Novel *Bacillus thuringiensis* Binary Insecticidal Crystal Proteins Active on Western Corn Rootworm, *Diabrotica virgifera virgifera* LeConte

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A new family of insecticidal crystal proteins was discovered by screening sporulated *Bacillus thuringiensis* cultures for oral activity against western corn rootworm (WCR) larvae. *B. thuringiensis* isolates PS80JJ1, PS149B1, and PS167H2 have WCR insecticidal activity attributable to parasporal inclusion bodies containing proteins with molecular masses of ca. 14 and 44 kDa. The genes encoding these polyepptides reside in apparent operons, and the 14-kDa protein open reading frame (ORF) precedes the 44-kDa protein ORF. Mutagenesis of either gene in the apparent operons dramatically reduced insecticidal activity of the corresponding recombinant *B. thuringiensis* strain. Bioassays performed with separately expressed, biochemically purified 14- and 44-kDa polypeptides also demonstrated that both proteins are required for WCR mortality. Sequence comparisons with other known *B. thuringiensis* insecticidal proteins failed to reveal homology with previously described Cry, Cyt, or Vip proteins. However, there is evidence that the 44-kDa polypeptide and the 41.9- and 51.4-kDa binary dipteran insecticidal proteins from *Bacillus sphaericus* are evolutionarily related. The 14- and 44-kDa polypeptides from isolates PS80JJ1, PS149B1, and PS167H2 have been designated Cry34Aa1, Cry34Ab1, and Cry34Ac1, respectively, and the 44-kDa polypeptides from these isolates have been designated Cry35Aa1, Cry35Ab1, and Cry35Ac1, respectively.

The various isolates and subspecies of *Bacillus thuringiensis* are best known as valuable sources of commercially important biopesticides (41). The most well-studied *B. thuringiensis* insecticidal proteins are the δ-endotoxins, which are parasporal crystalline inclusions that have diverse sizes, shapes, and protein compositions (21). The δ-endotoxins are proteins that belong to a number of sequence similarity groups and have oral activity against a wide range of insect pests (6, 11, 17, 22, 39). Advances in agricultural biotechnology have enabled expression of several *B. thuringiensis* proteins in transgenic plants, thereby imparting intrinsic insect resistance traits to a number of important crops (28, 35, 36, 43).

The larvae of western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), and related *Diabrotica* species are major coleopteran pests of corn (15). Crop rotation with soybeans, estimated to be used in 80% of north central United States acreage by the National Agricultural Statistics Service, is the most common pest management practice for corn rootworm control. However, newly identified rootworm behavioral adaptations potentially threaten the sustainability of crop rotation as an effective means of controlling this pest complex. In Minnesota a northern corn rootworm, *Diabrotica barberi* Smith & Lawrence biotype, has developed an extended diapause, in which eggs remain in the soil for an extra year and hatching is delayed until corn is planted again (20). In addition, the Illinois Natural History Survey and University of Illinois (http://www.staff.uiuc.edu/~s-isard/WCRStart.html) are tracking a strain of WCR beetles that have evolved behaviorally to lay eggs in soybean fields where corn is planted, and the eggs are ready to hatch the next year (19, 34).

Chemical pesticides are also used to control corn rootworms. The National Agricultural Statistics Service estimates that approximately 8 million pounds of soil insecticides are applied annually to 68 million corn acres in the United States for rootworm control. Some of these pesticides are currently being reviewed by the Environmental Protection Agency under the Food Quality Protection Act and could lose their registrations, while long-term use of some others is threatened by development of resistant rootworms or soil-enhanced microbial degradation (37, 44).

