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Alanine Scan of the Cry5B Toxin Domain Identifies Variants with Increased Activity Against the Nematodes Caenorhabditis elegans and Ancylostoma ceylanicum

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Alanine Scan of the Cry5B Toxin Domain Identifies Variants with Increased Activity
Against the Nematodes *Caenorhabditis elegans* and *Ancylostoma ceylanicum*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor
of Philosophy

in

Biology

by

Jillian Leah Sesar

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2016

The Dissertation of Jillian Leah Sesar is approved, and it is acceptable in quality and

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University of California, San Diego

2016

DEDICATION

I dedicate this dissertation to my family – who have supported and encouraged me throughout the years.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 5, currently unpublished, is, in part, co-authored. Sesar, Jillian; Hu, Yan; Aroian, Raffi. The dissertation author was the primary investigator and author of this material.

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ABSTRACT OF THE DISSERTATION

Alanine Scan of the Cry5B Toxin Domain Identifies Variants with Increased Activity
Against the Nematodes *Caenorhabditis elegans* and *Ancylostoma ceylanicum*

By

Jillian Leah Sesar

Doctor of Philosophy in Biology

University of California, San Diego, 2016

Professor Steven Wasserman, Chair
Professor Raffi V. Aroian, Co-Chair

Soil-transmitted helminths infect more than 2 billion people worldwide and only one drug (albendazole) is commonly available for use against intestinal parasite worms under conditions for mass drug administration. Recent studies have shown efficacy issues with this drug, stressing the importance of finding a new treatment option. Three-domain crystal (Cry) proteins produced from the soil bacterium *Bacillus thuringiensis* have been

used for decades as a means to control insects that destroy crops and transmit human diseases, and studies have shown many of these proteins to be safe to humans. Our lab has shown that the crystal protein Cry5B is able to kill both the free-living nematode *Caenorhabditis elegans*, as well as parasitic roundworms (e.g., *Ancylostoma ceylanicum*, hookworm). Cry proteins intoxicate invertebrates by acting as pore-forming proteins. We believe that the nematode-Cry5B system has potential to unlock mysteries surrounding Cry proteins and to help improve Cry5B as a therapeutic agent.

Here, I took a large scale systematic alanine-scanning approach and attempted to mutate all 698 amino acids in the toxin domain of Cry5B. I initially tested these mutants on *C. elegans* to assess for changes in toxicity levels, screening for variants with an increase in activity as compared to the wild type. Both the larger mutant set and the subset that had increased activity in my experiments are promising for future study, although the increased activity of the subset will need to be confirmed in assays with *C. elegans* and intestinal parasites. In the course of these studies, comparisons were made regarding the utility of CryB expressed *in E* coli, as purified protein, and as a spore/crystal mixture for activity assays and rescreening. Experiments were also carried out to address which system is best suited to test these variants in narrowing down those of interest and in predicting therapeutic potential *in vivo*. Overall, the Cry protein variants and approaches to their study described in this thesis hold promise for the development of therapeutics for treating one of the most neglected diseases of our time, parasitic worms.

CHAPTER 1

INTRODUCTION

1.1 Summary

Over 2 billion people worldwide are infected with soil-transmitted helminthes
(STH). Current treatment options are sub-optimal, displaying varied efficacies for the
different parasites, and resistance to the drugs may have begun to emerge. New drug
candidates are urgently needed, and Cry5B, a Crystal (Cry) Protein produced by the soil
bacterium *Bacillus thuringiensis* (Bt), is a candidate antihelminthic to address this global
problem. Cry proteins are pore-forming proteins and once ingested by an invertebrate
they attack the host intestine. Cry5B is toxic to *Caenorhabditis elegans* and Cry5B
activity against parasitic worms has been demonstrated in *in vitro* and *in vivo* studies
carried out by the Aroian lab. In particular, the worm burden for *Ancylostoma
ceylanicum*, *Heligmosomoides polygyrus*, and *Ascaris suum* in their respective hosts was
reduced by a treatment with Cry5B. While Cry5B is effective against these parasitic

nematodes, there is the potential to increase the activity of the protein by altering its amino acid sequence, which would allow for a better variant candidate as a treatment, as well as provide information regarding the structure and function of Cry5B.

