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A Broad-Host-Range Promoter-Probe Vector, pKU20, and Its Use in Promoter Cloning and Expression of *Bacillus thuringiensis* Crystal Protein Gene in *Pseudomonas putida*

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We have constructed a promoter-probe vector pKU20 using pKT230, a derivative of broad-host-range plsmid RSF1010, as a base. The pKU20 contains structural gene for aminoglycoside phosphotransferase (*aph*), without promoter, and a multiple cloning site upstream the *aph*. Using this vector,a 412base pairs (bp) *Pst*I fragment showing strong promoter activity both in *Escherichia coli* LE392 and *Pseudomonas putida* KCTC1644 has been cloned from *Pseudomonas fluorescens* chromosomal DNA on the basis of streptomycin resistance. The nucleotide sequence of the 412 bp fragment has been determined and the putative −35 and −10 region was observed. Insecticidal protein gene of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 inserted on downstream of the promoterlike DNA fragment was efficiently expressed in *E. coli* and *P. putida*. The toxin protein was efficiently synthesized in an insoluble form in both strains.

In recent years much attention has been paid to bacterial insecticides as alternatives to chemical pesticides for controlling insect pests. The major advantage of the bacterial insecticides resides in the elimination of hazards arising from persistance of the chemical pesticides in the environment. Among the insect-pathogenic bacteria, the sporeforming bacilli, especially *Bacillus thuringiensis* (*B.t.*) has the highest potential for use in the control of insect pests. Numerous isolates of *B.t.* strains belonging to over two dozen distinct flagella serotypes have been isolated and classified to date (5). Among them *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*), which produces a δ-endotoxin toxic to the most lepidopteran insects is one of the most intensively studied and commericially used subspecies. Formulations of the *B.t.k.* have been used for more than 20 years as biological insecticides to control lepidopteran insect pests. The *B.t.k.* commercial preparations, however, can be applied only to the exposed part of plants on the ground and repeated applications are necessary (10). In terms of application, therefore, it will be interesting to obtain a recombinant microorganism that can produce the toxin constitutively and is so closely related with plant that its habitat is in the feeding zone of the target insects. This organism would be used efficiently for controlling insect pests without repeated applications. From this point of view, the *Pseudomonas* species are thought to be one of the promising hosts for the development of a recombinant microbial pesticide producing the *B.t.* toxin.

Many of the crystal protein (*cry*) genes encoding δ-endotoxin have been cloned from different strains of *B.t.k.* such as HD-1 (19), HD-1 Dipel (8), HD-73 (1) and HD-224 (15) in *E. coli*, and the DNA sequences of the these genes have been characterized (for a recent review, see reference 9). Recently, introduction of the cloned toxin genes into other microbial hosts such as *B. subtilis* and *Pseudomonas fluorescens* has been reported (20, 17, 18), but in the *Pseudomonas* species, many of the available vectors have a low copy numbers and useful restriction sites were limited.

We report here on the construction of a broad-host-range promoter-probe vector from plasmid pKT230 to improve the utility as a cloning vector and, in addition,

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its application for promoter cloning and efficient synthesis of toxin protein in *P. putida*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Plasmids were constructed and characterized in the *Escherichia coli* LE392 (F^-, hsdR514(r_k^-, m_k^-), *sup*E44, *sup*F58, *lac*Y1 or Δ(lacIZY)6, *gal*K2, *gal*T22, *met*B1, *trp*R55, λ^-). *Pseudomonas putida* KCTC1644 and *P. fluorescens* KCTC 1767 were used in this study. LB medium was used for liquid culture and, with 1.5% agar, for solid medium. Antibiotics were used at the following concentrations: ampicillin (Ap), 50 μg/ml; kanamycin (Km), 25 μg/ml; streptomycin (Sm), 50 μg/ml for *E. coli* and Km and Sm, 100 μg/ml for *Pseudomonas* if not described otherwise.

DNA Manipulation

Plasmid DNA was isolated using the alkaline lysis procedure of Birnboim and Doly (4). Restriction enzymes, purchased from Promegra or Boerhinger Manheim were used as recommended by the suppliers. Protruding ends were filled in by using T4 DNA polymerase I, essentially as described by Maniatis *et al.* (14). *E. coli* strain was transformed by a calcium chloride method (13). Plasmid tranfers from *E. coli* to *P. putida* were performed by triparental filter matings (7) using *E. coli* HB101 carrying the pRK2013 as a source of helper plasmid. The transconjugants were selected on Pseudomonas isolation agar (Difco Laboratories) containing 100 μg/ml of Km.

