J. Microbiol. Biotechnol. (1998), **8**(5), 517–522

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Application of Multiplex PCR for Rapid Determination of *cryI* Gene Profiles of New *Bacillus thuringiensis* Isolates

MAHADI, NOR M.[1]*, SUGYO HASTOWO[2], BIBIANA LAY[2], AND DONALD H. DEAN[3]

[1]*Department of Microbiology, Faculty of Life Sciences, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Malaysia*
[2]*Bogor Agricultural University, Bogor 16144A, Bogor, Indonesia*
[3]*Department of Biochemistry, The Ohio State University, Columbus, OH 43210, USA*

Received: August 13, 1998

Abstract The *cryI* gene content of a collection of *Bacillus thuringiensis* strains, which include new isolates from Malaysia and Indonesia, was determined by a multiplex PCR using a set of eight oligonucleotide forward primers specific to *cryIAa, cryIAb, cryIAc, cryIBa, cryICa, cryIDa, cryIEa*, and *cryIFa* genes, and two reverse primers, one specific to *cryIAb* and the other common to the remaining *cryI* genes. Two-thirds of the 59 strains screened were *cryI* positive and contained one to four different genes. The *cry* gene profiles correlated well with toxicities of the strains to lepidopteran insects. The method can be used for rapid screening of a large number of new isolates as the total DNA extracted by boiling cells from single colonies can be used directly in the PCR. However, it is not suitable for follow-up monitoring of specific commercial strains after application in the field as the PCR product profiles of these strains could not be differentiated from those of new isolates.

Key words: *Bacillus thuringiensis*, PCR, *cryI* gene profile

Bacillus thuringiensis produces insecticidal protein toxins (δ-endotoxins) coded by *cry* genes [12] and to date, one hundred and twenty-four distinct *cry* genes have been characterized [8]. Several well-known *B. thuringiensis* strains, such as HD-1 and IPL-7, have been used for many years in conventional commercial insecticide formulations. *B. thuringiensis*-based bioinsecticides (BTBI) have been promoted as environment-friendly insecticides due to their biodegradability and lack of toxicity to mammals, birds, and nontarget organisms. However, over the last several years, there have been reports of development of resistance to BTBI among the target insect populations [9, 21, 28], indicating a continuing need to search for new strains with novel toxins. New isolates of *B. thuringiensis* can readily be obtained from soil [11, 20, 23], plant phylloplane [26], and animal feed mill residues [22]. However, the discovery of novel strains has been slow, due to the time-consuming and costly nature of insect bioassays.

The polymerase chain reaction (PCR) has been used extensively in studies of *cry* genes in *B. thuringiensis*. Carozzi *et al.* [4] and Ben-Dov *et al.* [1] have used PCR product profiles of *cry* genes as a tool to predict the insecticidal activity of *B. thuringiensis* strains. Bourque *et al.* [2] and Brousseau *et al.* [3] have recommended the use of PCR-based methods for monitoring the presence of specific commercialized strains after application in the field. Furthermore, the use of PCR-based approaches has led to the discovery of new variants of *cry* genes [1, 5, 6, 15, 16].

The present study was undertaken to determine the *cryI* gene profile of a range of *B. thuringiensis* strains obtained from different sources. The profiles were used to predict toxicities of the strains against lepidopteran insects and to identify novel strains.

MATERIALS AND METHODS

Bacterial Strains

Four groups of *B. thuringiensis* strains were used. The SN strains were obtained from the Department of Microbiology, Universiti Kebangsaan Malaysia. Strains SN-1 and SN-7 were reisolated from a commercial preparation (Bacillex, Shionogi, Japan). Strains SN-2, SN-3, and SN-4 are radiation-resistant mutant strains of SN-1 [14]. (In an earlier publication [14], SN-2, SN-3, and SN-4 were referred to as isolates 22, *a*, and 26, respectively; whereas SN-1 was referred to as BACILLEX.) The other SN strains were isolated from soil samples taken at various locations in Malaysia, using procedures