1.2 Soil transmitted helminths are a global issue

Soil transmitted helminths (STHs) are one of the leading causes of infection in the world. Estimates are that between 1.5-2.3 billion people worldwide are infected with STH, with children and pregnant women at the highest risk. (Bethony 2006, Hotez 2008). STH typically is used to refer to three categories of worms– the giant roundworm (*Ascaris lumbriocodies*), three hookworms (*Necator americanus, Ancylostoma duodenale,* and *Ancylostoma ceylanicum*), and the whipworm (*Trichuris trichiura*). Infections involving these worms affect some of the poorest people of the world, greatly impacting Africa, Southeast Asia, India, and Latin/South America (Bethony 2006, Hotez 2007).

STHs are a leading cause of morbidity and contribute to sustaining a cycle of poverty. People infected with these worms suffer from malnutrition, loss of appetite, intestinal issues, lethargy, and immune defects leading to increased susceptibility to diseases such as malaria, HIV, and tuberculosis, as well as vaccination failure (Bethony 2006, Hotez 2007, Hall 2007, Serene 2014, Francis 2012). As blood feeders,, hookworms have the additional effect of causing anemia. Children infected with STHs suffer from physical and cognitive stunting, as well as school absenteeism, which leads to a loss of future income. Co-infection with multiple parasites is common, and can increase and complicates the effects on the body. Previous studies have shown that the immune

response in the host can change with co-infection of parasites, and it has been suggested that this only helps to favor the survival of the worms and reduces the ability of the host to naturally expel the worms. A field study has shown that children co-infected with *Ascaris* and *Trichuris* had a lower processing speed index, a lower weight for age z-score, and lower standardized test scores (p<0.001 for all) as compared to children infected only with *Ascaris.* Additionally, field data from a study in 2016 has suggested that children with a higher intensity infection of *T. trichiura* had a greater chance of being infected with hookworms (Njaanake 2016, Gaze 2014, Liu 2015).

Currently, only one drug, albendazole, is considered to have good enough single-dose efficacy against most parasies for use in mass drug administration (generally speaking – not necessarily true for specific parasites) (Keiser 2008). Albendazole belongs to the benzimidizole drug family, which act on the microtubules, inhibiting polymerization (Goldsmith 1992). While this drug has high efficacy against *Ascaris*, it has moderate efficacy against hookworms, and poor efficacy against whipworms, and there are reports emerging of low or reduced efficacy to this drug (Gunawardena 2008, Adunga 2007, Humphries 2001). A study from 2011 showed reduced efficacy of albendazole in subjects from Ghana, and although multiple possibilities for this observation were explored, the authors point to the possibility of albendazole resistance (Humphries 2011). In addition, albendazole was identified in veterinary, not human, studies, which leaves an opening that there may be alternative methods with a higher efficacy in humans (Theodorides 1976). Currently, the WHO recommends treatment with antihelminthic drugs either once or twice a year to reduce STH burden, without individual diagnosis, to anyone who is living in an at-risk area (24). New drug candidates

are urgently needed, and we think that Cry5B, or potentially other crystal proteins, could be used as antihelminthic to address this global problem.

1.3 Crystal proteins

Crystal (Cry) proteins, a family of three-domain, alpha-helical pore forming proteins produced by the soil bacterium *Bacillus thuringiensis* (Bt), have been used for over 50 years in corn, cotton, rice, potatoes, and soybeans as a means to control insects that transmit diseases or harm crops (Betz 2000, Siegel 2001). Currently, 81% and 84% of acres of corn and cotton, respectively, planted in the United States are genetically engineered to contain Cry proteins (25). Bt corn and cotton are grown all over the world, and benefits include reduced insecticide use, leading to an associated reduction in both costs and insecticide poisonings, as well as an increase in yield (Toenniessen 2003). In addition, Cry proteins have a lack of toxicity in vertebrates and are safe for the environment, leading to a high level of safety for both humans and animals (Betz 2000,Siegel 2001). Because of the prevalence of Bt crops in agriculture today, numerous safety studies have been conducted on the effects of crystal proteins on humans, looking at toxicity and allergenic effects, and they have all concluded that these modified crops provide no cause for concern (Batista 2005, Betz 2000). Other commercial uses for Bt, however, have been employed long before transgenic crops. Bt *israelensis* was identified in 1976 as especially toxic to mosquitoes, and is used in water to help control mosquitoes (potentially carrying malaria) both in commercial and private use. Topical Bt sprays are found in local hardware stores and have been used commercially even longer than transgenic crops. Bt is available in a multitude of different formats for both commercial

and home use, including powders, sprays, liquids, dusts, and time- release rings (Sanahuja 2011).

