# Resistance to Cry toxins and epithelial healing

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**Abstract**: Resistance to Cry toxins can develop by alterations in any of the steps in the Cry toxin mode of action. Most characterized mechanisms of resistance to Cry toxins involve alterations in enzymatic toxin processing or toxin interaction with receptors in the insect midgut epithelium. Previous reports have suggested an alternative mechanism of resistance to Cry1Ac toxin in *Heliothis virescens* larvae involving enhanced midgut regeneration after toxin-induced injury. Our current hypothesis is that as midgut mature cells interact with Cry1Ac and undergo injury, stem cells divide and differentiate to replace damaged mature cells. Considering this hypothesis, resistant *H. virescens* larvae may display a more effective regenerative mechanism that prevents compromising epithelial integrity. We report the detection of differences in the proteins secreted by mature cells upon Cry toxin treatment and an efficient regenerative response to intoxication in midgut cells from Cry1Ac-resistant *H. virescens* larvae.

**Key words**: Cry toxins, resistance, *Heliothis virescens*, midgut regeneration, stem cells, flow cytometry, proteomics.

## Introduction

Cry toxins produced by the bacterium *Bacillus thuringiensis* (Bt) are used in insecticidal mixtures or expressed in transgenic plants (Bt crops) for environmentally-sound and effective insect control (Crickmore 2006). Upon ingestion by the insect, Cry toxins are solubilized and enzymatically processed in the insect midgut to an active toxin core. According to the model of Bravo et al. (Bravo et al. 2004), Cry toxin monomers bind to cadherin proteins on the midgut epithelium and undergo a conformational change that results in toxin oligomerization and an increased binding affinity towards aminopeptidase-N (APN) (Pardo-Lopez et al. 2006), and possibly alkaline phosphatase (ALP) (Jurat-Fuentes et al. 2004). Binding of toxin oligomers to these proteins results in accumulation of oligomers on lipid rafts, oligomer insertion, pore formation, and cell death by osmotic cell lysis. In an alternative model (Zhang et al. 2005), binding of toxin monomers to cadherin activates an intracellular oncotic signaling pathway resulting in cell death. Once midgut epithelium integrity is compromised, bacteria proliferate and invade the insect hemocoel causing septicemia and ultimately insect death (Broderick et al. 2006).

Even though insect resistance to Cry toxins can develop by alteration in any of the steps in the toxin mode of action, research efforts have focused on the most common mechanisms (Ferre et al. 2002): alteration of toxin proteolysis or changes in interactions between toxin and midgut receptors. In comparison, little is known on the role of epithelial regenerative mechanisms in resistance to Cry intoxication. This type of process would help explain cases of resistance to Cry toxins for which no mechanisms have been identified (Wang et al. 2007; Anilkumar et al. 2008). Enhanced midgut cell sloughing and regeneration has been proposed to result in resistance to baculoviral infection in *H. virescens* larvae (Hoover et al. 2000). Direct correlation between midgut regeneration and resistance to Cry1Ac was previously

reported in larvae from the CP73-3 and KCB strains of *H. virescens* (Forcada et al. 1999; Martinez-Ramirez et al. 1999). Clearly, further characterization of the regenerative response after Cry intoxication is needed to establish its potential role in resistance to Cry toxins.

In lepidopteran larvae, since differentiated cell types do not divide, midgut epithelium regeneration depends on stem cells (Hakim et al. 2001). Midgut stem cells respond to specific growth factors by proliferating and/or differentiating to mature cell types as needed. Isolation of primary midgut mature and stem cell cultures from *H. virescens* larvae (Loeb et al. 1996), has allowed the identification of several bioactive factors inducing a response in midgut stem cells in vitro (Loeb et al. 1999; Loeb et al. 2002; Blackburn et al. 2004). When primary midgut cell cultures from *H. virescens* larvae were treated with Cry toxins, a fast increase in the number of differentiating cells in response to dying mature cells was observed (Loeb et al. 2001a). Upon removal of Cry toxin from the culture, the proportion of each cell type returned to initial levels, suggesting a tightly regulated response to intoxication (Loeb et al. 2001b). Based on these observations from assays using isolated midgut cell cultures, our current hypothesis is that specific growth factors are produced during exposure of mature cell to Cry toxins that result in activation of the stem cell-mediated regenerative response. This process may be improved in Cry-resistant larvae resulting in complete recovery after intoxication.