Preparation of Cell Extracts for Protein Analysis

Cells grown in LB medium in the presence of an appropriate antibiotics were harvested, washed once in saline solution and resuspended in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA. The cells were disrupted by sonication, mixed with an equal volume of 2X sample buffer (0.1 M Tris-Cl (pH 6.8), 0.2 M dithiothreitol, 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue and 20% glycerol), and boiled for 3 min. The mixture was analyzed by SDS polyacrylamide gel electrophoresis.

Detection of Insecticidal Protein

Electrophoresis on 9% polyacrylamide gel was carried out as described by Laemmli (11). Protein bands were stained by 0.1% Coomassie Brilliant Blue R250 (Sigma) in 50% methanol-10% acetic acid. For immunological characterization the proteins were electrophoretically blotted onto a nitrocellulose filter and immunostained using antibody raised against crystal protein in rabbit and goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma). The bands were visualized in 0.1 M Tris-Cl; (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$ containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as colored substates. The enzymatic reaction was stopped at appropriate time using 20 mM Tris-Cl (pH 8.0), 5 mM EDTA.

RESULTS

Construction of Promoter-Probe Vector pKU 20

To construct a broad-host-range promoter-probe vector, we have chosen to modify plasmid pKT230 which was derived from the high-copy number, broad-host-range plasmid RSF1010 and carrying functional resistance genes for Sm and Km (2). As shown in Fig. 1, first we removed the regulatory seuqence of the *aph* conferring resistance to Sm (3) and introduced a multiple restriction site in that place (Fig. 1, A). In this step the *Eco*RI-*Bam*HI fragment of pUC19 was substituted for the small *Eco*RI-*Bam*HI fragment of pKT230. This substitution generated the plasmid pKU18, which confers Km and weak Sm resistance. Second, to avoid the transcriptional readthrough from the promoter of Km gene, we reversed the orientation of the small *Bam*HI-*Pst*I fragment of the pKU18 by fill-in the ends using T4 DNA polymerase and blunt-end ligation (Fig. 1, B). As a result of this construction pKU19 was obtained. The recombinant promoter-probe vector was further improved by introducing extra restriction enzyme sites from pUC19 (Fig. 1, C, D). The finally constructed plasmid pKU20 confers resistance to Km but very low resistance to Sm on both *E. coli* and *P. putida*; Minimal inhibitory concentration of Sm was 20 μg/ml against *E. coli* LE392 and 100 μg/ml against *P. putida* KCTC1644.

Cloning and Analysis of a Promoter from *P. fluorescens* Chromosomal DNA

Chromosomal DNA from *P. fluorescens* KCTC 1767 was digested with *Pst*I, and ligated to the pKU20 linearized with the same enzyme. *E. coli* LE392 was transformed with the ligation mixture. Several transformants showing high resistance against Sm were selected on LB plates containing Sm (from 50 μg/ml to 400 μg/ml), and plasmids isolated from Sm resistant cell were transferred into *P. putida* KCTC1644. One of the plasmids conferring high Sm resistance on both hosts that is up to 400 μg/ml in *E. coli* LE392 and to 5 mg/ml in *P. putida* KCTC1644 was selected and designated as pKP 120. The pKP120 carries 412 bp *Pst*I fragment and nucleotide sequence of the fragment was determined using Sanger's dideoxy chain termination method after subcloning into pUC18. As shown in Fig. 2, the fragment has putative −35 and −10 region in the sequence. Shine-Dalgano sequence and ATG initial codon could not observed.