Over 400 three-domain Cry proteins have been identified. Amino acid sequence comparisons reveal substantial diversity, although almost all such proteins contain five blocks of conserved amino acids on the N-terminal side of the protein. Crystal proteins are generally of two different sizes: either 130-140kDa, or 70kDa. During sporulation, *B. thuringiensis* naturally lyse and release both a spore and a crystal. The alpha-helical Cry proteins form pores that attack and destroy the intestines of invertebrates.

Crystal structures are known for nine different crystal proteins - Cry1Aa, Cry1Ac, Cry2Aa, Cry3A, Cry3Bb1, Cry4Aa, Cry4Ba, Cry5B, Cry8Ea1 (Grochulski 1995, Evdokimov 2014,Morse 2001, Sawaya 2014, Galitsky 2001, Boonserm 2005, Boonserm 2006, Guo 2009, Hui 2012). These structures reveal three separate and distinct domains. Domain I is composed of an alpha-helical bundle, and is the most similar structurally among the crystal proteins. It has been implicated in membrane insertion and pore formation based on its amphipathic nature, the length of the helices, and the similarities to the pore-forming domains of colicin, tetanus, diphtheria, and BAD toxins. Domain II is formed of β-sheets packaged together to form a β-prism with three-fold symmetry. Due to its loop regions, it is thought that domain II is involved in receptor recognition and binding. Domain II displays the most diversity among crystal proteins, due to the variability in the length of the apical loops and in amino acid sequence (De Maagd 2001). A wide range of previous studies in insects on Cry proteins have mutated specific residues in key surface loop regions of domain II, and assayed for changes in toxicity and binding affinity. These studies have demonstrated how disrupting amino acids in these

loop domains can have an effect on levels of both activity and binding. Targeted mutations in regions of domain II reduce toxicity, as well as binding (Rajamohan 1996). There is also data showing that mutations in the receptor binding loop can increase toxicity, perhaps, by increasing binding affinity with the receptor (Wu 2000). It is considered that receptor binding is a key determinant for specificity of Cry toxins. Domain III is composed of antiparallel β-sheets that form a 'jelly-roll' motif. It remains the least characterized of the domains, but it is thought to play a role in both pore formation and receptor binding, as well as structural conformation and stability (Burton 1999)., There have been many studies mutating specific, narrow, targeted regions of the protein and then looking at aspects of toxicity or function in insects. However, no large scale systematic study of an entire crystal protein has been published.

The general mechanism of action of crystal proteins has been worked out from studies in insects (Schnepf 1998, Palma 2014). Crystal proteins are produced upon sporulation from Bt packaged in inclusion bodies, and released upon cell lysis in the protoxin form. The protein is then ingested and solubilized in the midgut of the invertebrate. Next, the protoxin form is proteolytically cleaved at both ends by host proteases, converting it to the active toxin. The Cry protein then recognizes and binds to its receptor(s), oligomerizes, and inserts itself nonspecifically into the membrane, forming a pore. Following pore formation, death of the host invertebrate follows, most likely due to osmotic shock and the lysing of the midgut cells, resulting in a lack of feeding of the host. It is generally believed that the removal or disruption of any step – crystal formation, solubilization, processing, binding, oligomerization, or pore formation – will abolish toxicity. However, the relative importance of these steps is not well

described. With regards to receptor binding, the association between toxin and receptor may lead to the conformational change that allows the protein to insert into the membrane, may draw the toxin to the membrane surface, or may serve to promote oligomerization. Regardless, this interaction is clearly important, as shown by the relationship between toxicity and receptor binding. It is worth noting an alternative model for Cry protein action, in which crystal proteins are proposed to intoxicate cells via signal transduction events that occur independent of pore formation (Zhang 2006, Jenkins 2000, Aronson 2001)