The main goal of our project is to characterize the midgut regenerative response to assess its potential role as a mechanism of resistance to Cry toxins. In this report, we present preliminary data for the work that will be presented during our keynote presentation. Using primary midgut cell cultures and differential proteomics, we have been able to detect proteins differentially secreted in primary midgut cell cultures from *H. virescens* upon exposure to Cry1Ac. Primary midgut cell cultures from Cry1Ac-resistant larvae, but not from susceptible larvae, recovered from Cry1Ac intoxication.

## Material and methods

### *Insect rearing and bacterial strains*

*H. virescens* eggs were purchased from Benzon Research Inc. (Carlisle, PA). After hatching, larvae were reared on tobacco budworm artificial diet (Bio-Serv, Frenchtown, NJ) with a 8:16 (D:L) photoperiod at 26°C.

Cry1Ac and Cry3Aa protoxins were purified from cultures of *B. thuringiensis* HD-73 and var. *tenebrionis*, respectively.

### *Isolation of primary midgut cell cultures*

Larval midguts were dissected following methods described elsewhere (Loeb et al. 2001b; Loeb et al. 2003). Briefly, dissected guts were incubated in media (Grace's media (Gibco) diluted 3:1 in Ringer's solution) for 1 hour, then homogenized by pipetting. Tissue debris was discarded using 70µm filters. Mature and stem cells in the filtrate were separated by centrifugation (600 x *g* for 15 min.) in Ficoll-Paque (GE Healthcare). Recovered cells were washed in media twice by centrifugation (400 x *g* for 5 min.). Final cell pellets were resuspended in media and incubated in surface-treated 12-well plates (NUNC, Rochester, NY) at 26°C.

### *2D gel electrophoresis of midgut cell culture secretomes*

Midgut cell cultures were treated with buffer (20 mM carbonate buffer, pH 9.8), or 0.06 µg/ml of purified Cry1Ac or Cry3Aa protoxin for 1 day at 26°C, then collected by centrifugation (400 x *g* for 5 min.). Supernatants were processed and quantified for 2D electrophoresis as described elsewhere (Krishnamoorthy et al. 2007). Proteins (20 µg) were loaded on 7 cm, pH

3-10 Immobiline DryStrips (GE Healthcare) and resolved using isoelectrofocusing (IEF) as first dimension followed by SDS-8%PAGE as second dimension. After electrophoresis, gels were silver stained.

***Response to intoxication in midgut cell cultures from Cry1Ac-susceptible and resistant H. virescens larvae***

Midgut cell cultures from larvae of susceptible (YDK) and Cry1Ac-resistant (CXC) strains. Cell cultures were treated with a dose of Cry1Ac protoxin representing a sublethal concentration for CXC larvae (6.7 μg /ml) and incubated at 26°C. Cultures were observed at days one and four after intoxication. To determine cell mortality, sub-cultures were stained with trypan blue before microscopic observation.

## Results and discussion

***Discrimination of H. virescens midgut mature and stem cells***

While mature cells had a complex morphology with the presence of brush border on some cells, stem cells were round and semi-translucent (Fig. 1). Separation of stem and mature cells allowed us to test for the expression of ALP and cadherin. Alkaline phosphatase has been described as a stem cell marker in mammalian systems (Nunomura et al. 2005), while cadherin use as stem cell marker depends on the tissue (Watt, 1998). As shown in Fig. 1, stem cells had much lower levels of both ALP and cadherin than mature cells. Lower expression of an adhesion protein such as cadherin would be expected for midgut stem cells, which are loosely attached to the epithelium. In contrast, the low levels of ALP in midgut stem cells may suggest differences in protein expression between mammalian and invertebrate stem cells. These data identify both cadherin and ALP as negative markers for insect midgut stem cells. Additionally, since both ALP and cadherin are proposed functional Cry1Ac receptors, our data help explain lower susceptibility of midgut stem cells to Cry1Ac toxin (Loeb et al. 2001b).

