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# NOTES

## Only Part of the Protoxin Gene of *Bacillus thuringiensis* subsp. *berliner* 1715 Is Necessary for Insecticidal Activity†

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***Escherichia coli* strains harboring deletion mutations of the insecticidal protoxin gene of *Bacillus thuringiensis* subsp. *berliner* 1715 were constructed. Although these strains did not produce intact protoxin, cell extracts from one of the mutants were extremely toxic to tobacco hornworm (*Manduca sexta*) larvae, indicating that only a part of the protoxin gene is required for insecticidal activity.**

The gram-positive bacterium *Bacillus thuringiensis* produces an insecticidal proteinaceous parasporal crystal during sporulation (4). The crystal is composed of a monomeric glycoprotein subunit ($M_r$ = 134 kilodalton [kDa]), a protoxin (1) that is converted to a lower-molecular-weight toxin ($M_r$ = 68 kDa) upon ingestion by a susceptible insect (1, 2, 5, 9). The toxin possesses the same specific toxicity as the protoxin (2, 3), suggesting that a part of the protoxin is inconsequential to insecticidal activity.

To genetically characterize the protoxin and toxin and to better understand the regulation of their syntheses, chromosomal and plasmid genes encoding for protoxin have been isolated from several *B. thuringiensis* subspecies (6, 7, 11). In the present paper, we describe an *Escherichia coli* clone that contains only a part of the protoxin gene of *B. thuringiensis* subsp. *berliner* 1715 but that synthesizes proteins whose toxicity is virtually identical to that of native protoxin.

The protoxin gene was isolated from a 42-megadalton plasmid of *B. thuringiensis* subsp. *berliner* 1715 (kindly provided by G. Rapoport, Institut Pasteur) as described by Klier et al. (7) except that we used pUC13 (14) as a cloning vector and *E. coli* JM83 (14) as a host. The clone, designated pHW13, contains 14.5 kilobase pairs (kbp) of *Bam*HI insert derived from the *B. thuringiensis* subsp. *berliner* 1715 plasmid. A physical map is presented in Fig. 1. It has been shown (7) that the major coding region of the protoxin resides in a 3.0-kbp *Pst*I-*Sst*I subfragment of a 5.2-kbp *Sst*I fragment (Fig. 1). Therefore, for further subcloning, pHW13 DNA was digested completely with either *Pst*I or *Sst*I and religated with T4 DNA ligase. *E. coli* JM83 was transformed with the ligated DNAs with $CaCl_2$ (10), and transformants were screened for ampicillin (100 μg $ml^{-1}$) resistance on L-agar plates. Thus, clones pP1 from *Pst*I digestion and pS11 from *Sst*I digestion were obtained (Fig. 1). Both clones contained the 3.0-kbp *Pst*I-*Sst*I insert. Intact protoxin was produced by the pP1 clone but not by pS11 (see below).

To further truncate pP1 DNA, the pP1 clone was sequentially deleted beginning at the *Pst*I site by partial digestion with *Hin*dIII. Although it has been reported (7) that the 14.5-kbp *Bam*HI fragment does not contain a *Hin*dIII cleavage site, we found three such sites, two of which were located in pP1 DNA (Fig. 1). The 14.5-kbp *Bam*HI insert in pHW13 is probably different from that in pBT42-1 isolated by Klier et al. (7), although the restriction pattern, other than that for *Hin*dIII, is quite similar. An excess amount of pP1 DNA (about 20 μg) was cleaved with *Hin*dIII (8 U) for a limited period (20 min) at 37°C. The cleavage products were separated by gel electrophoresis with 0.6% low-gelling-temperature agarose (Sigma Chemical Co., St. Louis, Mo.). Portions of agarose containing relevant sizes of DNA were cut and melted at 65°C, and the DNA was extracted by phenol treatment at 37°C and ethanol precipitated. DNA obtained in such a manner was self-ligated and used to transform strain JM83. Ampicillin-resistant transformants were selected as described above, and clones pH2 and pH15 were obtained (Fig. 1).

To examine the expression of the genes subcloned in *E. coli* with respect to protoxin antigen production and insect toxicity, we prepared cell extracts of *E. coli*. Early-stationary-phase cells (grown in L broth containing 100 μg of ampicillin $ml^{-1}$ at 37°C) of strain JM83 harboring each subcloned plasmid were concentrated 25-fold in a solution of lysozyme (1 mg $ml^{-1}$ in 10 mM EDTA, pH 8.5), incubated for 30 min at 25°C, and then sonicated (Branson sonifier, model 350, output control 3, 30% duty, 10 bursts). The resulting lysates were adjusted to pH 11.7 with 1 N NaOH. After the lysates were stirred for 2.5 h at 25°C, 1/20 volume of 2 M Tris hydrochloride (pH 8.3) was added to them, and they were cleared by centrifugation (13,000 × *g* for 5 min). The proteins in the extracts were separated by sodium dodecyl sulfate–7% polyacrylamide gel electrophoresis as described by Laemmli (8). The proteins in the gel were transferred electrophoretically (5 W for 3 h) to a nitrocellulose filter (Schleicher & Schuell, Inc., Keene, N.H.). The presence of protoxin was detected by enzyme-linked immunochemical staining (12), with the following modifications. Gelatin (3% [wt/vol]) was used instead of Tween 20 to prevent nonspecific binding of the antibody to the nitrocellulose filter. Phosphate buffer plus saline was replaced by 20 mM Tris hydrochloride (pH 7.5) and 500 mM NaCl through-

