sure. Had the pillar been completely immersed in a solution or in soil, the rate of corrosion of the Delhi pillar iron would have been comparable to modern iron and steels. This has been verified by corrosion studies on other ancient Indian phosphoric irons (for example, see ref. 25). The passive film prevents ingress of atmospheric corrosion due to the benign nature of the exposure environment.

The wetting time (due to rainfall and atmospheric conditions) of the Delhi iron pillar has been estimated for a period of one year. A non-steady state heat-transfer mathematical model has been applied to determine the wetting of the Delhi iron pillar based on environmental conditions. The estimated wetting times due to environmental conditions were two orders of magnitude lower than those due to rainfall. Based on the known wetting times, the anticipated film thickness on the surface of the Delhi iron pillar has been predicted, which is much higher than the actual rust thickness on the pillar. The importance of the protective passive film mechanism of corrosion resistance of the Delhi iron pillar has, therefore, been emphasized.

1. Balasubramaniam, R., *Delhi Iron Pillar: New Insights*, Indian Institute of Advanced Study, Shimla, 2002.
2. Balasubramaniam, R., On the corrosion resistance of the Delhi iron pillar. *Corros. Sci.*, 2000, **42**, 2103.
3. Balasubramaniam, R. and Ramesh Kumar, A. V., Characterization of Delhi iron pillar rust by X-ray diffraction, Fourier infrared spectroscopy and Mössbauer spectroscopy. *Corros. Sci.*, 2000, **42**, 2085.
4. Balasubramaniam, R., On the growth kinetics of the protective passive film of the Delhi iron pillar. *Curr. Sci.*, 2002, **82**, 1357.
5. Hudson, J. C., The Delhi iron pillar. *Nature*, 1953, **172**, 499.
6. Wranglen, G., The rustless iron pillar at Delhi. *Corros. Sci.*, 1970, **10**, 761.
7. Sanyal, B. and Preston, R., *Note on Delhi Pillar*, Chemical Research Laboratory, London, 1952.
8. Bardgett, W. E. and Stanners, J. F., Delhi iron pillar – A study of the corrosion aspects. *J. Iron Steel Inst.*, 1963, **210**, 3; *NML Tech. J.*, 1963, **5**, 24.
9. Holman, J. P., *Heat Transfer*, McGraw Hill, New York, 1997, 8th edn.
10. Evans, U. R. and Taylor, C. A. J., Mechanism of atmospheric rusting. *Corros. Sci.*, 1972, **12**, 227.
11. Stratmann, M., The investigation of the corrosion properties of metals covered with adsorbed electrolyte layers – a new experimental technique. *Corros. Sci.*, 1987, **27**, 869.
12. Stratmann, M. and Streckel, H., On the atmospheric corrosion of metals which are covered with thin electrolyte layers – I. Verification of the experimental technique. *Corros. Sci.* 1990, **30**, 681.
13. Stratmann, M. and Streckel, H., On the atmospheric corrosion of metals which are covered with thin electrolyte layers – II. Experimental results. *Corros. Sci.*, 1990, **30**, 697.
14. Stratmann, M. and Streckel, H., On the atmospheric corrosion of metals which are covered with thin electrolyte layers – III. The measurement of polarization curves on metal surfaces which are covered by thin electrolyte layers. *Corros. Sci.*, 1990, **30**, 715.
15. Balasubramaniam, R., New insights on the corrosion of the Delhi iron pillar based on historical and dimensional analysis. *Curr. Sci.*, 1997, **73**, 1057.
16. http://www.geocities.com/CapeCanaveral/Galaxy/4707/Aeronotes/ATMOprog.htm, November 2002.
17. ASM Metals Reference Book, 1993, 3rd edn, p. 152.
18. http: //www.newportus.com/Products/Techncal/MetlEmty.htm, November 2002.
19. Sastry, S. S., *Introductory Methods of Numerical Analysis*, Prentice Hall of India Pvt Ltd, New Delhi, September 1999, 3rd edn.
20. Ghoshdastidar, P. S., *Computer Simulation of Flow and Heat Transfer*, Tata McGraw Hill, New Delhi, 1998, 1st edn.
21. http://www.polymath-software.com, November 2002.
22. http://www.underground.com, November 2002.
23. Ghosh, M. K., The Delhi iron pillar and its iron. *NML Techn. J.*, 1963, **5**, 31.
24. Leygraf, C., Atmospheric corrosion. In *Corrosion Mechanisms in Theory and Practice* (ed. Marcus, P.), Marcel Dekker, New York, 2002, 2nd edn, pp. 529–562.
25. Balasubramaniam, R. and Ramesh Kumar, A. V., Corrosion resistance of the Dhar iron pillar. *Corros. Sci.*, 2003, **45**, 2451.

