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Insecticidal Effect of New Strains of *Bacillus thuringiensis* on the Diamondback Moth, *Plutella xylostella*

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Twenty–eight strains of *Bacillus thuringiensis* (*Bt*) that were obtained from soil and are currently deposited in the Environmental Bacillus Collection at IBL were screened for toxicity against a susceptible strain of the diamondback moth (DBM), *Plutella xylostella*. Eight of the strains [IBL 24, 136, 156, 194, 425, 465, 745 and 455 (Dipel®, a commercial strain)] caused high levels of larval mortality after 2-4 days of feeding. When these eight Bt strains were tested on the Cry1A toxin-resistant (Dipel®-resistant) strain of DBM, the LC_{50} was lowest for 425, followed by 24 and 194. Thus, 425 was the most promising for development as a bioinsecticide against the Dipel®-resistant strain of DBM. Seven of the eight strains (not IBL 24) were found to have a cry1 gene (cry1Aa), while IBL 425 also had a cry1Ba gene.

Introduction

The diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae), is a major agricultural insect pest that only attacks cruciferous plants in the family Brassicaceae (eg., cabbage, cauliflower, kale, turnip and Brussels sprouts). Several subspecies of *Bacillus thuringiensis* (*Bt*), in conjunction with spore formation, produce crystal proteins that are toxic to members of various orders of insects, including Lepidoptera, Coleoptera and Diptera. To be effective, *Bt* crystal protein is solublized in the insect gut, and the Cry protein(s) that are released (after enzymatic digestion) bind to specific midgut cell membrane receptors, resulting in the destruction of the gut followed by the death of the larva. *Bt* var. *kurstaki* (*Btk*) is effective in killing DBM and has been commercialized under the name Dipel®, but DBM is reported to be the only pest to evolve *Btk* resistance in open field populations (1, 8), in part due to their rapid generation time (3). This study was conducted to identify and select other strains of *Bt* that are highly toxic to DBM, and thus, would be good candidates for use as biological control agents against this economically important pest.

Materials and Methods

Twenty-eight strains of Insect Biocontrol Laboratory (IBL)-collected *Bt* strains that were collected from soil, primarily in the U.S., were screened for toxicity against early 2nd instar *P. xylostella* larvae. Thirty µl aliquots of one of four treatments were pipetted onto the surface of cubes (approximately 3 mm^3) of artificial diet (7) that had been placed in wells of a 16-well bioassay tray (C-D International, Potman, NJ). These four treatments were: 1) a whole culture suspension (6.6 $x10^{-5}$ plate equivalents/µl), 2) a solublized crystal protein solution (0.298±0.02 µg/µl) prepared by incubating whole culture suspensions with NaOH for 24 h, centrifuging, collecting the supernatant and neutralizing with HCl, 3) a washed spore suspension (2.86±0.57 x 10^5 spores/µl), i.e., the washed precipitate after NaOH treatment, and 4) 30 µl of water (controls). For each treatment, 7 to 10 DBM larvae were introduced into each of 8-12 wells of the tray, and trays were stored in an incubator at 25±2°C, 60% R.H. and at 16h L:8h D light regime. Mortality for each well (1 replicate for a given treatment) was recorded each day for 10 days or until larval mortality reached 100%.

The presence of the genes encoding Cry1Aa and Cry1Ba in the eight most efficacious strains was determined using PCR. To obtain the DNA for extraction for PCR experiments, *Bt* was grown in 50 ml of liquid broth for 16 h at 25±2°C. After centrifugation, pellets were washed once in 5 ml TES (25mM Tris, 10mM EDTA, 25% sucrose) followed by resuspension in 5 ml TES with lysozyme (4 mg/ml) and incubated for 45 min at 37°C. A standard alkaline lysis preparation was performed (6) followed by resuspension of the DNA in 1.0 ml final volume of 0.1 x TE (10 mM Tris, 1 mM EDTA) pH 8.0. Initially, universal primers Un1(d) and Un1(r) were used to determine if selected *Bt* strains

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contained the *cry1* gene (2). Later, specific primers for *cry1Aa* [IAa and I(-)] and *cry1B* (IB and I(-)] were used to detect the presence of these genes (5). Gradient PCR with an annealing temperature range of 53-61°C yielded fragments that were sequenced with Big Dye protocols (Applied Biosystems, Foster City, CA) on an ABI 3100 automated sequencing machine (Applied Biosystems).

Results and discussion

On day 4 post-treatment, whole culture suspensions and spores of all the strains caused >96% mortality (data not shown). Control larvae remained healthy and pupated approximately 6 days after being transferred to wells of the diet tray. The whole culture suspension, which contained both spores and crystals, was the most effective of the three treatments, and the spore preparation was more effective than the dissolved crystal protein in killing DBMs. The latter preparation did not cause 100% mortality until day 6 after treatment with *Bt* had been initiated.

The eight most active *Bt* strains were also tested for toxicity against Cry1A toxin-resistant, i.e., the Dipel®-resistant strain, of DBM (Benzon Research, Carlisle, PA). In these experiments, the screening method was similar to that described earlier in Materials and Methods (7). After 4 days of exposure to *Bt*, mortality caused by whole culture suspensions of IBL 24, 156, 194 and 425 was between 96 and 100%. Mortality due to treatment with either spores or dissolved crystal protein was also considerable. Of these four most active *Bt* strains, IBL 425, 24 and 194 were found to cause the highest mortality in DBMs, and thus, these 3 strains are the most promising for development as a bioinsecticide against DBM.