Construction of Recombinant Plasmid pKUC20

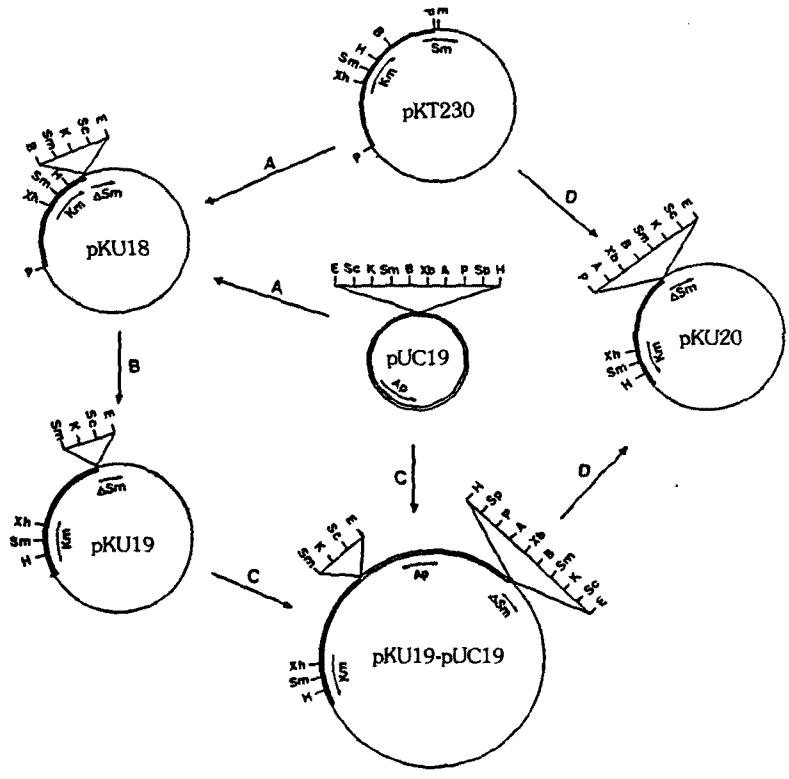

Fig. 1. Construction of the broad-host-range promoter-probe vector pKU20.
The restriction map of pKT230 is derived from Bagdasarian *et al.* (2). The heavy line represents sequences derived from pACYC177. In constructing pKU20, pKU19 and pUC19 plasmids are digested with *Eco*RI and ligated (C). Then this hybrid plasmid is digested with *Pst*I and *Xho*I and ligated with *Pst*I-*Xho*I fragment of pKT230 (D). Abbreviation: E, *Eco*RI; Sc, *Sac*I; K, *Kpn*I; Sm, *Sma*I; B, *Bam*HI; Xb, *Xba*I; A, *Acc*I; P, *Pst*I; Sp, *Sph*I; H, *Hin*dIII; Xh, *Xho*I. ΔSm designates Sm resistance gene deleted promoter.

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1          11         21         31         41         51
CTGCAGAGTTACAGAAGGGCAGACAAAGGTGAAGTCCAGCGGGTAGAAAAACACCAGGCC
61         71         81         91         101        111
GTATTTGCCTTTGATGGCTTCAGACAATTTGAAGCTGTCAACGATTTCGCCATTGCCGAG
121        131        141        151        161        171
GACGGCTGGGACGTCGAAATCCCGGGCTTGTTTACCGACGAGTACGCTCATGGGTTATCT
181        191        201        211        221        231
CCTGATGTAGGGGTGACGATTGAGTAAAGGACCGGCCTCGATCTGCGCACCTAACGACAG
241        251        261        271        281        291
ACCTGTGACGCAGTCACGCTTCTGAAGACCGAACATCATACACTGAAAAAACCGTTCGTC
301        311        321        331        341        351
AGGCGGCGGGGGTTTAGCCTAAAGACCTACGATTCTACTTTGACAATCATTCTGTTAA
361        371        381        391        401        411
CATTAAGATCTGCGAGCGCACAACGCCCTTGTCATCGATCTCTAGAGCTGCAG
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Fig. 2. Nucleotide sequence of the 412 bp *Pst*I fragment having promoter activity.
Putative −35 and −10 regions were underlined.

and pKPC120 Containing the *cryIA(c)* Gene

The *B.t.k.* HP73 crystal protein gene, *cry*IA(c) (8) contained in a 3.7 Kb *Bam*HI fragment was isolated from the recombinant plasmid pMK74 (16) after *Bam*HI digestion and inserted into the same site of pKU20 and pKP120 in the same orientation as the Sm gene (Fig. 3). The resulting plasmids were designated as pKUC20 and pKPC120 respectively. In pKPC120 the *cry*IA(c) is located on downstream the 412 bp *Pst*I insert having strong promoter activity.