Received 15 July 2003; revised accepted 17 September 2003

Analysis of Cry2A proteins encoded by genes cloned from indigenous isolates of *Bacillus thuringiensis* for toxicity against *Helicoverpa armigera*

Shantanu Kumar[†], V. Udayasuriyan[#,*], P. Sangeetha[#] and M. Bharathi[#]

[†]Centre of Advanced Studies in Agriculture Microbiology, and [#]Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore 641 003, India

The Cry2A proteins of *Bacillus thuringiensis* are promising candidates for management of resistance development in insects due to their differences from the currently used Cry1A proteins, in structure and insecticidal mechanism. The Cry2Aa and Cry2Ab proteins isolated from recombinant *Escherichia coli* strains harbouring indigenous *cry2Aa* and *cry2Ab* genes under the control of T7 promoter were tested for toxicity against *Helicoverpa armigera* (Hubner). In artificial diet bioassay, inclusions containing Cry2Aa and urea-solubilized Cry2Aa protein showed 100% mortality of *H. armigera* at a concentration of 650 and 100 ng ml^{-1} after 48 and 72 h respectively. On the other hand, the Cry2Ab protein inclusions as well as urea-solubilized Cry2Ab protein were not toxic to *H. armigera*, probably due to lack of solubility in alkaline condition and improper folding after urea solubilization.

BACILLUS thuringiensis (*Bt*) is a soil bacterium that produces one or more crystalline inclusion bodies containing specific insecticidal protein(s) or δendotoxin(s)[1]. The final toxicity of *Bt* is the result of a series of events, including solubilization of crystal, activation of protoxin by gut

*For correspondence. (e-mail: udayvar@hotmail.com)

proteases, recognition of binding sites on the brush-border membrane and post binding events such as channel formation and intracellular signalling[2,3].

Transgenic *Bt*-cotton expressing Cry1Ac has been permitted for commercial cultivation in India during the year 2002; it primarily targets *Helicoverpa armigera*. Continuous exposure to a single kind of *Bt* toxin can lead to resistance development in insects. Kranthi *et al.*[4] reported 76-fold resistance development in an Indian population of *H. armigera* against Cry1Ac after ten generations of selection. However, the Cry1Ac-resistant *H. armigera* was not cross-resistant to Cry2Aa[5]. Routine replacement of *cry* genes or pyramiding of *cry* genes could be useful for effective control of insect pests by *Bt* transgenic plants. Due to the difference in structure and insecticidal mechanism[6], *cry2A* genes are promising candidates for management of resistance development in insects. The combination of *cry2Ab* and *cry1Ac* gene in Bollgard® II cotton has been permitted for commercial use in USA. It has been shown that Bollgard® II provides superior control of lepidopteran pest and is expected to have positive implication for resistance management, especially with respect to cotton bollworm[7]. To the best of our knowledge the toxicity of Cry2Ab protein against the Indian population of *H. armigera* has not been studied so far. The Indian populations of *H. armigera* are significantly less susceptible to Cry2Aa than Cry1Ac[8,9]. Variations of a single amino acid can significantly influence the level of toxicity in Cry proteins[10,11]. Hence, the Cry2A proteins of new *Bt* isolates may have more toxicity due to sequence variation. Therefore, in the present study Cry2Aa and Cry2Ab proteins encoded by genes cloned from new isolates of *Bt* were tested for toxicity against cotton bollworm, *H. armigera*.