The challenges to corn rootworm management mentioned above reinforce the need to diversify rootworm control measures, and an option that we have explored is to use transgenic corn hybrids expressing *B. thuringiensis*-derived proteins in their roots for protection against larval damage. A first step towards this goal was the discovery of *B. thuringiensis* proteins with effective levels of activity against the economically important rootworm pest complex, and it is thought that the gene(s) encoding these proteins could be engineered for expression in commercial corn hybrids to provide an additional management tool for rootworm control. In this report we describe a novel class of binary *B. thuringiensis* insecticidal crystal proteins that form the basis for highly efficacious control of corn rootworms when the proteins are expressed in transgenic corn hybrids (29).

(Some aspects of this work have been described in patent applications [PCT international applications WO9740162 {30 October 1997}, and WO0114417 {March 2001}] and related U.S. patents [31, 32].)
### TABLE 1. \textit{B. thuringiensis} and \textit{P. fluorescens} bacteria evaluated for insecticidal activity against WCR in this study

<table>
<thead>
<tr>
<th>Organism</th>
<th>Isolate</th>
<th>Phenotype and/or genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{B. thuringiensis}</td>
<td>PS80J1</td>
<td>Wild type (NRRL B-18679)</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{B. thuringiensis}</td>
<td>PS149B1</td>
<td>Wild type (NRRL B-21553)</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{B. thuringiensis}</td>
<td>PS167H2</td>
<td>Wild type (NRRL B-21554)</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{B. thuringiensis}</td>
<td>Cry B</td>
<td>Acrylaliphilic cloning host (40)</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{B. thuringiensis}</td>
<td>MR541</td>
<td>Cry B (pMYC2424); PS80J1</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{B. thuringiensis}</td>
<td>MR542</td>
<td>Cry B (pMYC2425); PS80J1</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{B. thuringiensis}</td>
<td>MR543</td>
<td>Cry B (pMYC2426); PS80J1</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{P. fluorescens}</td>
<td>MR839</td>
<td>MB214 containing proprietary cloning vector</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{P. fluorescens}</td>
<td>MR1240</td>
<td>MB214 (pMYC2580); PS80J1</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{P. fluorescens}</td>
<td>MR1242</td>
<td>MB214 (pMYC2581); PS80J1</td>
<td>This study</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Deposited in the permanent collection of the Patent Culture Collection (NRRL), Regional Research Center, Peoria, Ill. \textsuperscript{b} MB214 is a \textit{P. fluorescens} host strain of Dow AgroSciences.

### MATERIALS AND METHODS

**Native bacterial isolates and culture.** The bacterial isolates used in this study are listed in Table 1. \textit{B. thuringiensis} isolates PS80J1, PS149B1, and PS167H2 were obtained from the Dow AgroSciences proprietary collection of bacteria and were deposited in the permanent collection of the Agricultural Research Service Patent Culture Collection, Regional Research Center, Peoria, Ill. \textit{B. thuringiensis} isolates were grown to lysis in cross-bagged flasks at 30°C with shaking at 250 rpm in PGSM medium (7) supplemented with 10 µg of erythromycin per ml when appropriate. Lysed cultures were centrifuged at 10,880 × g for 25 min at 4°C, and the cell pellets were resuspended in small volumes of sterile, deionized water. Sample aliquots were stored on ice at 4°C and used for biossays immediately, and the remaining portions were either stored at −80°C or lyophilized, stored at room temperature, and used for further purification. Two proteins, which had molecular masses of 14 and 44 kDa, were quantified by densitometry of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels by using a Molecular Dynamics densitometer and bovine serum albumin as the standard.

**Bioassays.** Test materials were applied to the surface of casen and wheat germ modified artificial diet for corn rootworm (26). Forty-eight wells were plated with a surface area of 0.78 cm\(^2\) were treated by using an application rate of 125 µl of treatment suspension per well. Treated bioassay plates were surface dried in a laminar flow hood. The bioassay negative controls were Cry B (40), a \textit{B. thuringiensis} strain lacking insecticidal protein genes that was tested at a cell mass equivalent to that of other relevant commercial isolates PS80JJ1, PS149B1, and PS167H2 inserts, we identified one clone that contained an approximately 4.0- to 4.4-kbp HindIII fragment that hybridized to the PS80J1 44-kDa protein gene probe. This DNA restriction fragment was isolated, purified, and subcloned into pHT370 to generate pMYC2427.

