B. subtilis* BD170 (pBT313); 6, *B. subtilis* BD224 (pBT313); 7, *B. subtilis* RM125 (pBT313).

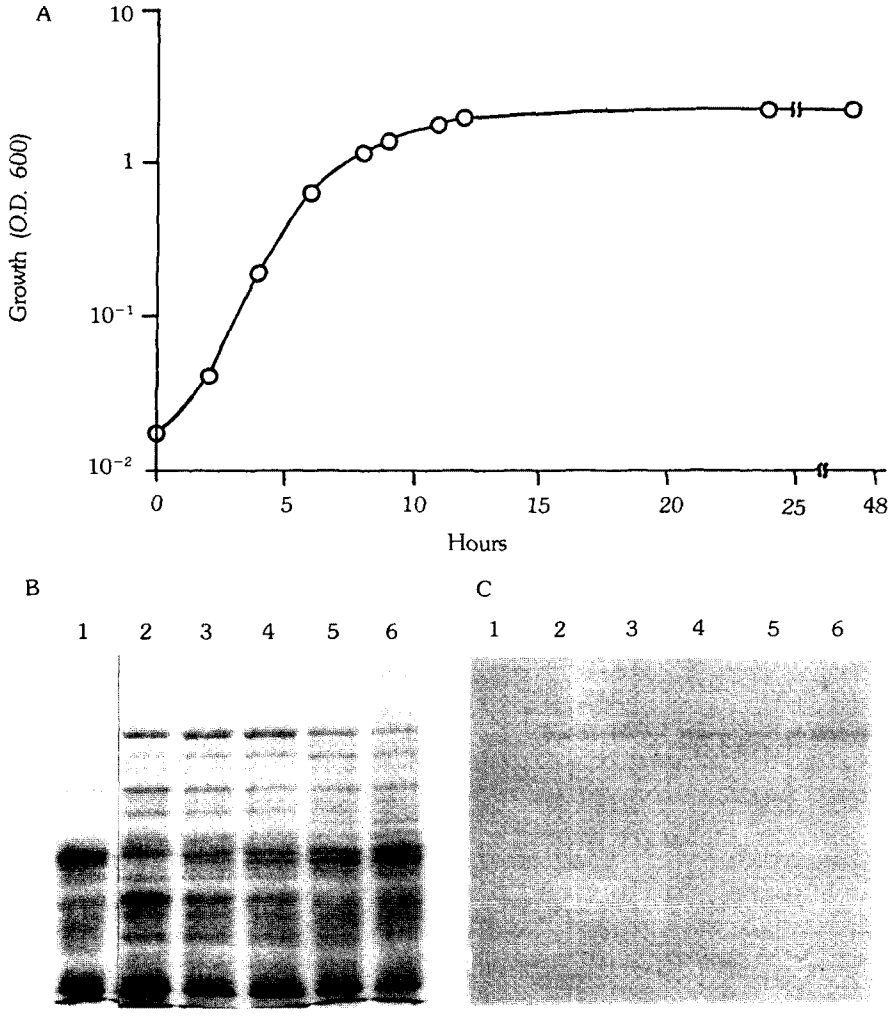

Fig. 5. Expression of *cp* gene in *B. subtilis* RM125 having pBT313 at different periods of growth.
Growth curve (A), SDS-polyacrylamide gel electrophoresis pattern of total cell extracts (B) and corresponding protein immunoblot (C) are shown. Lane 1, *B. subtilis* RM125 (pEB203) harvested after overnight growth; Lanes 2 to 6, *B. subtilis* RM125 (pBT313) harvested at 6 hr, 9 hr, 12 hr, 24 hr, and 48 hr, respectively, after inoculation.

al. (34). They described that the expression level of the 6.6 gene with its own promoter in *B. subtilis* was about 5% of the total cellular proteins. Another recombinant plasmid which has the *EB* promoter and *cp* gene but in opposite orientation to that of the pBT313 was also constructed, and the expression of the *cp* gene in *E. coli* and *B. subtilis* was observed. The expression level in them, however, was very low (data not shown). From these results we confirmed that the *EB* promoter worked efficiently for expression of the *cp* gene in both hosts. We also observed the *cp* gene expression in *B. subtilis* at different growth times. *B. subtilis* RM125 containing pBT313, which was storaged at −70℃ with 15% glycerol, was inoculated in LB medium containing Km (10 μg/ml). After overnight incubation at 30℃ the culture was diluted 20-fold into fresh medium and incubated 7 h, and then inoculated for the main culture. Culture broth were removed at different growth times and processed for protein analysis as described in Materials and Methods. As shown in Fig. 5 *cp* gene expression in *B. subtilis* by the *EB* promoter was evident at all stages, and the amount of crystal protein appeared to be reached at a high level at 6 h, already. The *EB* promoter, therefore, thought to be a promoter which exerts on vegetative growth phase.

Bioassays of Cloned Extracts

The lysates of recombinant cells of *E. coli* and *B. subtilis* RM125 carrying pBT313 showed more than 90%

mortality within five days against larvae of *H. cunea*, but that of the host cells containing pEBP313 did not show any toxicity.

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