Cloning of *cry2Aa* orf3 and *cry2Ab* orf from indigenous *Bt* isolates (22-4 and 22-11 respectively) and their expression in *Escherichia coli* were confirmed by partial sequencing and by Western analysis respectively[12]. Recombinant *E. coli*, BL21 (λDE3) harbouring pET2Aa (pET29a + *cry2Aa* orf3 of *Bt* strain 22-4), pET2Ab (pET29a + *cry2Ab* orf of *Bt* strain 22-11) or pET29a vector alone were grown in LB broth containing kanamycin 50 μg/ml at 37°C till OD_{600} = ~0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to each flask at a final concentration of 1 mM. Cells were further grown at 37°C for another 6 h (OD_{600} = ~1.3). This was followed by centrifugation and sonication of the cells in TE buffer containing 1 mM PMSF, until more than 90% of the cells were broken. Sonicated cells were centrifuged at 7000 *g* for 15 min at 4°C. The pellet was suspended in the TE buffer containing 1.0% triton X-100 and washed twice in the same buffer. Finally, the pellet was dissolved in a small volume of sterile double distilled water. SDS–PAGE analysis[13] of proteins purified from recombinant *E. coli* strains showed a prominent band of ~65 kDa in case of pET2Aa and pET2Ab, whereas there was no distinct band of that size in case of pET control (Figure 1). Other prominent protein bands (below ~65 kDa) as visualized in Figure 1, were similar to the control. Alpha imager gel documentation system (AlphaInnotech, USA) was used to quantify the expressed Cry2Aa/Cry2Ab proteins (prominent band of ~65 kDa) in the Coomassie brilliant blue-stained gel by densitometric scanning in comparison with different concentrations (0.5 to 3.0 μg lane^{-1}) of BSA as standard. Based on the concentration of CryAa/Cry2Ab in the cell extracts of *E. coli*, dilutions were made with sterile double distilled water to get 650 and 1300 ng ml^{-1} of Cry2Aa/Cry2Ab protein.

For bioassay experiments, a laboratory culture of *H. armigera* which was originally initiated from field-collected larvae, was obtained from the Department of Entomology, Tamil Nadu Agricultural University (TNAU), Coimbatore and reared on a semi-synthetic diet[14]. Ten micro-litres of Cry2Aa/Cry2Ab inclusions (650 or 1300 ng ml^{-1} protein) was applied on the artificial diet contained in 1.8 ml cryovial (Tarson®) and allowed to dry for 30 min. Neonate larvae were released at the rate of one per vial and they were closed halfway with a screw cap, leaving space for gas exchange. All these processes were done in a laminar airflow chamber. A treatment without Cry protein served as control. Thirty larvae were used per treatment and each treatment was replicated thrice. Mortality of larvae was scored every 24 h for seven days. The surviving larvae were weighed on the 7th day. All the experiments were carried out in a room with a photoperiod of 14 : 10 (L : D) and at an average temperature of 27°C; 60% RH. Higher dose of Cry2Aa protein (1300 ng ml^{-1}) resulted in 100% mortality after 24 h, whereas lower dose (650 ng ml^{-1}) re-

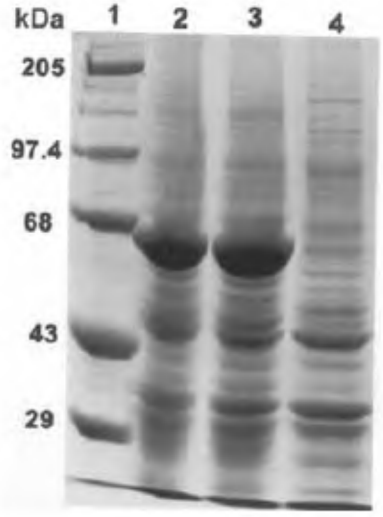

Figure 1. Recombinant *E. coli* harbouring pET2Aa, pET2Ab and pET29a (control) was grown in 25 ml LB broth with IPTG induction. Cells were harvested by centrifugation and sonicated in TE buffer. After partial purification, inclusions were suspended in 250 μl double distilled sterile water. Each sample (3.0 μl) was analysed on 0.8% SDS–PAGE and stained with Coomassie brilliant blue R-250. Lane 1, Protein molecular weight marker; lanes 2–4, Proteins from *E. coli* harbouring pET2Aa; pET2Ab; and pET29a respectively.