*Corresponding author
Phone: 60-3-8292697; Fax: 60-3-8252698;
E-mail: mahadi@pkrisc.cc.ukm.my

of Ohba and Aizawa [23]. The HD strains, except HD-1 and HD-9, were obtained from the Northern Regional Research Laboratory (NRRL), the US Department of Agriculture, Peoria, IL, U.S.A. HD-1 was obtained from the *Bacillus* Genetic Stock Center, The Ohio State University, Columbus, OH, U.S.A. HD-9 was obtained from Professor K. Aizawa, Kyushu University, Japan. The INA strains were isolated from soil samples taken from the following locations in Indonesia: Irian Jaya, Java, South Sulawesi, and Timor, as described previously [11]. The Dipel, Florbac, and Thuricide strains were reisolated from the respective commercial preparations (Dipel WP, Abbott Laboratories, U.S.A.; Florbac FC, Novo Nordisk, Denmark; Thuricide HP, Sandoz Ltd, Swilzerland). The serotypes of some of the SN isolates and all of the INA isolates were determined by Dr. Michio Ohba of the Institute of Biological Control, Kyushu University, Japan.

DNA Extraction

The template DNA was extracted directly from single colonies on solid growth medium [4]. For each strain, cells from a single overnight colony on nutrient agar (Difco) were resuspended in 10 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and boiled for 10 min. After centrifugation in a microfuge (15,000 rpm, 1 min), the supernatant fluid was used directly as a source of DNA template.

Oligonucleotide Primers

The primer mixture (kindly provided by Dr. Takashi Yamamoto, Sandoz Agro, Inc., Palo Alto, CA, U.S.A.) contained eight forward primers and two reverse primers and the sequences are as previously reported in Kalman *et al.* [16]. The forward primers are specific to eight *cryl* genes indicated below. One of the reverse primers is unique to *cry1Ab*, whereas the other is universal for the other seven *cryl* genes. The expected sizes of the PCR products are as follows: *cry1Aa*, 724 bp; *cry1Ab*, 238 bp; *cry1Ac*, 487 bp; *cry1Ba*, 830 bp; *cry1Ca*, 288 bp; *cry1Da*, 414 bp; *cry1Ea*, 883 bp; *cry1Fa*, 368 bp.

Polymerase Chain Reaction

Each PCR mixture was 10 µl in volume and contained the ten primers at 1 µM each, 200 µM deoxynucleoside triphosphates (Boehringer Mannheim, Germany), 1 µl 10× reaction buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin, pH 8.3), 0.25 unit *Taq* DNA Polymerase, and 1 µl crude DNA extract. The template DNA (kindly provided by Dr. Takashi Yamamoto) for control reactions consisted of mixed DNAs extracted from various *B. thuringiensis* strains and contained all the *cryl* genes screened except *cry1Ea* and *cry1Fa*. The reactions were performed in a thermal cycler (Perkin Elmer Cetus) for 25 cycles of 1 min at 94°C, 2 min at 52°C, and 3 min at 72°C with the final extension at 72°C for 7 min. The products of the reactions were analyzed by electrophoresis in 2.0% agarose.

Toxicity Test

The activity of the INA strains on *Bombyx mori* larvae was determined as described previously [11]. Ten 3rd instar larvae were fed with mulberry leaves smeared with 0.3 ml suspension of one or two loopfuls of sporulated culture from agar plates. Mortality was scored at 48 h as percent mortality.

RESULTS AND DISCUSSION

The multiplex PCR generated amplified DNA products that separated as clear and distinct bands in the agarose gel, confirming the specificity of the primers. Some of the results are shown in Fig. 1, and the profiles generated for all strains tested are given in Tables 1 and 2. HD-1, the reference strain used in this study and known to contain all three *cry1A* genes [12], produced the expected profile containing the three respective products of 724 bp, 238 bp, and 487 bp (Fig. 1A). Both Dipel and Thuricide formulations also contain HD-1 [17, 29]. However, the Thuricide strain screened in this study demonstrated a loss of the *cry1Ab* gene (Fig. 1B). Such a loss has been reported before for both Dipel [17] and Thuricide strains [29]; and in their screening using multiplex PCR, Bourque *et al.* [2] did not detect *cry1Ab* in either Dipel or Thuricide strain.