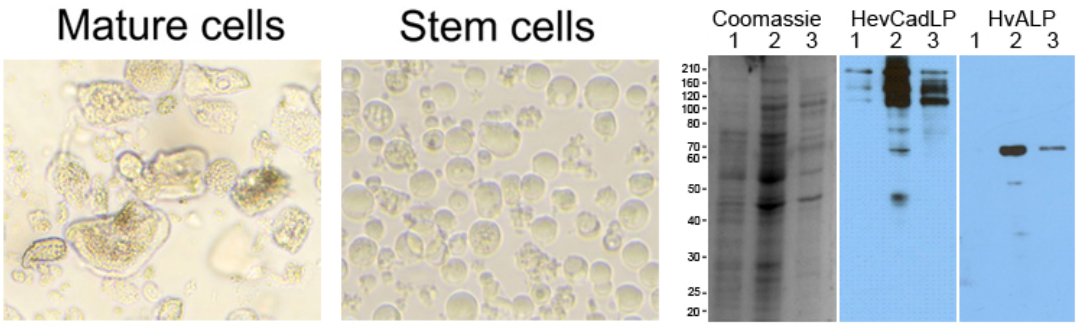


Figure 1. Morphological characteristics of mature and stem cells (left and center panels). Detection of expression of cadherin (HevCadLP) and alkaline phosphatase (HvALP) in stem cells (1), mature cells (2), or brush border membrane vesicles (3).

***H. virescens midgut cells secrete specific proteins during Cry1Ac intoxication***

Our current hypothesis is that upon exposure to sublethal levels of Cry1Ac protoxin, dying cells express and secrete specific growth factors that activate stem cell proliferation and/or differentiation. A prediction from this hypothesis would be that mature cells treated with Cry1Ac would secrete proteins to induce stem cell-based epithelial regeneration. We treated *H. virescens* midgut cell cultures with buffer or a sublethal concentration of purified Cry1Ac

or Cry3Aa, the later being inactive against *H. virescens*. After 1 day, we collected secreted proteins by centrifugation and compared the secretome pattern induced by each treatment using 2D electrophoresis. Several protein spots appeared to be specifically expressed after treatment with Cry1Ac (Fig. 2B). These proteins were not observed in control cultures treated with buffer (Fig. 2A) or with Cry3Aa (Fig. 2C). A group of proteins between 120- to 160-kDa in size were preferentially expressed when midgut cells were treated with Cry1Ac. Additionally, a protein spot smaller than 30-kDa was not detected in cultures treated with Cry1Ac, evidence that treatment with this toxin induces complex alterations in the secretome.

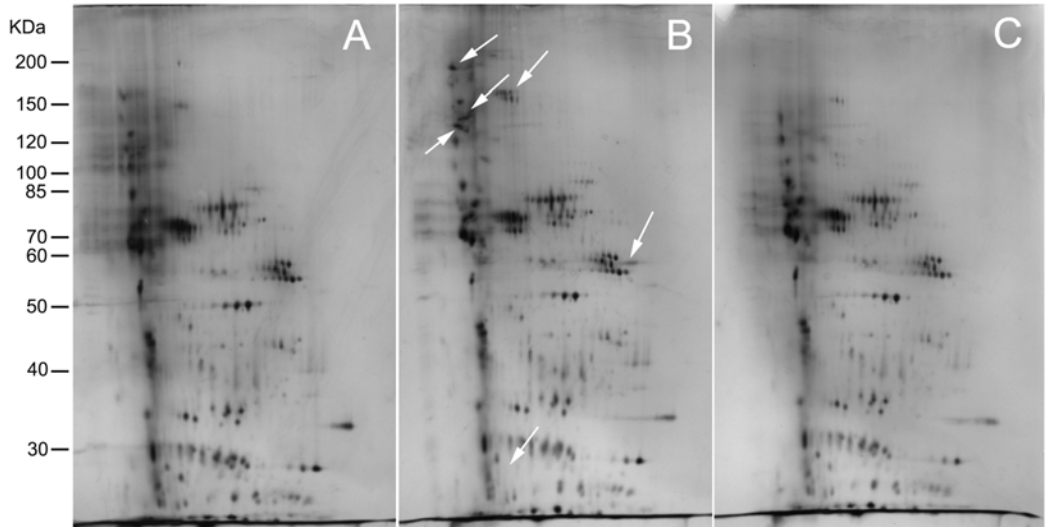


Figure 2. Secretomes of primary midgut cells treated with buffer (A), Cry1Ac (B), or Cry3Aa (C) as indicated. White arrows indicate some of the differentially expressed proteins.

***Response to Cry1Ac intoxication in midgut cells from susceptible and resistant H. virescens larvae***

To test whether this enhanced regenerative response could be reproduced in our in vitro system, we treated midgut cell cultures prepared from larvae of a susceptible (YDK) and Cry1Ac-resistant (CXC) strain of *H. virescens*. Cell cultures were treated with a dose of Cry1Ac protoxin representing a sublethal concentration for CXC larvae (6.7 μg /ml). After staining sub-cultures with trypan blue to detect dead cells, we observed the cultures after one and four days (Fig. 3). In YDK cultures, mostly ghost and dead cells were observed at both time intervals. In contrast, while most cells appeared dead in CXC cultures during the first day of incubation, some live cells were also detected. After 4 days, no live cells were detected in YDK cultures, while live cells were observed in CXC cultures among some dead and ghost cells. These observations suggest midgut regeneration by a more effective regenerative response in CXC larvae. This mechanism would explain the cross-resistance phenotype to Cry1Ac and Cry2Aa toxins observed in this strain (Jurat-Fuentes et al. 2003).