Table 1. Toxicity of Cry2Aa and Cry2Ab protein inclusions against *H. armigera*

Treatment	Mortality[a] (per cent)			Surviving larval weight on day 7 (mg)
	Day 1	Day 2	Day 7	Mean ± SD
Cry2Aa (650 ng ml^{-1})	76.67 ± 3.5	100 ± 0	100 ± 0	–
Cry2Aa (1300 ng ml^{-1})	100 ± 0	100 ± 0	100 ± 0	–
Cry2Ab (650 ng ml^{-1})	0.0	0.0	0.0	74.53 ± 4.5
Cry2Ab (1300 ng ml^{-1})	0.0	0.0	0.0	69.06 ± 3.4
Control	0.0	0.0	0.0	71.67 ± 7.8

[a]Mean of three replications, 30 larvae for each replication.

sulted in 100% mortality of *H. armigera* neonates after 48 h. The Cry2Ab and control treatments did not show any mortality of *H. armigera* till the 7th day. There was no significant difference in the surviving larval weight between the Cry2Ab and control treatments (Table 1). These observations conclude that Cry2Aa proteins produced by recombinant *E. coli* was toxic, whereas Cry2Ab proteins were non-toxic to *H. armigera* neonates.

In order to know the reasons for non-toxicity of Cry2Ab protein, attempts were made to study the solubility of Cry2Aa/Cry2Ab proteins in alkali and urea buffers. Initially, Cry2Aa/Cry2Ab protein inclusions were incubated with 50 mM NaOH, 1 mM phenyl methyl sulphonyl fluoride (PMSF) at pH 12.0 for 30 min and 60 min at 37°C, with shaking. The samples were centrifuged at 10000 *g* to remove undissolved material and the supernatant was analysed on SDS–PAGE for the presence of soluble Cry2Aa/Cry2Ab proteins. Solubilized Cry2Aa protein of ~65 kDa was noticed in the supernatant after 30 and 60 min incubation whereas solubilized Cry2Ab protein of ~65 kDa was not noticed (Figure 2). Other prominent bands below 65 kDa (Figure 2) are similar to those of control (Figure 1, lane 4). The Cry2Ab protein was further incubated for 6 h in NaOH buffer, but solubilized protein was not detected even after 6 h of incubation (data not shown).

Another attempt was made to solubilize the Cry2Ab protein in carbonate/bicarbonate buffer (50 mM Na_2CO_3/$NaHCO_3$, 10 mM dithiothreitol) at different pH values, viz. 8.2, 10.0, 10.5 and 11.2 for a maximum of 6 h at 37°C. But Cry2Ab protein was not solubilized and hence not detected in SDS–PAGE (data not shown). In order to increase the solubility of Cry2Ab protein expressed in *E. coli*, it was allowed to grow at a relatively low temperature (25 and 30°C) and with low concentration of IPTG induction (0.5 mM). Proteins harvested from *E. coli* grown at low temperature and with less concentration of IPTG induction were subjected to solubilization in NaOH buffer as described earlier. Again, solubilized Cry2Ab protein could not be detected in SDS–PAGE (data not shown). Alternatively, Cry2Aa/Cry2Ab protein inclusions were solubilized in 8 M urea, 200 mM NaCl, 10 mM dithiothreitol, 0.1 mM PMSF, 50 mM $NaHCO_3$ (pH 10.0) for 6 h. The samples were centrifuged at 10000 *g* to remove