The other strains studied contained from one to four of the genes screened (Tables 1 and 2). Only six genes were detected and none of the strains contained *cry1Ea* or *cry1Fa*. For most strains belonging to serotype *aizawai* and *kurstaki*, the *cryl* gene profiles are similar to those reported in the literature [12]. Most of the *kurstaki* strains contained at least two *cry1A* genes whilst most of the *aizawai* strains contained only one of these genes. The majority of the *aizawai* strains also carry *cry1Ca* and *cry1Da*, whereas none of the *kurstaki* strains carry either of these genes. However, two of the INA strains (INA-02 and INA-03) belonging to *kurstaki* serotypes are quite unique. They contained only the *cry1Aa* gene. The INA-02 is indeed unique. It was reported earlier by Sasaki *et al.* [24] that, in addition to *cry1Aa*, INA-02 also contained the *cryV* gene.

In the study carried out by Carozzi *et al.* [4], HD-9, HD-11, and HD-68 were found to contain *cry1Aa*. This is supported by the present study. In addition, the use of specific primers for the various *cryl* genes in the present study also revealed that HD-9, HD-11, and HD-68 did not contain the other two *cry1A* genes (*cry1Ab* and *cry1Ac*),

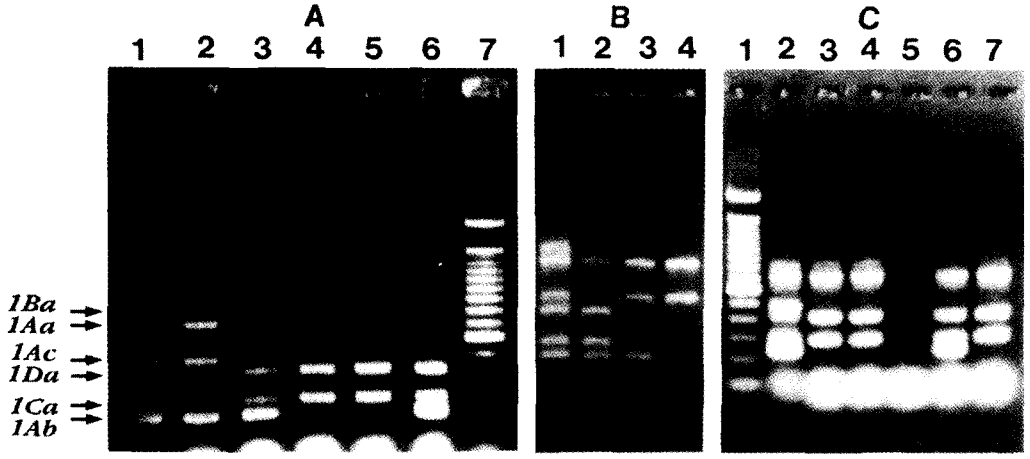

Fig. 1. Agarose gel (2.0%) electrophoresis of PCR products of DNA templates extracted from various strains of *Bacillus thuringiensis.*

A. Selected SN strains: 1, control templates; 2, HD-1 control; 3, SN-2; 4, SN-3; 5, SN-4; 6, SN-6; 7, 100 bp ladder. B. Commercial strains: 1, control templates; 2, Florbac strain; 3, Dipel strain; 4, Thuricide strain. C. Selected HD strains: 1, 100-bp ladder; 2, control templates; 3, HD-67; 4, HD-68; 5, HD-111, 6, HD-112; 7, HD-113.

Table 1. *cryI* gene content of SN and HD strains of *Bacillus thuringiensis.*

Strain	Serotype*	*cryI* gene							
		1Aa	*1Ab*	*1Ac*	*1Ba*	*1Ca*	*1Da*	*1Ea*	*1Fa*
SN-1	*aizawai*	-	+	-	-	+	+	-	-
SN-2	*aizawai*	-	+	-	-	+	+	-	-
SN-3	*aizawai*	-	-	-	-	+	+	-	-
SN-4	*aizawai*	-	-	-	-	+	+	-	-
SN-5	*kurstaki*	+	+	+	-	-	-	-	-
SN-6	*aizawai*	-	+	-	-	+	+	-	-
SN-7	*kurstaki*	+	+	+	-	-	-	-	-
SN-9	ND	-	-	-	-	-	-	-	-
SN-10	ND	-	-	-	-	-	-	-	-
SN-11	ND	-	+	-	-	+	+	-	-
SN-13	ND	-	-	-	-	+	+	-	-
SN-14	ND	+	+	+	-	-	-	-	-
SN-15	ND	+	-	+	-	-	-	-	-
SN-16	ND	-	-	-	-	-	-	-	-
SN-17	ND	-	-	+	-	-	-	-	-
SN-18	ND	-	-	-	-	-	-	-	-
SN-19	ND	+	-	+	-	-	-	-	-
HD-1	*kurstaki*	+	+	+	-	-	-	-	-
HD-9	*entomocidus*	+	-	-	+	+	+	-	-
HD-11	*aizawai*	+	-	-	-	-	-	-	-
HD-52	*aizawai*	+	-	-	-	+	+	-	-
HD-67	*aizawai*	+	-	-	-	+	+	-	-
HD-68	*aizawai*	+	-	-	-	+	+	-	-
HD-111	*aizawai*	-	-	-	-	-	-	-	-
HD-112	*aizawai*	+	+	-	-	+	+	-	-
HD-113	*aizawai*	+	-	-	-	+	+	-	-
HD-114	*aizawai*	+	-	-	-	-	-	-	-
HD-115	*aizawai*	+	-	-	-	+	+	-	-
HD-122	*aizawai*	+	-	+	-	+	+	-	-
HD-126	*aizawai*	+	-	-	-	-	-	-	-