Using Southern blot analysis of DNA from recombinant phase containing PS167H2 inserts, we identified one clone that contained an approximately 4.0- to 4.4-kbp HindIII fragment suitable for subcloning the PS80J1 44-kDa protein gene. The SacI site flanking the PS80J1 gene is a phage vector cloning site, while the flanking XbaI site is located within the PS80J1 DNA insert. This DNA restriction fragment was subcloned by standard methods into pBR322 as a 1.2-kb XbaI-SacI fragment suitable for subcloning the PS80J1 44-kDa protein gene. The XbaI site flanked the 500-bp HindIII fragment that hybridized to the PS80J1 44-kDa protein gene probe. This DNA restriction fragment was isolated, purified, and subcloned into pHT370 to generate pMYC2427.

Using Southern blot analysis of DNA from recombinant phase containing PS80J1 inserts, we identified one clone that contained an approximately 5.9-kbp DNA fragment that hybridized to the PS80J1 44-kDa protein gene probe. Complete C11 digests of PS149B1 genomic DNA were size fractionated on agarose gels, cloned into pHT370, and screened by hybridization. One representative clone containing the PS149B1 genomic DNA was designated pMYC2429.

DNA insert containing genes in pMYC2421, pMYC2427, and pMYC2429 were sequenced by using ABI373 or ABI377 automated sequencers and software (PE Biosystems, Foster City, Calif.). Additional sequence analysis was performed by using Wisconsin Package, version 10.2 (Genetics Computer Group, Madison, Wis.).

**Heterologous expression and protein purification.** The 14- and 44-kDa proteins were overexpressed in a Dow AgroSciences proprietary inducible \textit{Pseudo- monas fluorescens} plasmid expression system. The 14- and 44-kDa protein genes were first separately engineered into a plasmid vector under control of an inducible promoter by standard DNA cloning methods; the recombinant plasmids were then transformed by electroporation into \textit{P. fluorescens} host strain MB214 to obtain strains MR1242 and MR1240, respectively. Following growth and induction, a portion of either an MR1240 culture or an MR1242 culture was lysed in lysis buffer to obtain protein mixtures. These isolates were then resuspended in 50 mM sodium citrate (pH 3.3) by gentle rocking at 4°C for 1 h. This buffer completely solubilized the 14-kDa protein and partially solubilized the 44-kDa protein. The preparations were then centrifuged at 20,000 × g for 20 min, and the supernatants were fractionated by agarose gel electrophoresis. DNA fragments that were 9.3 to 23 kbp in size were excised from the gel, electrophoresed from the gel slice, purified on an Elutip-D ion-exchange column (Schleicher and Schuell, Keene, N.H.), and used for hybridization of phage plaques and Southern blot analysis by standard methods (25). Hybridizing phage were plaque purified and used to infect liquid cultures of \textit{E. coli} K12 cells for isolation of DNA by standard procedures (25).

To prepare insecticidal proteins from \textit{B. thuringiensis}, 1 g of a lyophilized...
TABLE 2. Sequence accession numbers and designations of the binary insecticidal crystal proteins

<table>
<thead>
<tr>
<th>Designation</th>
<th>GenBank accession no.</th>
<th>Gene sequence</th>
<th>Protein sequence</th>
<th>Strain</th>
<th>Size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry34Aa1</td>
<td>AYO16411</td>
<td>AAG50341</td>
<td>PS80JJ1</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Cry35Aa1</td>
<td>AYO16411</td>
<td>AAG50342</td>
<td>PS80JJ1</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Cry34Ab1</td>
<td>AYO11120</td>
<td>AAG41671</td>
<td>PS149B1</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Cry35Ab1</td>
<td>AYO11120</td>
<td>AAG41672</td>
<td>PS149B1</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Cry34Ac1</td>
<td>AYO16410</td>
<td>AAG50118</td>
<td>PS167H2</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Cry35Ac1</td>
<td>AYO16410</td>
<td>AAG50117</td>
<td>PS167H2</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