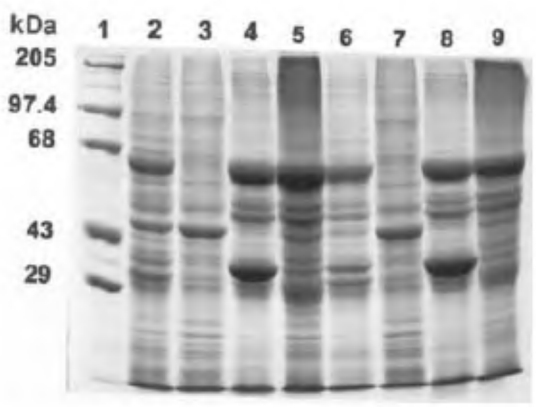

Figure 2. Four milligram pellet of *E. coli* extract containing Cry2Aa or Cry2Ab protein was mixed with 50 µl NaOH buffer and incubated for 30 and 60 min. After incubation, samples were centrifuged and supernatant was taken out. Residual pellet was resuspended in 50 µl of double distilled sterile water. Ten micro-litres of soluble and insoluble protein fraction was analysed on 0.8% SDS–PAGE and stained with Coomassie brilliant blue R-250. Lane 1, Protein molecular weight marker; lanes 2 and 3, Solubilized Cry2Aa and Cry2Ab protein respectively, after 30 min of incubation; lanes 4 and 5, Residual pellet of Cry2Aa and Cry2Ab obtained after 30 min of incubation; lanes 6 and 7, Solubilized Cry2Aa and Cry2Ab proteins obtained after 1 h of incubation; lanes 8 and 9, Residual pellet of Cry2Aa and Cry2Ab after 1 h of incubation.

undissolved material and the supernatant was dialysed against the same buffer without urea for 16 h at 4°C. Solubilized proteins were analysed on SDS–PAGE. An intact band of ~65 kDa was recorded in the case of Cry2Aa and Cry2Ab proteins (Figure 3). Other prominent bands below 65 kDa shown in Figure 3 are similar to those of the control (Figure 1, lane 4). Estimation of proteins and their dilutions was made (as described earlier) with sterile double distilled water to get 100 and 400 ng ml^{-1} of Cry2Aa/Cry2Ab protein.

Another bioassay experiment was conducted with urea-solubilized and dialysed Cry2Aa/Cry2Ab proteins using *H. armigera* neonates, as described earlier. *H. armigera* neonates fed on Cry2Aa protein (100 ng ml^{-1})-coated diet showed 100% mortality after 48 h. On the other hand, all the larvae survived till the 7th day in case of solubilized Cry2Ab protein treatments (100 and 400 ng ml^{-1}) as well as control treatments. Larval weight recorded after seven days did not show much variation between the control and Cry2Ab treatments (Table 2). These observations conclu-

Table 2. Toxicity of urea-solubilized Cry2Aa and Cry2Ab proteins against *H. armigera*

Treatment	Mortality[a] (per cent)				Surviving larval weight on day 7 (mg)
	Day 1	Day 2	Day 3	Day 7	Mean ± SD
Cry2Aa (100 ng ml^{-1})	35.63 ± 4.9	71.13 ± 6.7	100 ± 0	100 ± 0	–
Cry2Ab (100 ng ml^{-1})	0.0	0.0	0.0	0.0	65.86 ± 6.4
Cry2Ab (400 ng ml^{-1})	0.0	0.0	0.0	0.0	70.86 ± 6.1
Buffer control[b]	0.0	0.0	0.0	0.0	69.67 ± 9.0
Control	0.0	0.0	0.0	0.0	72.43 ± 7.1

[a]Mean of three replications, 30 larvae for each replication.
[b]Urea buffer dialysed to remove urea.