***Summary***

Our data suggest the expression and secretion of specific proteins when midgut cells are exposed to an active Cry toxin. These proteins are not observed when the Cry toxin is inactive against the cells. Regeneration of primary cell cultures from CXC larvae suggest the presence of an enhanced regenerative mechanism in larvae of this strain, which may contribute to resistance. Additional data on the characterization of the regenerative response as well as the role of enhanced regeneration in resistance will be displayed during our presentation.

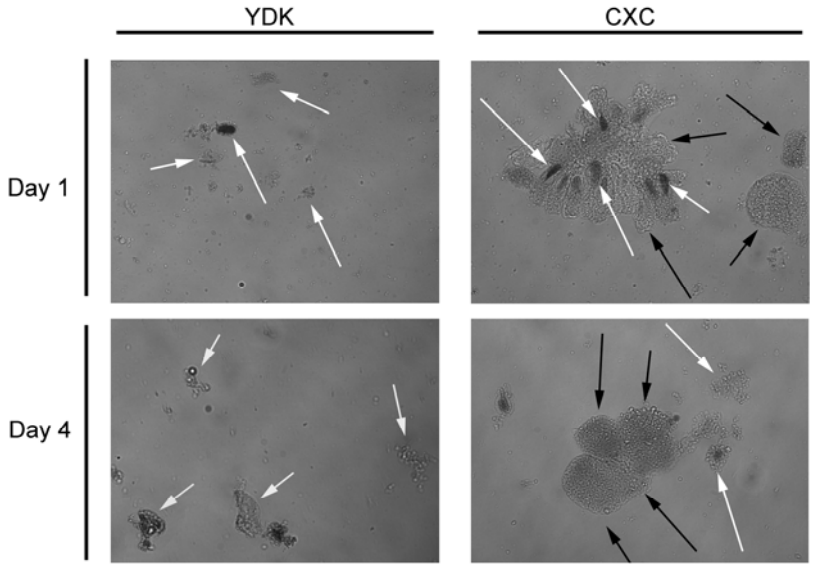


Figure 3. Effect of Cry1Ac treatment on midgut cells from susceptible (YDK) and Cry1Ac-resistant (CXC) larvae. White arrows indicate dead cells, black arrows indicate live cells.

## Acknowledgements

This research was supported by grant I0S0718807 from the National Science Foundation to Juan Luis Jurat-Fuentes.

## References

Anilkumar, K.J., Rodrigo-Simon, A., Ferre, J., Pusztai-Carey, M., Sivasupramaniam, S., Moar, W.J., 2008. Production and characterization of *Bacillus thuringiensis* Cry1Ac-resistant cotton bollworm *Helicoverpa zea* (Boddie). Appl. Environ. Microbiol. 74, 462-469.

Blackburn, M.B., Loeb, M.J., Clark, E., Jaffe, H., 2004. Stimulation of midgut stem cell proliferation by *Manduca sexta* alpha-arylphorin. Arch. Insect Biochem. Physiol. 55, 26-32.

Bravo, A., Gomez, I., Conde, J., Munoz-Garay, C., Sanchez, J., Miranda, R., Zhuang, M., Gill, S.S., Soberon, M., 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochim. Biophys. Acta 1667, 38-46.

Broderick, N.A., Raffa, K.F., Handelsman, J., 2006. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. Proc. Natl. Acad. Sci. U. S. A. 103, 15196-15199.

Crickmore, N., 2006. Beyond the spore--past and future developments of *Bacillus thuringiensis* as a biopesticide. J. Appl. Microbiol. 101, 616-619.

Ferre, J., Van Rie, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Ann. Rev. Entomol. 47, 501-533.

Forcada, C., Alcacer, E., Garcerá, M.D., Tato, A., Martinez, R., 1999. Resistance to *Bacillus thuringiensis* Cry1Ac toxin in three strains of *Heliothis virescens*: proteolytic and SEM study of the larval midgut. Arch. Insect Biochem. Physiol. 42, 51-63.