*See Crickmore et al., http://www.bios.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html.*

PS80JJ1 or MR543 wet cell pellet was suspended in a wash buffer containing 80 mM Tris-Cl, 5 mM Na2EDTA, 100 μg of leupeptin per ml, 0.7 mg of pepstatin per ml, and 40 μg of bestatin per ml (pH 7.8). The resulting suspension was centrifuged at 5,000 × g for 30 min. The resulting supernatant was discarded, and the wash procedure was repeated an additional four times. The final pellet was resuspended in 10 ml of the wash buffer. The materials were then processed as described above for the proteins expressed in *P. fluorescens*.

**Gene mutagenesis.** The PS80JJ1 genes were separately inactivated on two plasmid constructs. The mutated operons were maintained under control of the native promoter for expression in *B. thuringiensis*. First, the 44-kDa protein gene was mutated by truncation at the *EcoRI* site at base position 587 of the open reading frame (ORF). The resulting operon, which included the intact 14-kDa protein gene and the truncated 44-kDa protein gene, was subcloned into pHT370 to generate pMYC2424. Transformation of pMYC2424 into CryB by electroporation generated recombinant *B. thuringiensis* strain MR541. Next, the 14-kDa protein gene was mutated by insertion of an oligonucleotide linker containing termination codons in all possible reading frames at the *NruI* site at base position 11 of the 14-kDa protein ORF. The sequence of the mutagenic linker was 5′-TGAGTAACTAGATCTATTCAATTA 3′. The linker introduced a *BglII* site for screening putative mutants by hybridization with the gene probe for the PS80JJ1 44-kDa protein N-terminal sequence. The operon insert which encoded the 14-kDa protein nonsense mutation was subcloned into pHT370 to generate pMYC2424. Transformation of pMYC2424 into CryB by electroporation generated recombinant *B. thuringiensis* strain MR542.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the nucleotide and protein sequences, as well as the designations of the PS80JJ1, PS167H2, and PS149B1 binary insecticidal crystal proteins, are shown in Table 2.

**RESULTS**

*B. thuringiensis* isolates active on WCR. *B. thuringiensis* isolates PS80JJ1, PS167H2, and PS149B1 all produced parasporal inclusions comprised primarily of proteins whose molecular masses were approximately 13 to 14 and 44 kDa, as determined by SDS-PAGE (Fig. 1). The *B. thuringiensis* strains were determined to have activity on WCR neonates in surface-applied bioassays. The estimated effective LC50 of total 14- and 44-kDa proteins from spore crystal preparations from the three *B. thuringiensis* isolates for WCR were 6 to 8 μg/cm2 (Table 3). Tests of these *B. thuringiensis* isolates at estimated concentrations of 100 μg/cm2 with the primary lepidopteran corn pests, including European corn borer (*Ostrinia nubilalis* Hubner) (Lepidoptera: Crambidae), corn earworm (*Helicoverpa zea* Boddie), and black cutworm (*Agrotis ipsilon* Hufnagel) (Lepidoptera: Noctuidae), revealed no observable insecticidal effect (data not shown).

**Nucleotide and deduced amino acid sequences for the insecticidal protein operons.** Southern blot restriction fragment length polymorphism analyses of PS80JJ1 total DNA that was hybridized with oligonucleotide probes based on the N-terminal sequences of the 14- and 44-kDa proteins suggested that the genes encoding these proteins were located adjacent to each other (data not shown). Therefore, plasmid clones encoding the 44-kDa crystal protein from each *B. thuringiensis* isolate were identified by hybridization with the gene probe for the PS80JJ1 44-kDa protein N-terminal sequence. Sequence analyses of the 44-kDa protein loci revealed that all three *B. thuringiensis* isolates active on WCR have the genes encoding the 14- and 44-kDa crystal proteins arranged in an apparent operon. The nucleotide and deduced polypeptide sequences for the PS80JJ1 operon are shown in Fig. 2. The predicted molecular masses of the deduced polypeptides from *B. thuringiensis* isolate PS80JJ1 are 13.2 and 44.3 kDa, whereas the molecular masses of the homologous proteins from both PS149B1 and PS167H2 are 13.6 and 43.8 kDa. The size differences for the ca. 14-kDa proteins are apparent in Fig. 1, lanes 2 to 4. Size comparisons of the ca. 44-kDa proteins were com-