LC_{50} values for the eight *Bt* strains that were most active against the susceptible strain of DBM were calculated from dose-response curves generated 6 days after the exposure of DBMs to *Bt* preparations (Table 1). In these tests, DBMs were reared on rehydrated diet pellets [diet was prepared in 96-well format and then freeze-dried (4)] or on diet cubes as described previously. The type of diet used did not affect mortality. Values are expressed as plate equivalents (added to the diet) required to kill 50% of the DBMs. The LC_{50} of whole culture suspensions was lower than that of either spore or crystal protein preparations. Among the eight strains (of the 28 tested) giving the highest mortality, the LC_{50} values of *Bt* for whole culture suspensions and crystal protein against susceptible DBM were the lowest for IBL 465, while for resistant DBM, the LC_{50} values were the lowest for IBL 425. IBL 465, which showed the highest efficacy against susceptible DBM, as well as IBL 136, 455, and 745 were not toxic to resistant DBMs (Table 1).

Using PCR, IBL 425 was found to have both *cry1Aa* and *cry1Ba* genes (Fig. 1b, c), whereas for IBL 136, 156, 194, 455, 465 and 745, we only identified *cry1Aa*. IBL 24 and 1003 (the negative control, known not to have a *cry1* gene) did not exhibit a *cry1* band (Fig. 1a).

TABLE 1. LC_{50} values (in plate equivalents) for whole culture (WCS), spore and solublized *Bt* crystal preparations for susceptible (S) and resistant (R) DBMs.

Bt IBL #	LC_{50} (x 10^{-6}) [1] 6 days post-treatment					
	WCS		Spores		Crystal protein	
	S	R	S	R	S	R
24	66±14	330±181	605±274	2,026±47	829±212	139±33
194	29±3	337±61	337±139	1,320±0	223±49	380±156
425	13±5	19±5	267±130	136±56	109±2	60±5
465	1±0	1,223±451	261±190	0	59±15	0
136	14±4	0	316±24	0	3,478±797	0
156	75±12	789±296	1,027±292	1,651±331	1,867±318	1,897±260
455	2±0	2,638±334	432±30	0	1,235±3	0
745	2±1	0	149±33	0	2,045±47	0

[1]For simplicity, actual values have been multiplied by10^6.

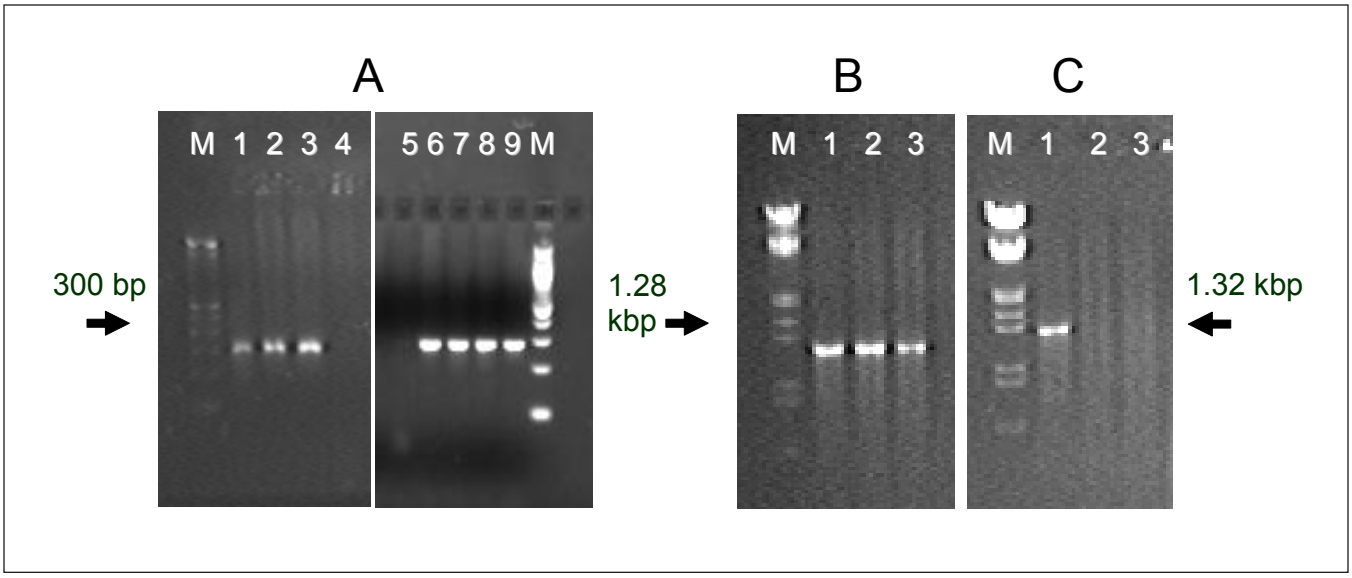

FIG. 1. Detection of *cry1* genes in *Bt* strains (A) using a universal primer [Un1(d) and Un1(r) (2)], (B) using a specific primer for *cry1Aa* [IAa and I(-) (5)], (C) using a specific primer for *cry1B* (IB and I(-) (5)]. 1 = IBL 425, 2 = IBL 455, 3 = IBL 465, 4 = IBL 1003, 5 = IBL 24, 6 = IBL 136, 7 = IBL 156, 8 = IBL 194, 9 = IBL 745, M=Marker.

Conclusion

Seven new strains of IBL-isolated *Bt* exhibited significant toxicity against *P. xylostella.* Of these, IBL 465 was even more effective against susceptible DBM than IBL 455 (equivalent to Dipel®). Of the 3 strains (IBL 24, 194 and 425) that were active against the Cry1A toxin-resistant DBM, IBL 425 was the most potent. Thus, IBL 465, 425, 194 and 24 are good candidates for development as possible biocontrol products. Experiments are in progress to completely sequence the *cry* genes of these four strains (three active against resistant DBM, one active against susceptible DBM) in the hopes of identifying those genes whose products are highly toxic to DBM.

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