1.4 The potential of Cry5B against human parasites

Although the majority of crystal proteins target insects, it has currently been shown that several crystal proteins can kill/intoxicate nematodes – Cry21, Cry14A, Cry3Bb, Cry6A, and Cry5B (Wei 2003). The most current and extensive studies have focused on Cry5B, and this protein is the focus of my work. Cry5B has been shown to be one of the most structurally divergent Cry proteins, specifically with regards to domain II, which is more similar to a banana lectin than to domain II of other Cry proteins (Meagher 2005). This suggests that this domain is most likely responsible for binding, especially considering that the receptor for Cry5B is an invertebrate-specific glycolipid, and most likely provides the specificity to targeting nematodes (Griffitts 2005). Another important piece of information that this receptor helps to confirm is the level of safety of Cry5B, as it has been shown that when knocked out in the bre mutants, the *C. elegans* are resistant to Cry5B (Griffitts 2005). This invertebrate specificity makes Cry5B an excellent candidate to pursue as a future antihelminthic.

In the laboratory, Cry5B has been shown in multiple assays to be toxic to *C. elegans* (Wei 2004). Additionally, Cry5B has been found to be effective against three of the parasitic nematodes tested in *in vivo* experiments. In mice infected with *Heligmosomoides polygyrus*, it was shown that a single dose of 100mg/kg of Cry5B reduced the worm burden approximately 70%, (Hu 2010). Furthermore, Cry5B was shown to rapidly degrade in simulated gastric fluids, suggesting that the actual dose to reach these worms was far less than what was administered during treatment, supporting its efficacy as a therapeutic (Hu 2010). In hamsters infected with *Ancylostoma ceylanicum*, it was shown that a single dose treatment with Cry5B of either 10 mg in water or 3 mg in Tris, pH 8.5, buffer led to a 93% and 97% reduction in worm burden, respectively (Hu 2012). *Ascaris suum,* which infects pigs*,* is generally considered to be closely related to the Ascaris that infects people, *Ascaris lumbricoides* (Leles 2012). In pigs infected with *A. suum*, two 20mg/kg Cry5B treatments reduced the worm burden 97% (Urban 2013). It was also observed that when Cry5B expressed in *E. coli* was tested against the free-living stages of the nematode *Nipponstrongylus brasiliensis*, a rat intestinal parasite (and the same family of worms as Haemonchus and Ostertagia), both the development and overall health of the worms were compromised (Wei 2003).

Plant parasitic nematodes are also sensitive to Cry proteins. In tomato roots expressing Cry6A and Cry5B separately, it was found that for the root-knot nematode *Meloidogyne incognita*, this nematode was intoxicated after ingestion of the roots, as measured by a decrease in progeny (Li 2007, Li 2008). This data only adds to the widespread applicability of Cry proteins as nematicides, not solely against human or

animal parasites, but against plant-parasitic nematodes as well. Additionally, the Aroian group has demonstrated that Cry5B has a synergistic effect when combined with other known antihelminthics on *C. elegans* (Hu 2010). From the current data, Cry5B has great potential as a potent antihelminthic. We known that this protein is toxic to helminths, but there is the potential to change its activity to make it even better and more efficient than it already is.

1.5 Crystal protein variants exist and are useful

Previous mutagenesis studies performed on insect Cry proteins have shown that single amino acid changes can increase toxicity. Specifically, it was demonstrated that changes in loop 1 (domain II) in Cry3A lead to two variants which have increased toxicity against three coleopteran species as compared to the wild-type (Wu 2000). Site-directed mutagenesis on Cry1Ac targeting the hydrophobic motif of α-helix 7 (domain I) created a mutant that was eight times more toxic than the wild type when tested on neonate larvae of the cotton bollworm (Rajamohan 1996). Based on these studies, it seems highly likely that single amino acid mutations in Cry5B could lead to variants that greatly increase activity.

Mutated Cry variants are commonly used in transgenic crops. For example, six amino acids were changed in Cry3Bb1 to produce a variant with a 200-fold increase in toxicity, which was then granted FDA approval to be used in genetically engineered corn. Without these changes, this crystal protein would most likely not be used in the field (43).The benefits of generating such a variant of Cry 5B would include reduced quantity needed for treatment, lower costs, and increase efficacy against STHs.

1.6 References

1. Yan Hu, Sophia B. Kelleher, Alan J. Kelleher, Raffi V. Aroian, *Bacillus thuringiensis* Cry5B Protein is Highly Efficacious as a Single Dose Therapy against an Intestinal Roundworm Infection in Mice. *PLOS Neglected Tropical Diseases* **4**, (2010).