**TABLE 3. Insecticidal activity of *B. thuringiensis* isolates against WCR neonates in three top load artificial diet bioassays**

<table>
<thead>
<tr>
<th>Isolate or treatment</th>
<th>n</th>
<th>LC50 (μg/cm2)</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS80JJ1</td>
<td>403</td>
<td>6 (4–8)</td>
<td>1.5</td>
</tr>
<tr>
<td>PS167H2</td>
<td>369</td>
<td>6 (4–9)</td>
<td>1.6</td>
</tr>
<tr>
<td>PS149B1</td>
<td>369</td>
<td>8 (4–12)</td>
<td>1.8</td>
</tr>
<tr>
<td>Water</td>
<td>228</td>
<td>—</td>
<td>NA²</td>
</tr>
</tbody>
</table>

*LC50 was defined as the concentration that caused 50% mortality of the test population. It was expressed in micrograms of protein per square centimeter of diet surface. LC50 was calculated by Probit analysis by using POLO-PC for treatments in which larval mortality was observed.

¹ The values in parentheses are 95% confidence intervals.

² The effects of negative control treatments were expressed in percent WCR mortality; water treatment resulted in 4% mortality.

³ NA, not applicable.
plicated by apparent proteolytic processing (Fig. 1, lane 4; also see below). The genes are arranged with the 14-kDa protein ORF upstream of the 44-kDa protein ORF. Both the 14-kDa protein ORF and the 44-kDa protein ORF are preceded by putative ribosome binding sites. Intergenic spacers separating the 14- and 44-kDa protein ORFs were 121 bp long (PS80JJ1) or 107 bp long (PS149B1 and PS167H2). The intergenic region of the PS80JJ1 sequence contains inverted repeats (Fig. 2) that have the potential to form a secondary structure with a \\n\[ \Delta G^{\text{folding}} = 18.8 \text{ kcal/mol} \] (estimated at 37°C). Similar patterns, lacking the outermost repeat, occur in the shorter spacers found in PS149B1 and PS167H2 (data not shown). Sequence comparisons of the proteins from the three \textit{B. thuringiensis} isolates revealed that the 14- and 44-kDa proteins comprise two new sequence families and that the PS80JJ1 proteins are more distantly related to the homologous proteins of either PS149B1 or PS167H2. For example, the PS80JJ1 14-kDa protein is 77% identical to the PS149B1 14-kDa protein, whereas the PS80JJ1 44-kDa protein is 80% identical to the PS149B1 44-kDa protein. The homologous 14- and 44-kDa protein pairs for PS149B1 and PS167H2 both exhibit approximately 94% sequence identity. In the case of the PS80JJ1 operon, a portion of an \textit{IS}\textsubscript{240}-like insertion element, similar to that in the GenBank accession number M23741 sequence, is present in the sequence distal to the gene for the 44-kDa protein, including an inverted repeat and a portion of a transposase (Fig. 2). While this apparent insertion element may have disrupted the normal transcription terminator for the PS80JJ1 sequence, a strong terminator-mRNA stabilizer sequence of the type found for the Cry1 genes (reviewed in reference 39) also appears to be absent in the 120 to 200 bp 3' to the 44-kDa protein coding sequences in the other two strains.