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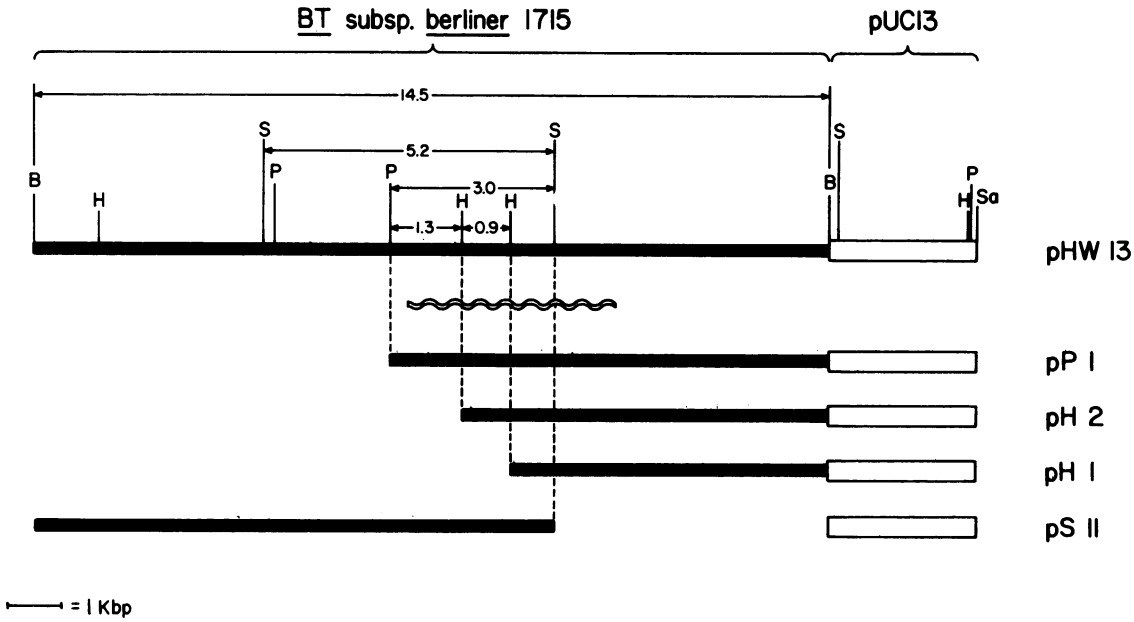


FIG. 1. Plasmids containing *B. thuringiensis* subsp. *berliner* 1715 protoxin gene and its deletion mutations. The plasmid pHW13 carried a 14.5-kbp *Bam*HI fragment derived from a 42-megadalton *B. thuringiensis* subsp. *berliner* 1715 plasmid. It is shown as a linearized form with a unique *Sal*I (Sa) site in pUC13 as reference. Subclone pP1 was obtained from pHW13 by *Pst*I digestion. Deletion derivatives pS11, pH1, and pH2 were constructed from pP1 by using either *Sst*I or *Hin*dIII digestion as described in the text. Solid bar, *B. thuringiensis* subsp. *berliner* DNA; open bar, pUC13 DNA; cleavage sites, B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sst*I; wavy bar, predicted protoxin gene locus. Numbers represent kilobase pairs of DNA.