*The serotyping for the SN strains was done by Dr. M. Ohba of the Institute of Biological Control, Kyushu University, Japan.
ND: not determined.

whilst HD-9 also contained *cry1Ba, cry1Ca*, and *cry1Da*, and HD-68 also contained *cry1Ca* and *cry1Da*.

Among the strains screened, nineteen contained none of the *cry1* genes used in the search. The 19 strains included two *aizawai* strains (INA-118 and HD-111) and all of the INA strains belonging to *entomocidus* serotype, which normally contain one or more *cry1* genes [12]. Thus, these strains may contain other or novel *cry* genes.

None of the strains screened gave PCR products of unexpected sizes. Thus, the *cry* genes detected are very likely to be similar in size to the holotype *cry* genes. However, this does not necessarily mean that the strains do not also carry variants of the known genes. Novel sequences present within or flanking the respective primer sets would not be detected using the primers employed in the present study. To enable such further characterization, Carozzi *et al.* [4] proposed the use of five to seven primer pairs of 200 bp apart and specific to the 1.8-kb variable region of a given *cry1* gene. Using such an approach, Kalman *et al.* [16] found a new variant of *cry1Ca*, and Chak *et al.* [6] found two distinct *cry1Ca*-type genes.

Bourque *et al.* [2] claimed that multiplex PCR can be used to monitor the presence of specific commercial strains in the field after application. To investigate this further, several commercial strains were screened and the resulting profiles were compared with those of other strains tested in this study. It was found that the Dipel strain profile was identical to that of a number of new isolates (SN-5, SN-14, and INA-76) as well as to one of the Bacillex strains (SN-7). Similarly, the profile of the Thuricide strain was identical to those of several other new isolates (SN-15, SN-19, INA-105, INA-106, INA-107, INA-108, and INA-109), and the Florbac strain profile was identical to that of HD-112. The profile shown by the other Bacillex strain (SN-1) is also shown by a number of other strains. Thus, the *cry1* gene profile as determined by the multiplex PCR used in this study appears to be insufficient for the monitoring stated above. Bourque *et al.* [2] also encountered similar difficulties as even different commercial strains produced similar profiles. In this regard, the proposal of Brousseau *et al.* [3], which involves the use of arbitrary primer PCR to determine fingerprints of different strains, is probably more feasible.

Twelve of the twenty-six INA strains were toxic to *B. mori* (Table 2). This toxicity correlates with their *cry1* profiles, as these strains contain either *cry1Aa* or *cry1Da* whose products are toxic to *B. mori* [7]. Incidentally, INA-02 was earlier reported to be toxic to two other lepidopteran species, *Spodoptera litura* and *Plutella xylostella*, and the toxicity to *S. litura* was found to be due to CryV protein [24].

Table 2. *cry1* gene content of INA strains of *Bacillus thuringiensis*.