**Expression and mutational analysis of the PS80JJ1 insecticidal protein operon.** Recombinant \textit{B. thuringiensis} isolates expressing both the 14- and 44-kDa proteins encoded by the PS80JJ1 operon or these proteins separately were constructed (Fig. 3) to determine if one or both proteins were responsible for WCR mortality. Wild-type and mutant genes were subcloned into an \textit{E. coli}-\textit{B. thuringiensis} shuttle vector (pHT370)
and transformed into the Cry
\(^*/^\text{H11002}\)

B host. Expression of the 14-
\(^{kDa}\) protein (Fig. 4, lane 2), the 44-kDa protein (Fig. 4, lane 3),
or both proteins together (Fig. 4, lane 4) was observed when recombinant isolates were grown to the sporulation stage. Recombinant
\(\text{B. thuringiensis}
\)

Cry
\(^*/^\text{H11002}\)

B strains containing the operons from both PS149B1 and PS167H2 also expressed both 14- and 44-kDa proteins (data not shown).

The bioassay results for recombinants expressing proteins derived from isolate PS80JJ1 showed that only the strain expressing both the 14- and 44-kDa proteins, MR543, was insecticidal for WCR larvae. The LC\(_{50}\) for MR543 containing co-expressed proteins was estimated to be 37 \(\mu\)g/cm\(^2\) (90% confidence interval, 17 to 366 \(\mu\)g/cm\(^2\); slope, 0.8). The activities of the recombinants that expressed only individual proteins (e.g., MR541 expressing the 14-kDa protein and MR542 expressing the 44-kDa protein) were indistinguishable from the activities of negative controls at similar doses. The negative controls, including water and acrystalliferous Cry
\(^*/^\text{H11002}\)

B applied at similar biomass rates, caused 4% WCR mortality.

**Heterologous insecticidal protein expression.** The PS80JJ1 14- and 44-kDa proteins were also expressed individually in recombinant
\(\text{P. fluorescens}
\)

strains designated MR1240 and MR1242 (Fig. 5, lanes 4 and 5, respectively). Doublet bands were observed for the 44-kDa protein expressed in MR1240 (Fig. 5, lane 4), and these bands had slightly slower mobility than the corresponding polypeptide produced in either the native or recombinant
\(\text{B. thuringiensis}
\)

strain (Fig. 5, lanes 2 and 3). The N-terminal sequences of both bands of the doublet from MR1240 were determined by using Edman degradation (27), and the results showed that both protein species in this doublet have the same N-terminal sequence as the deduced 44-kDa polypeptide sequence and the crystals obtained from
\(\text{B. thuringiensis}
\).

The mobilities of the 44-kDa proteins from the native and recombinant bacterial sources (Fig. 5, lanes 2 to 4) provide indirect evidence that there is C-terminal proteolytic processing of the 44-kDa polypeptides obtained from both sources.

The inclusions produced by the recombinant
\(\text{P. fluorescens}
\)

strains were purified and quantified by gel densitometry for use in bioassays with WCR. Purified recombinant MR1240 expressing the 14-kDa protein and purified recombinant MR1242 expressing the 44-kDa protein were tested with WCR individually and at a protein concentration ratio of 1:1. This mass ratio mimics the 3:1 molar ratio of the 14- and 44-kDa proteins observed for wild-type strain PS80JJ1. Similar to the results of the gene knockout experiment, oral activity against WCR was observed when recombinant proteins were added to the WCR...
TABLE 4. Activity of 14- and 44-kDa proteins derived from PS80JJ1 coexpressed in *B. thuringiensis* (MR543) or expressed in *P. fluorescens* separately or at a mass ratio of 1:1 (MR1240 and MR242) against WCR neonates in top load bioassays

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (µg/cm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>% Mortality&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS80JJ1</td>
<td>8 (6–10)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>MR543</td>
<td>35 (24–50)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>MR1240 + MR1242</td>
<td>138 (88–257)</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>MR1240</td>
<td>10 NA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6 NA</td>
<td></td>
</tr>
<tr>
<td>MR1242</td>
<td>10 NA</td>
<td>6 NA</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>6 NA</td>
<td>6 NA</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> The effects of treatments for which LC<sub>50</sub> could not be calculated are expressed in percent mortality for rates of ≥100 µg/cm<sup>2</sup>.