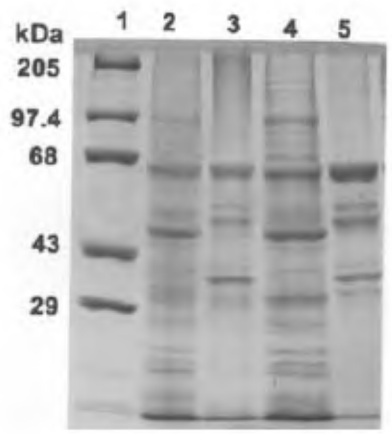

Figure 3. Pellet of *E. coli* cell extract containing Cry2Aa or Cry2Ab was solubilized in urea buffer (in 1 : 1 w/v ratio) for 6 h and centrifuged to collect the supernatant. Residual pellets were resuspended in double distilled sterile water. Supernatant was dialysed against a buffer without urea. Dialysed Cry2A proteins and insoluble Cry2A proteins were analysed on 0.8% SDS–PAGE and stained with Coomasie brilliant blue R-250. Lane 1, Protein molecular weight marker; lanes 2 and 3, Urea-solubilized Cry2Aa and Cry2Ab proteins; lanes 4 and 5, Residual Cry2Aa and Cry2Ab proteins after urea solubilization.

ded that urea-solubilized Cry2Aa (produced by recombinant *E. coli*) was toxic at the concentration of 100 ng ml^{-1}, but four times higher concentration of Cry2Ab was not toxic to *H. armigera* neonates.

In the present study, we determined the toxicity variation of Cry2Aa and Cry2Ab proteins (87% homologus) on the Indian population of *H. armigera*. In this context, the *cry2Aa* and *cry2Ab* genes from new isolates of *Bt*, 22-4 and 22-11 were expressed in *E. coli* under the control of the T7 promoter. Bioassay experiment using cell extract of recombinant *E. coli* expressing Cry2Aa protein showed toxicity to *H. armigera* neonates, whereas cell extract of recombinant *E. coli* expressing Cry2Ab protein did not show mortality or growth inhibition of *H. armigera* neonates. Solubility of Cry proteins at alkaline pH is the major step that determines toxicity towards insects. Therefore, solubilization studies were performed and the results showed that Cry2Aa protein was soluble at alkaline pH, but Cry2Ab protein was not soluble at alkaline pH ranging from 8.2 to 12.0 at different time intervals (30 min – 6 h). Solubility of foreign protein expressed in *E. coli* can be improved by reducing the growth temperature and inducer concentration[15]. In this regard *E. coli* cells expressing the Cry2Ab protein were grown at 25 and 30°C with reduced IPTG concentration (0.5 mM), but Cry2Ab protein expressed at low temperature with reduced IPTG concentration also could not be solubilized in alkali buffer.

Widner and Whiteley[16] reported the expression of *cry2Aa* and *cry2Ab* genes by *lac* promoter in *E. coli*. Earlier workers[17,18] have also expressed the *cry2Aa* genes in *E. coli* under the control of the T7 promoter, and the expressed Cry2Aa protein was biologically active. When a foreign gene is expressed under the control of the T7 promoter, a high-level expression could be achieved within 3–6 h after induction. Therefore, we preferred T7 promoter-based vector system for expressing *cry2Aa* and *cry2Ab* genes. In the present investigation also *cry2Aa* and *cry2Ab* were successfully expressed by the T7 promoter, but the Cry2Ab inclusions were not soluble at alkaline pH, probably due to the amino acid sequence variation between the Cry2Aa and Cry2Ab proteins.

High-level expression of protein in *E. coli* may result in the formation of insoluble aggregates. To obtain soluble active protein, the inclusion has to be denatured and the released denatured protein is subsequently allowed to refold[19]. Similarly, aggregates isolated from *E. coli* cells expressing Cry2Aa and Cry2Ab proteins were solublized in a buffer containing 8 M urea. The Cry2Aa and Cry2Ab proteins remain in solution after dialysis. The dialysed sample of Cry2Aa protein (100 ng ml^{-1}) was toxic to *H. armigera* neonates. In contrast, solubilized Cry2Ab protein (400 ng ml^{-1}) proved nontoxic to *H. armigera* neonates. This observation suggests that the Cry2Ab protein does not refold correctly after urea solubilization.