Strain	Serotype	*cry1* gene								Toxicity to *Bombyx mori*
		1Aa	*1Ab*	*1Ac*	*1Ba*	*1Ca*	*1Da*	*1Ea*	*1Fa*	
INA-02	*kurstaki*	+	-	-	-	-	-	-	-	+
INA-03	*kurstaki*	+	-	-	-	-	-	-	-	+
INA-06	*tohokuensis*	-	-	-	-	-	-	-	-	-
INA-24	*entomocidus*	-	-	-	-	-	-	-	-	-
INA-26	*entomocidus*	-	-	-	-	-	-	-	-	-
INA-27	*entomocidus*	-	-	-	-	-	-	-	-	-
INA-29	*morrisoni*	-	+	-	-	-	+	-	-	+
INA-33	*morrisoni*	-	+	-	-	-	+	-	-	+
INA-37	*entomocidus*	-	-	-	-	-	-	-	-	-
INA-40	*entomocidus*	-	-	-	-	-	-	-	-	-
INA-45	*entomocidus*	-	-	-	-	-	-	-	-	-
INA-49	*entomocidus*	-	-	-	-	-	-	-	-	-
INA-57	*morrisoni*	-	+	-	-	-	+	-	-	+
INA-58	*entomocidus*	-	-	-	-	-	-	-	-	-
INA-67	*morrisoni*	-	+	-	-	-	+	-	-	+
INA-69	*entomocidus*	-	-	-	-	-	-	-	-	-
INA-76	*kurstaki*	+	+	+	-	-	-	-	-	+
INA-105	*kurstaki*	+	-	+	-	-	-	-	-	+
INA-106	*kurstaki*	+	-	+	-	-	-	-	-	+
INA-107	*kurstaki*	+	-	+	-	-	-	-	-	+
INA-108	*kurstaki*	+	-	+	-	-	-	-	-	+
INA-109	*kurstaki*	+	-	+	-	-	-	-	-	+
INA-113	NT	-	-	-	-	-	-	-	-	-
INA-114	NT	-	-	-	-	-	-	-	-	-
INA-118	*aizawai*	-	-	-	-	-	-	-	-	-
INA-136	*tohokuensis*	-	-	-	-	-	-	-	-	-

NT: not typable.

The toxicities of a few of the SN strains are known from previous studies, and examination of the *cry* gene profiles of these strains (Table 1) shows good correlation between the toxicities and the profiles. SN-2, which is highly toxic to *P. xylostella* larvae but shows low toxicity to *B. mori* [14], contained *cry1Ab*, but not *cry1Aa*. Cry1Ab protein has been shown to be highly toxic to *P. xylostella* [25], whereas Cry1Aa protein has been shown to contain a receptor-binding domain for the gut brush border membrane of *B. mori* [10, 18]. SN-3 and SN-4, which are mutant strains of SN-1, are less toxic than SN-2 towards *P. xylostella* [14]. This is also consistent with their *cry1* profile in that both mutant strains have lost *cry1Ab*, but still have *cry1Ca*. Cry1Ca protein is toxic to *P. xylostella* [7]. SN-5 has been found to be more toxic than HD-1 toward *P. xylostella* [19], *B. mori*, and *Choristoneura fumiferana* (van Frankenhuyzen, personal communications). Like HD-1, this strain contains all three *cry1A* genes and the products of all three genes are toxic to both *P. xylostella* and *C. fumiferana* [7]. The higher toxicity of SN-5 could be due to the presence of other Cry toxins or differences in the amount of the three Cry1A toxins expressed compared with HD-1.

The toxicity of the strains used in this study against other lepidopteran insects can be predicted based on their *cry1* gene profiles. Thus, half of the SN and HD strains screened should be toxic to *Spodoptera exigua* and *S. littoralis*, as they contain *cry1Ca* [31]. It can also be predicted that strains, such as SN-2 and HD-112, which contain a high number of different classes of *cry1* genes, are good candidates for prolonged use in the field because target insects will take a long time to develop resistance to such strains. It has been demonstrated that insects can quickly become resistant to a single toxin BTBI [27], whereas the use of multi-gene strains, such as *B. thuringiensis* subsp. *israelensis*, or mixtures of BTBIs containing different toxins, could delay development of resistance [30].

In the majority of cases, insect bioassays will probably confirm the prediction of toxicity based on *cry* gene profiles. However, since toxicity depends on the expression of the genes, some predictions may not be accurate. For example, the results of the present study would predict that HD-68 should be highly toxic to *S. littoralis* as it has the *cry1Ca* gene. However, using monoclonal antibodies, Höfte *et al.* [13] did not detect Cry1Ca protein (type C protein) in the parasporal crystals of HD-68 and the crystals were not toxic to *S. littoralis*. Thus, to select the most effective SN or HD strain for the control of *Spodoptera* species, bioassays are still needed.