<sup>b</sup> The values in parentheses are 95% confidence intervals.

<sup>c</sup> NA, not applicable.

diet in a mixture (LC<sub>50</sub> 138 µg/cm<sup>2</sup>), whereas the proteins added individually were not lethal (Table 4).

The bioassay experiments (Tables 3 and 4; also see above) suggested that the levels of activity observed for the 14- and 44-kDa proteins when they were expressed by *B. thuringiensis* clone MR543 and *P. fluorescens* and administered in a mixture were not as high as the levels of activity observed when parent isolate PS80JJ1 was used. However, the combined results of the gene knockout experiment and the heterologous expression experiment provide convincing evidence that both the 14- and 44-kDa proteins were responsible for the lethal effect observed with WCR. This led to further expression experiments performed with the genes from isolates PS167H2 and PS149B1 to determine if insecticidal activity could be optimized with different combinations (data not shown). The results of studies in which we used *B. thuringiensis* operon clones and separately expressed 14- and 44-kDa proteins from PS149B1 in *P. fluorescens* revealed a level of activity that was approximately the same as the level of activity of the PS149B1 *B. thuringiensis* isolate. Based on the higher level of activity of the PS149B1 proteins, we decided to transform corn with the PS149B1 genes for rootworm protection (29).

**Sequence similarities between the insecticidal proteins and other proteins.** Sequence comparisons failed to show a convincing similarity between the 14- and 44-kDa proteins and any of the previously described *B. thuringiensis* Cry, Cyt, or Vip proteins. However, a BLAST (2) database search using the PS149B1 44-kDa protein (as a representative of this family) revealed matches with the 42-kDa *Bacillus sphaericus* crystal inclusion protein (expectation score, 3 \times 10^{-14}) and the 51-kDa *B. sphaericus* crystal inclusion protein (expectation score, 3 \times 10^{-18}). An alignment of the 44-kDa PS149B1 peptide sequence with the 42-kDa *B. sphaericus* crystal inclusion protein sequence revealed 26% identity over 325 residues. A similar comparison of the 44-kDa PS149B1 peptide sequence with the 51-kDa *B. sphaericus* crystal inclusion protein sequence revealed 29% identity over 229 residues. Similar results were obtained when the PS80JJ1 and PS167H2 proteins were used. As shown in the multiple-sequence alignment in Fig. 6, a number of conserved sequence motifs were identified in the three proteins by the MEME algorithm (4). Four of these conserved motifs overlapped with conserved blocks A, B, C, and D identified by Baumann et al. (5) between the 51- and 42-kDa *B. sphaericus* crystal proteins. An additional conserved motif was identified between the B and C motifs due to the greater similarity of the 44-kDa *B. thuringiensis* proteins to the *B. sphaericus* 42-kDa protein in this region. The N termini of the 44-kDa *B. thuringiensis* proteins are aligned near the 10th residue of the *B. sphaericus* 42-kDa protein and the 25th residue of the *B. sphaericus* 51-kDa protein (Fig. 6). This region is close to the segment defining the required functional N termini of both *B. sphaericus* proteins, as determined by protease cleavage sites or deletion analysis (5, 12, 33), and could indicate that there is a minimal dispensable sequence at the N terminus. The position of the C termini of the 44-kDa *B. thuringiensis* proteins in the alignment is intermediate between the positions of the C termini of the corresponding *B. sphaericus* proteins and well past the positions of the segments required for minimal function. The latter finding is consistent with the apparent C-terminal proteolytic processing of the 44-kDa proteins described above.