Expression of *cryIA(c)* in *E. coli* and *P. putida*

To test the fuctionality of the newly constructed plasmid pKPC120 it was introduced into *E. coli* LE392 and *P. putida* KCTC1644. The LE392 and KCTC1644 tranformants carrying one of the recombinant plasmids were grown for 15 h in LB medium containing appropriate concentration of Km at 37℃ and 30℃, respectively. The protein extracts were prepared from the cells as described in Materials and Methods and were separated by

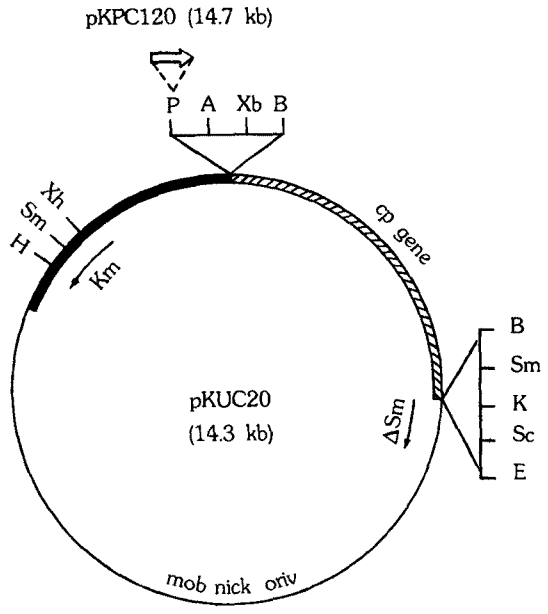

Fig. 3. Restriction map of pKUC20 and pKPC120.
The 3.7 Kb *Bam*HI fragment containing *cry*IA(c) was cloned in the same orientation relative to the Sm gene both in pKU20 and pKP120. Open arrow indicate DNA fragment having promoter activity cloned from *P. fluorescens* chromosomal DNA. Abbreviations are same as Fig. 1.

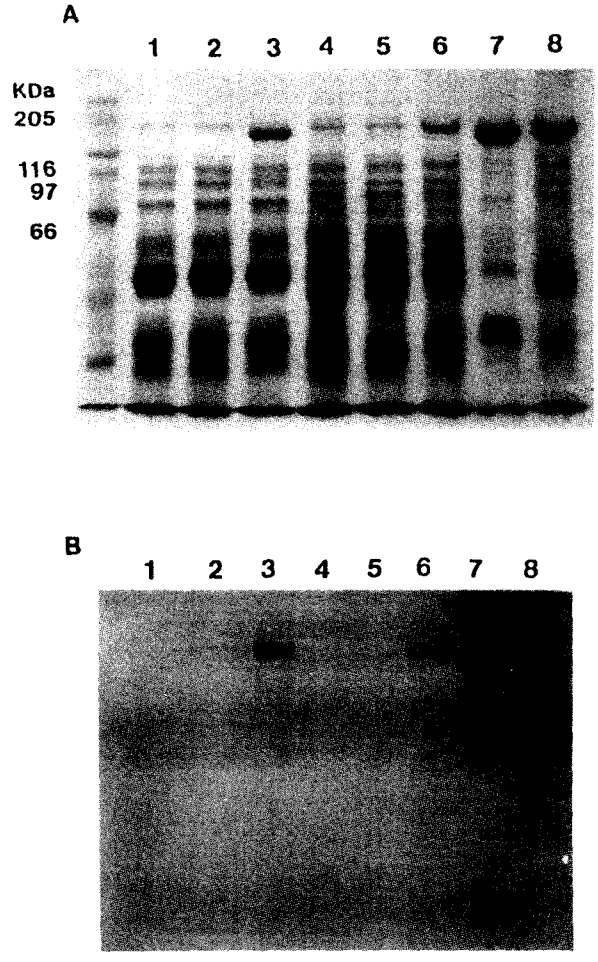

Fig. 4. The crystal protein gene expression in *E. coli* and *P. putida* transformants.
SDS-polyacrylamide gel electrophoerogram (A) and corresponding protein immunoblot (B) are shown. Lanes: 1, LE 392; 2, LE392(pKUC20); 3, LE392(pKPC120); 4, KCTC 1644, 5, KCTC1644(pKUC20); 6, KCTC1644(pKPC120). Lane 7, and 8 show the centrifuged pellet of LE392(pKPC 120) and KCTC1644(pKPC120) after sonication (see Results). MW standards are also shown.