Several Cry proteins have been expressed in heterologous hosts like *Bacillus polymixa*[20] and *E. coli*[21,22], and the proteins expressed in *E. coli* showed biological activity. To the best of our knowledge, there is no earlier report where native (wild type) *cry2Aa* or *cry2Ab* gene expressed in *E. coli* resulted in biologically inactive proteins. However, Bosch *et al.*[23] reported that the engineered

Cry1A protein becomes insoluble and biologically inactive when expressed in *E. coli*. Here we report that the wild type Cry2Ab protein when expressed in *E. coli* was not toxic, probably due to the lack of its solubility in alkaline pH and improper refolding after solubilization in urea. Under similar conditions, when the *cry2Aa* gene was expressed in *E. coli* it showed biological activity. This information suggests that the amino acid sequence variation between these two highly homologous proteins plays an important role in their solubility and refolding after solubilization in urea. Since *cry2Ab* gene is cryptic in nature, another way to get biologically active Cry2Ab protein is by expressing under the control of *cry3A*[24] promoter in acrystalliferous *Bt* strain.

Proteins of indigenous *cry2Aa* genes cloned by Misra *et al.*[17] did not show significant toxicity against *H. armigera*, but were active against *Spodoptera litura*. The protein encoded by *cry2Aa* gene in our study showed 100% mortality of *H. armigera*, and hence it could be useful for the control of polyphagous *H. armigera* in Indian agriculture. Further studies are being carried out to compare toxicity of the newly cloned Cry2Aa with that of Cry1Ac as well as other Cry2Aa proteins against important crop pests such as *H. armigera*, *S. litura*, *Plutella xylostella*, etc.