A BLAST database (2) search performed with the 14-kDa proteins described here detected no sequences that had significant similarity beyond the similarities of related *B. thuringiensis* crystal protein sequences that were described in recent patent applications (PCT international patent applications WO0114417 [1 March 2001] and WO0066742 [9 November 2000]).

**DISCUSSION**

*B. thuringiensis* δ-endotoxins are well known for their ability to control a variety of insect pests, including members of the Lepidoptera, Coleoptera, and Diptera (41). However, only a few previously described *B. thuringiensis* proteins have significant levels of efficacy against WCR. These proteins include the insecticidal crystal proteins Cry3 (13, 14, 18, 23) and Cry6Aa1 (30, 42) and soluble proteins expressed during the vegetative growth phase, Vip1 and Vip2 (9). In addition, genetically engineered derivatives of Cry3Bb1 (16) have been reported to have improved activity against rootworms. The binary insecticidal proteins described here represent new families of insecticidal crystal proteins that are effective against WCR larvae. In tests conducted so far, activity of these insecticidal proteins was observed with other members of the genus *Diabrotica* and certain other members of the Coleoptera, but no insecticidal activity at comparable rates has been observed with primary

![FIG. 6. Comparison of the 44-kDa proteins of PS149B1, PS167H2, and PS80JJ1 with the 51.4- and 41.9-kDa proteins of *P. fluorescens*. (GenBank accession number M20390). Previously observed features of the *B. sphaericus* proteins are: blocks of sequence similarity (5) (A to D), protease cleavage sites (5, 11) near the ends (arrows, underlined), and deletion endpoints from a functional analysis (32), indicated by boxed sections near the ends, where terminal endpoints retained toxicity, and internal endpoints were nontoxic, when combined with the intact binary partner. Overlined regions are conserved regions found in register in at least four of the five proteins by the MEME program (4). Black highlighting indicates that conserved residues are present in all five sequences, while gray highlighting indicates that conserved residues are present in four of the five sequences.](image-url)
lepidopteran pests of corn. Recently, the PS149B1 14- and 44-kDa proteins were cotransformed into corn and were shown to provide excellent protection against corn rootworm damage (29); therefore, these proteins provide a biotechnological option for corn rootworm pest control.

The 14- and 44-kDa protein families are unique among the insecticidal proteins both because of their sequence relatedness and because two separate proteins are required for insecticidal activity. While neither of these protein families is related to any previously described Cry proteins, the 44-kDa proteins have significant homology with the B. sphaericus 41.9- and 51.4-kDa polypeptides comprising the binary insecticidal proteins active against mosquitoes (5). The B. sphaericus proteins affect mosquito midgut cells in a 1:1 association; the 51.4-kDa protein provides the binding function, while the 41.9-kDa protein is required for activity (12, 33). While the B. thuringiensis binary corn rootworm insecticidal crystal proteins appear to act on the larval midgut after ingestion (29), further work is necessary to determine the role of each protein component in the intoxication process. Also, mosquito screening assays of the type used to identify mosquitocidal B. sphaericus and B. thuringiensis strains have not identified a similar level of activity for these B. thuringiensis binary toxin proteins.

We concluded that the binary toxins described here are encoded in apparent operons based on their close proximity, coordinate function, and coordinate appearance in crystals. A 14-kDa protein is required for activity (12, 33). While the 44-kDa proteins were cotransformed into corn and were shown to provide excellent protection against corn rootworm damage (29); therefore, these proteins provide a biotechnological option for corn rootworm pest control.

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ADDENDUM

During the revision of the manuscript, the binary insecticidal crystal proteins described here were designated Cry34Aa1, Cry34Ab1, and Cry34Ac1 (for the 14-kDa polypeptide components) and Cry35Aa1, Cry35Ab1, and Cry35Ac1 (for the 44-kDa polypeptide components), as shown in Table 2 (N. Crickmore, D. R. Zeigler, E. Schnepp, J. Van Rie, D. Lereclus, J. Baum, A. Bravo, and D. H. Dean, http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bi/index.html).

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