Hakim, R.S., Baldwin, K.M., Loeb, M., 2001. The role of stem cells in midgut growth and regeneration. In Vitro Cell Dev. Biol. Anim. 37, 338-342.

Hoover, K., Washburn, J.O., Volkman, L.E., 2000. Midgut-based resistance of *Heliothis virescens* to baculovirus infection mediated by phytochemicals in cotton. J. Insect Physiol. 46, 999-1007.

Jurat-Fuentes, J.L., Adang, M.J., 2004. Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. Eur. J. Biochem. 271, 3127-3135.

Jurat-Fuentes, J.L., Gould, F.L., Adang, M.J., 2003. Dual resistance to *Bacillus thuringiensis* Cry1Ac and Cry2Aa toxins in *Heliothis virescens* suggests multiple mechanisms of resistance. Appl. Environ. Microbiol. 69, 5898-5906.

Krishnamoorthy, M., Jurat-Fuentes, J.L., McNall, R.J., Andacht, T., Adang, M., 2007. Identification of novel Cry1Ac binding proteins in midgut membranes from *Heliothis virescens* using proteomic analyses. Insect Biochem. Molec. Biol. 37, 189-201.

Loeb, M.J., Clark, E.A., Blackburn, M., Hakim, R.S., Elsen, K., Smagghe, G., 2003. Stem cells from midguts of Lepidopteran larvae: clues to the regulation of stem cell fate. Arch. Insect Biochem. Physiol. 53, 186-198.

Loeb, M.J., Hakim, R.S., 1996. Insect midgut epithelium in vitro: an insect stem cell system. J. Insect Physiol. 42, 1103-1111.

Loeb, M.J., Jaffe, H., 2002. Peptides that elicit midgut stem cell differentiation isolated from chymotryptic digests of hemolymph from *Lymantria dispar* pupae. Arch. Insect Biochem. Physiol. 50, 85-96.

Loeb, M.J., Jaffe, H., Gelman, D.B., Hakim, R.S., 1999. Two polypeptide factors that promote differentiation of insect midgut stem cells in vitro. Arch. Insect Biochem. Physiol. 40, 129-140.

Loeb, M.J., Martin, P.A., Hakim, R.S., Goto, S., Takeda, M., 2001a. Regeneration of cultured midgut cells after exposure to sublethal doses of toxin from two strains of *Bacillus thuringiensis*. J. Insect Physiol. 47, 599-606.

Loeb, M.J., Martin, P.A., Narang, N., Hakim, R.S., Goto, S., Takeda, M., 2001b. Control of life, death, and differentiation in cultured midgut cells of the lepidopteran, *Heliothis virescens*. In Vitro Cell Dev. Biol. Anim. 37, 348-352.

Martinez-Ramirez, A.C., Gould, F., Ferre, J., 1999. Histopathological effects and growth reduction in a susceptible and a resistant strain of *Heliothis virescens* (Lepidoptera : Noctuidae) caused by sublethal doses of pure Cry1A crystal proteins from *Bacillus thuringiensis*. Biocont. Sci. Tech. 9, 239-246.

Nunomura, K., Nagano, K., Itagaki, C., Taoka, M., Okamura, N., Yamauchi, Y., Sugano, S., Takahashi, N., Izumi, T., Isobe, T., 2005. Cell surface labeling and mass spectrometry reveal diversity of cell surface markers and signaling molecules expressed in undifferentiated mouse embryonic stem cells. Mol. Cell. Prot. 4, 1968-1976.

Pardo-Lopez, L., Gomez, I., Rausell, C., Sanchez, J., Soberon, M., Bravo, A., 2006. Structural changes of the Cry1Ac oligomeric pre-pore from *Bacillus thuringiensis* induced by N-acetylgalactosamine facilitates toxin membrane insertion. Biochemistry 45, 10329-10336.

Wang, P., Zhao, J.Z., Rodrigo-Simon, A., Kain, W., Janmaat, A.F., Shelton, A.M., Ferre, J., Myers, J., 2007. Mechanism of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a greenhouse population of the cabbage looper, *Trichoplusia ni*. Appl. Environ. Microbiol. 73, 1199-1207.

Watt, F.M., 1998. Epidermal stem cells: markers, patterning and the control of stem cell fate. Philos Trans. R. Soc. Lond. B Biol. Sci. 353, 831-837.

Zhang, X., Candas, M., Griko, N.B., Rose-Young, L., Bulla, L.A., 2005. Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R(1) expressed in insect cells. Cell Death Differ. 12, 1407-1416