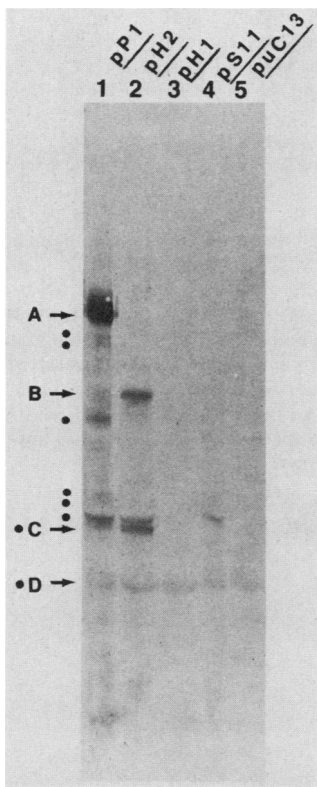


FIG. 2. Immunochemical detection of proteins in *E. coli* cell extracts. Early-stationary-phase cells of strain JM83 harboring each plasmid as described in the legend to Fig. 1 were concentrated 25-fold. Extracts were prepared as described in the text. About 80 μg of protein was separated by sodium dodecyl sulfate–7% polyacrylamide gel electrophoresis. After the proteins were transferred to a nitrocellulose filter, the filter was treated with antibody against *B. thuringiensis* subsp. *kurstaki* solubilized crystal which is known to react with *B. thuringiensis* subsp. *berliner* solubilized protoxin (13). The proteins that reacted with the antibody were stained as described in the text. Lanes: 1, pP1; 2, pH2; 3, pH1; 4, pS11; 5, pUC13. Molecular masses (kilodaltons) of the proteins indicated by arrows are: A, 140; B, 100; C, 65; D, 54. These values were estimated by comparing their relative mobilities with those of the following standard proteins (obtained from Bio-Rad Laboratories, Richmond, Calif.) stained with Coomassie blue: myoglobin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase b; 93 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa. The symbol ● represents proteins that reacted with antibody from the pP1 clone, except protein A (lane 1).

out the procedure. The blot nitrocellulose sheet was incubated with goat antibody produced against solubilized parasporal crystal protoxin. The sheet was then treated with rabbit anti-goat immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). The protoxin antigen was visualized finally by oxidation of 3,3′-diaminobenzidine (Sigma) catalyzed by peroxidase in antigen–antibody–anti-IgG antibody complexes. The result is shown in Fig. 2. The pP1 clone (Fig. 2, lane 1), as well as pHW13 (not shown), produced protoxin antigen with a molecular mass of approximately 140 kDa (band A, lane 1), which is very similar to that of the native *B. thuringiensis* subsp. *berliner* protoxin (13). In addition to the major 140-kDa protein, approximately eight proteins (indicated by closed circles) reacted with the antibody (Fig. 2). Except for the 54-kDa protein (Fig. 2, band D, lanes 1 through 5), these proteins were not present in the control strain JM83 harboring pUC13 (lane 5).

Apparently, the 54-kDa protein cross-reacted nonspecifically with the antibody, because this protein was present in every clone including strain JM83 containing pUC13 alone (lane 5). The eight proteins in question could be degradation products of the intact protoxin. In the case of the pH2 clone, a 100-kDa protein was produced (Fig. 2, band B, lane 2) instead of the 140-kDa protein. If this 100-kDa protein is a truncated protoxin gene product, the size of the deletion in the pH2 protoxin gene should correspond to approximately 1 kbp of DNA. Therefore, we believe that one terminal of the protoxin gene resides at about 0.3 kbp from the *Pst*I site in the pP1 clone (Fig. 1). Furthermore, a 65-kDa protein (Fig. 2, band C, lanes 1 and 2) in deletion mutant pH2 is more prominent than in pP1. This protein may be incomplete protoxin or a degradation product of the complete protoxin. It should be noted that the size of this 65-kDa protein is very close to that of the toxin ($M_r$ = 68 kDa; see reference 3). When further deletion was accomplished, such as in pH1, none of the specific proteins mentioned above was obvious (Fig. 2, lane 3). No protoxin antigen was produced by the pS11 clone (Fig. 2, lane 4). Because the 3.0-kbp *Pst*I-*Sst*I fragment was common to both pP1 and pS11 (Fig. 1) and the pP1 clone produced intact protoxin, we believe that the other terminal of the gene is outside the *Sst*I fragment of pS11 (Fig. 1).

Insect toxicity of the *E. coli* cell extracts was tested with tobacco hornworm (*Manduca sexta*) larvae by the method of Bulla et al. (3). One larva was placed on 10 ml of solid artificial diet (Carolina Biological Supply Co., Burlington, N.C.) in a single cup (4 by 7 cm) onto which the cell extracts had been uniformly distributed. The cups containing larvae were incubated at 25°C. The extracts from pHW13, pP1, and pH2 were toxic. In fact, the specific toxicity of each clone, deduced from the amount of protoxin in *E. coli* cell extracts (0.1 to 0.5% of the total protein), was comparable to that of solubilized parasporal crystal (50% lethal concentration, 1.36 ng $cm^{-2}$, as calculated by Tyrell et al. [13]). Conversely, no toxicity was observed with extracts of pH1, pS11, and pUC13 clones under the same conditions. This result demonstrates that the pH2 clone has a toxicity nearly identical to that of the original pHW13 plasmid and to that of the pP1 clone and that only part of the protoxin gene is required for toxicity. Possibly, the 100- or 65-kDa protein or both were responsible for the toxicity of the pH2 clone. Because pH1 did not exhibit toxicity and because the 0.9-kbp *Hin*dIII fragment was absent in this clone (Fig. 1), this particular fragment apparently contains the site that delimits the portion of DNA required for insecticidal activity in the entire protoxin gene. We are currently attempting to localize more precisely the region of DNA that contains the protoxin gene.

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