SDS-polyacrylamide gel electrophoresis. As shown in Fig. 4, in LE392(pKPC120), the *cry*IA(c) flanked by the 412 bp promoter fragment was expressed efficiently so that the crystal protein (Cry) of approximate 135 Kdal was produced to a high level (Fig. 4, A). The amount of Cry produced corresponds to about 16% of the total cellular proteins. In KCTC1644 carrying the pKPC120, the *cry*IA (c) was also well expressed and showed a prominent Cry band (Fig. 4, A). The amount of Cry corresponds to about 7% of the total cellular protein. However, the *cry*IA(c) cloned in pKUC20 having only a part of its promoter was expressed very poorly in both *E. coli* and *P. putida* (Fig. 4, A). In both hosts carrying the pKPC120 the Cry was present in the form in inclusion bodies. This fact was confirmed by microscopic observation and by following experiment. Recombinant cells of LE392 (pKPC120) and KCTC1644(pKPC120) were disrupted by sonication and the insoluble material was collected by centrifugation and dissolved by boiling in sample buffer. The bulk of the Cry was present in the pellet fraction when analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4, A). So we concluded that most of the Cry synthesized by the recombinant cells is present in an insoluble form.

Immunological activity of the newly synthesized protein was tested by blotting the polyacrylamide gel onto a nitrocellulose filter paper as described in Materials and Methods. The filter paper was incubated with purified antiserum and alkaline-phosphatase-conjugated antibody. One major band could be visualized in LE392 (pKPC120) and KCTC1644(pKPC120) lanes, while only trace of corresponding bands could be seen in LE392 (pKUC20) and KCTC1644(pKUC20) lanes. There seems to be a number of degraded crystal proteins which are recognized by the immunoblot (Fig. 4, B).

The biological activities of the Cry produced by the recombinant cells were determined. Cell lysates of both

LE392(pKPC120) and KCTC1644(pKPC120) gave 100 % mortality against larvae of the fall webworm, *Hyphantria cunea* (data not shown). Cell lysates prepared from pKUC20 harboring cell had very weak activity against *H. cunea*.

DISCUSSION

It is well known that the broad-host-range IncQ/P-4 group plasmids including RSF1010, R1162 and R300B are potentially useful for development of *Pseudomonas* vectors. They are multicopy, relatively small in size, and can be transferred among different bacterial stains by mobilization if conjugal transfer functions are provided by a coexisting transfer-proficient plasmid, such as RK2.

In this research, we have constructed a broad-host-range promoter-probe vector pKU20 using pKT230, a derivative of the RSF1010, as a base, and then cloned a 412 bp DNA fragment having strong promoter activity from *P. fluorescens* chromosomal DNA using the pKU20 and introduced the *cry*IA(c) into the *P. putida* using the finally constructed expression vector pKPC120. In these processes the initial DNA manipulation and cloning work were performed using *E. coli* LE392 as the host due to the low frequency of *Pseudomonas putida* transformation by plasmid DNA, and the plasmids prepared in *E. coli* were transferred into *Pseudomonas putida* by triparental matings. Plasmid pKU20 and its derivatives retain the *mob* region which encodes mobilization function and were efficiently mobilized between *E. coli* and *P. putida* by the function of pRK2013. The mobilization of the broad-host-range cloning vector appears to be of great use for gene cloning in a variety of Gram-negative bacteria, especially in a strain which is not readily transformed with plasmid DNA.

It has been reported that the *cry*IA(c) of *B.t.k.* HD-1 Dipel is expressed from two developmentally regulated overlapping promoters during sporulation, but, transcription of the *cry*IA(c) in a recombinant strain of *E. coli* started from a site located between the two promoters and was independent of the growth phase (21). By the way, our *cry*IA(c), which was cloned from 75 Kb plasmid of *B.t.k* HD-73 using *Nde*I (16) retains only the promoter sequence started from Ec (transcription start site in *E. coli*) of Wong *et al.*, and do not contain BtlI (one of the two transcription start sites in *Bacillus*), −10-like and −35-like sequences (1). This *cry*IA(c) cloned in pKUC20 was expressed very poorly in both *E. coli* and *P. putida*. The 412 bp promoter fragment cloned from *P. fluorescens* chromosomal DNA, however, raised expression level of the *cry*IA(c) in both hosts highly so that inclusion bodies could be formed. When the *cry*IA(c) was oriented in opposite direction to the 412 bp DNA fragment there was no detectable Cry band (data not shown). Therefore, it is likely that RNA polymerase of *E. coli* and *P. putida* use the 412 bp DNA fragment of pKP120 efficiently for starting of transcription.

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