Screening by PCR has allowed rapid determination of *cry* gene profiles of a large number of *B. thuringiensis* strains. The profiles can significantly reduce the number of strains that need to be bioassayed when the objective is to choose the most suitable strains for specific target insects. The profiles obtained with primers used in this study, however, are unsuitable for use in monitoring the presence of specific strains after application in the field. This screening also detected several possible novel strains which can be further tested with regard to their toxicity and for the presence of novel *cry* genes. These strains may provide additional tools for management of insect resistance to *B. thuringiensis* or for controlling insects which are currently not susceptible to BTBIs.

Acknowledgments

This work was supported in part by IRPA Grant No. 1-07-03-003 from the Ministry of Science, Technology and Environment, Malaysia. We are grateful to Mohd Jasmali Yusop and Zarina Samsudin for their technical assistance. The authors wish to thank Dr. Sheila Nathan for reading the manuscript.

REFERENCES

1. Ben-Dov, E., A. Zaritsky, E. Dahan, Z. Barak, R. Sinai, R. Manasherob, A. Khamraev, E. Troitskaya, A. Dubitsky, N. Berezina, and Y. Margalith. 1997. Extended screening by PCR for seven *cry*-group genes from field collected strains of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **63:** 4883–4890.
2. Bourque, S. N., J. R. Valero, J. Mercier, M. C. Lavoie, and L. C. Levesque. 1993. Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **59:** 523–527.
3. Brousseau, R., A. Saint-Onge, G. Prefontaine, L. Masson, and J. Cabana. 1993. Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. *Appl. Environ. Microbiol.* **59:** 114–119.
4. Carozzi, N. B., V. C. Kramer, G. W. Warren, S. Evola, and M. G. Koziel. 1991. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. *Appl. Environ. Microbiol.* **57:** 3057–3061.
5. Ceron, J., A. Ortis, R. Quintero, L. Guereca, and A. Bravo. 1995. Specific PCR primers to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. *Appl. Environ. Microbiol.* **61:** 3826–3831.
6. Chak, K.-F., D.-C. Chao, M.-Y. Tseng, S.-S. Kao, S. J. Tuan, and T.-Y. Feng. 1994. Determination and distribution of *cry*-type genes of *Bacillus thuringiensis* isolates from Taiwan. *Appl. Environ. Microbiol.* **60:** 2415–2420.
7. Chilcott, C. N. and P. J. Wigley. 1994. Insecticidal activity of *Bacillus thuringiensis* crystal proteins, pp. 43–52. *In* R. J. Akhurst (ed.), Proceedings of the 2nd Canberra Meeting on *Bacillus thuringiensis*. CSIRO, Canberra.

8. Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, and D. H. Dean. 1998. *Bacillus thuringiensis* toxin nomenclature. http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html (April 30, 1998).
9. Ferre, J., M. D. Real, J. VanRie, S. Jansens, and M. Peferoen. 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. *Proc. Natl. Acad. Sci. USA* **88:** 5119–5123.
10. Ge, A. Z., N. I. Shivarova, and D. H. Dean. 1989. Location of the *Bombyx mori* specificity domain on a *Bacillus thuringiensis* delta-endotoxin. *Proc. Natl. Acad. Sci. USA* **86:** 4037–4041.
11. Hastowo, S., B. W. Lay, and M. Ohba. 1992. Naturally occurring *Bacillus thuringiensis* in Indonesia. *J. Appl. Bacteriol.* **73:** 108–113.
12. Höfte, H. and H. R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis. Microbiol. Rev.* **53:** 242–255.
13. Höfte, H., J. VanRie, S. Jansens, A. Van Houtven, H. Vanderbruggen, and M. Vaeck. 1988. Monoclonal antibody analysis and insecticidal spectrum of three types of lepidopteran-specific insecticidal crystal proteins of *Bacillus thuringiensis. Appl. Environ. Microbiol.* **54:** 2010–2017.
14. Jangi, M. S. and H. Ibrahim. 1983. Toxicity of radiation-resistant strains of *Bacillus thuringiensis* (Berl.) to larval *Plutella xylostella* (L.). *Can. J. Microbiol.* **29:** 552–557.
15. Juarez-Perez, V. M., M. D. Ferrandis, and R. Frutos. 1997. PCR-based approach for detection of novel *Bacillus thuringiensis cry* genes. *Appl. Environ. Microbiol.* **63:** 2997–3002.
16. Kalman, S., K. L. Kiehne, J. L. Libs, and T. Yamamoto. 1993. Cloning of a novel *cry1Ca*-type gene from a strain of *Bacillus thuringiensis* subsp. *galleriae. Appl. Environ. Microbiol.* **59:** 1131–1137.
17. Kronstad, J. W., H. E. Schnepf, and H. R. Whiteley. 1983. Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *J. Bacteriol.* **154:** 419–428.
18. Lee, M. K., R. E. Milne, A. Z. Ge, and D. H. Dean. 1992. Location of *Bombyx mori* receptor binding region on a *Bacillus thuringiensis* δ-endotoxin. *J. Biol. Chem.* **267:** 3115–3121.
19. Mahadi, N. M., M. S. Jangi, and W. M. Yusof. 1991. Production of the bioinsecticides *Bacillus thuringiensis* and baculovirus, pp. 30–38. *In* A. H. Zakri, S. Ismail, and M. N. Embi (eds.), Research Priorities for Development of Science and Technology. Universiti Kebangsaan Malaysia Publisher, Bangi.
20. Martin, P. A. W. and R. S. Travers. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl. Environ. Microbiol.* **55:** 2437–2442.
21. McGaughey, W. H. and M. E. Whalon. 1992. Managing insect resistance to *Bacillus thuringiensis* toxins. *Science* **258:** 1451–1455.
22. Meadows, M. P., D. J. Ellis, J. Butt, P. Jarrett, and H. D. Burges. 1992. Distribution, frequency, and diversity of *Bacillus thuringiensis* in animal feed mill. *Appl. Environ. Microbiol.* **58:** 1344–1350.
23. Ohba, M. and K. Aizawa. 1986. Insect toxicity of *Bacillus thuringiensis* isolated from soils of Japan. *J. Invertebr. Pathol.* **47:** 12–20.
24. Sasaki, J., S. Asano, T. Iizuka, H. Bando, B. W. Lay, S. Hastowo, G. K. Powell, and T. Yamamoto. 1996. Insecticidal activity of the protein encoded by the *cryV* gene of *Bacillus thuringiensis kurstaki* INA-02. *Curr. Microbiol.* **32:** 195–200.
25. Shimizu, Y., K. Oshie, K. Nakamura, Y. Takada, K. Oeda, and H. Ohkawa. 1988. Cloning and expression in *Escherichia coli* of the 135-kDa insecticidal protein gene from *Bacillus thuringiensis* subsp. *aizawai* IPL7. *Agric. Biol. Chem.* **52:** 1565–1573.
26. Smith, R. A. and G. A. Couche. 1991. The phylloplane as a source of *Bacillus thuringiensis* variants. *Appl. Environ. Microbiol.* **57:** 311–315.
27. Stone, T. B., S. R. Sims, and P. G. Marrone. 1989. Selection of tobacco budworm for resistance to a genetically engineered *Pseudomonas fluorescens* containing the delta-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki. J. Invertebr. Pathol.* **53:** 228–234.
28. Tabashnik, B. E., N. L. Cushing, N. Finson, and M. W. Johnson. 1990. Field development of resistance to *Bacillus thuringiensis* in diamond-back moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* **83:** 1671–1676.
29. Van Frankenhuyzen, K., R. Milne, R. Brousseau, and L. Mason. 1992. Comparative toxicity of the HD-1 and NRD-12 strains of *Bacillus thuringiensis* subsp. *kurstaki* to defoliating forest lepidoptera. *J. Invertebr. Pathol.* **59:** 149–154.
30. VanRie, J. 1994. Biochemical mechanism of insect resistance to *Bacillus thuringiensis*, pp. 31–36. *In* R. J. Akhurst (ed.), Proceedings of the 2nd Canberra Meeting on *Bacillus thuringiensis*. CSIRO, Canberra.
31. Visser, B., T. van der Salm, W. van den Brink, and G. Folkers. 1988. Genes from *Bacillus thuringiensis entomocidus* 60.5 coding for insect-specific crystal proteins. *Mol. Gen. Genet.* **212:** 219–224.