1. Hofte, H. and Whiteley, H. R., Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.*, 1989, **53**, 242–255.
2. English, L. and Slatin, S. L., Mode of action of delta-endotoxins from *Bacillus thuringiensis*: a comparison of other bacterial toxins. *Insect Biochem. Mol. Biol.*, 1992, **22**, 1–7.
3. Agrawal, N., Malhotra, P. and Bhatnagar, R. K., Interaction of gene-cloned and insect cell-expressed aminopeptidase N of *Spodoptera litura* with insecticidal crystal protein Cry1C. *Appl. Environ. Microbiol.*, 2002, **68**, 4583–4592.
4. Kranthi, K. R., Kranthi, S., Ali, S. and Banerjee, S. K., Resistance of Cry1Ac δendotoxin of *Bacillus thuringiensis* in a laboratory selected strain of *Helicoverpa armigera* (Hubner). *Curr. Sci.*, 2000, **78**, 1001–1004.
5. Akhurst, R., Games, B. and Bird, L., Proceedings of the 10th Australian Cotton Conference, Brisbane, 2000.
6. Morse, R. J., Yamamoto, T. and Stroud, R. M., Structure of Cry2Aa suggests an unexpected receptor binding epitope. *Structure*, 2001, **9**, 409–417.
7. Perlak, F. J. *et al.*, Development and commercial use of Bollgard® cotton in the USA – early promises versus today's reality. *Plant J.*, 2001, **27**, 489–501.
8. Chakrabarti, S. K., Mandaokar, A., Kumar, A. P. and Sharma, R. P., Efficacy of lepidopteran-specific delta-endotoxin of *Bacillus thuringiensis* against *Helicoverpa armigera*. *J. Invertebr. Pathol.*, 1998, **72**, 336–337.
9. Babu, B. G., Udayasuriyan, V., Asia Mariam, M., Sivakumar, N., Bharathi, M. and Balasubramanian, G., Comparative toxicity of Cry1Ac and Cry2Aa δendotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera* (H.). *Crop Protect.*, 2002, **21**, 817–822.
10. Rajamohan, F., Hussain, S. R. A., Cotrill, J. A., Gould, F. and Dean, H. D., Mutation in domain II, loop 3 of *Bacillus thuringiensis cry1Aa* and *cry1Ab* delta endotoxin suggested loop 3 is involved in initial binding of lepidopteran midgets. *J. Biol. Chem.*, 1996, **271**, 25220–25225.
11. Udayasuriyan, V., Nakamura, A., Mori, A., Masaki, H. and Uozumi, T., Cloning of a new *crylA(a)* gene from *Bacillus thuringiensis* strain FU-2-7 and analysis of chimeric *cry1A(a)* proteins of toxicity. *Biosci. Biotechnol. Biochem.*, 1994, **58**, 830–835.
12. Shantanu Kumar and Udayasuriyan, V., Cloning of *cry2Aa* and *cry2Ab* genes from new isolates of *Bacillus thuringiensis* and their expression in recombinant *Bacillus thuringiensis* and *Escherichia coli* strains. *World J. Microbiol. Biotechnol.*, 2004, **20**, 11–17.
13. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, **227**, 680–685.
14. Patel, R. C., Patel, J. K., Patel, P. B. and Singh, R., Mass breeding of *Heliothis armigera* (H.). *Indian J. Entomol.*, 1968, **30**, 272–280.
15. Schien, C. H., Production of soluble recombinant proteins in bacteria. *Bio/Technology*, 1989, **7**, 1141–1148.
16. Widner, W. R. and Whiteley, H. R., Two highly related crystal proteins of *Bacillus thuringiensis*. *J. Bacteriol.*, 1989, **171**, 965–974.
17. Misra, H. S., Khairnar, N. P., Mathur, M., Vijayalakshmi, N., Hire, R. S., Dongre, T. K. and Mahajan, S. K., Cloning and characterization of an insecticidal crystal protein gene from *Bacillus thuringiensis* subspecies *Kenyae*. *J. Genet.*, 2002, **81**, 5–11.
18. Moar, W. J., Trumble, J. H., Hice, R. H. and Backman, P. A., Insecticidal activity of the CryIIA protein from NRD-12 isolates of *Bacillus thuringiensis* subsp. *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf colonizing strain of *Bacillus cereus*. *Appl. Environ. Microbiol.*, 1994, **60**, 896–902.
19. Rudolph, R. and Lilie, H., *In vitro* folding of inclusion body proteins. *FASEB J.*, 1996, **10**, 49–56.
20. Sudha, S. N., Jayakumar, R. and Sekar, V., Introduction and expression of the *cry1Ac* gene of *Bacillus thuringiensis* in a cereal-associated bacterium, *Bacillus polymyxa*. *Curr. Microbiol.*, 1999, **38**, 163–167.
21. Tuli, R., Saluja, J. and Notani, N. K., Cloning and expression in *Escherichia coli* of entomotoxic protein gene from *Bacillus thuringiensis* subspecies *kurstaki*. *J. Genet.*, 1989, **68**, 147–160.
22. Schnepf, H. E. and Whiteley, H. R., Cloning and expression of the *Bacillus thuringiensis* protein gene in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, 1981, **78**, 2989–2897.
23. Bosch, D., Visser, B. and Stiekema, W. J., Analysis of non-active engineered *Bacillus thuringiensis* crystal proteins. *FEMS Microbiol. Lett.*, 1994, **118**, 129–134.
24. Dankocsik, C., Donovan, W. P. and Jany, C. S., Activation of a cryptic protein gene of *Bacillus thuringiensis* subspecies *kurstaki* by gene fusion and determination of crystal protein insecticidal specificity. *Mol. Microbiol.*, 1990, **4**, 2087–2094.

ACKNOWLEDGEMENTS. We are grateful to Dr K. Ramasamy, Director, Centre for Plant Molecular Biology, TNAU, Coimbatore for facilities provided and constant encouragement during the course of study. This research is supported by the Rockefeller Foundation, New York, USA and Department of Biotechnology, Government of India, New Delhi, S.K. thanks the University Grants Commission, Government of India, New Delhi, for the Junior Research Fellowship. We thank Mrs A. Kalpana for help in the maintenance of *H. armigera* culture.

Received 9 June 2003; revised accepted 29 October 2003