2. Joel S. Griffitts, Sophia B. Kelleher, Tinglu Yang, Stephan F. Garczynski, Barbara Mulloy, Howard Morris, Paul S. Cremer, Anne Dell, Michael J. Adang, Raffi V. Aroian, Glycolipids as Receptors for *Bacillus thuringiensis* Crystal Toxin. *Science* **307**, 922-925 (2005).

3. Xiang-Qian Li, Jun-Zhi Wei, Anderson Tan, Raffi V. Aroian, Resistance to root-knot nematode in tomato roots expressing a nematicidal *Bacillus thuringiensis* crystal protein. *Plant Biotechnology Journal* **5**, 455-464 (2007).

4. Jun-Zhi Wei, Kristina Hale, Lynn Carta, Edward Platzer, Cynthie Wong, Su-Chiung Fang, Raffi V, Aroian, *Bacillus thuringiensis* crystal proteins that target nematodes. *PNAS* **100**, 2760-2765 (2003).

5. Debbie Humpries, Emily Mosites, Joseph Otchere, Welbeck Amoani Twum, Lauren Woo, Hinkley Jones-Sanpei, Lisa M. Harrison, Richard D. Bungiro, Blair Benham-Pyle, Langbong Bimi, Dominic Edoh, Kwabena Bosompem, Michael Wilson, Michael Cappello, Epidemiology of Hookworm Infection in Kintampo North Municipality,Ghana: Patterns of Malaria Coinfection, Anemia, and Albendazole Treatment Failure. *The American Journal of Tropical Medicine and Hygiene* **84(5)**, 792-800 (2011).

6. Joseph F, Urban, Jr., Yan Hu, Jr., Melanie M. Miller, Ulrike Scheib, Ying Y. Yiu, Raffi V. Aroian, *Bacillus thuringiensis*-derived Cry5B Has Potent Anthelmintic Activity against *Ascaris suum*. *PLOS Neglected Tropical Diseases* **7**, 2263 (2013).

7. Daniela Leles, Scott L Gardner, Karl Reinhard, Alena Iniguez, Adauto Araujo, Are *Ascaris lumbricoides* and *Ascaris suum* a single species? *Parasites & Vectors* **5(42)**, (2012).

8. Jeffrey Bethony, Simon Brooker, Marco Albonico, Stefan M Geiger, Alex Loukas, David Diemert, Peter J Hotez, Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *The Lancet* **367(9521)**, 1521-1532 (2006).

9. Peter J. Hotez, Neglected Infections on Poverty in the United States of America. *PLOS Neglected Tropical Diseases* **2(6)**, e256 (2008).

10. Peter J. Hotez, David H. Molyneux, Alan Fenwick, Jacob Kumareasan, Sonia Ehrlich Sachs, Jeffrey D. Sachs, Lorenzo Savioli, Control of neglected tropical diseases. *The New England Journal of Medicine* **357**, 1018-1027 (2007).

11. Andrew Hall, Micronutrient supplements for children after deworming. *The Lancet* **7**, 297-302 (2007).

12. Jennifer Keiser, Jurg Utzinger, Efficacy of Current Drugs Against Soil-Transmitted Helminth Infections. *Jama* **299(16)**, 1937-1948 (2008).

13. NK Gunawardena, ND Amarasekera, A Pathmeswaran, NR de Silva, Effect of repeated mass chemotherapy for filariasis control on soil-transmitted helmnths infects in Sri Lanka *Ceylon Med Journal* **53**, 13-16 (2008).

14. Yenew Kebede, Solomon Adunga, Feleke Moges, Moges Tiruneh, Efficacy of mebendazole and albendazole for Ascaris lumbriocoides and hookworm infections in an area with long time exposure for antihelminths, Northwest Ethiopia. *Ethiopian Medical Journal* **45(3)**, 301-306 (2007).

15. Debbie Humphries, Emily Mosites, Joseph Otchere, Welbeck Amoani Twum, Lauren Woo, Hinckley Jones-Sanpei, Lisa M. Harrison, Richard D. Bungiro, Blair Benham-Pyle, Langbong Bimi, Dominic Edoh, Kwabena Bosompem, Michael Wilson and Michael Cappello, Epidemiology of Hookworm Infection in Kintampo North Municipality, Ghana: Patterns of Malaria Coinfection, Anemia, and Albendazole Treatment Failure. *The American Society of Tropical Medicine and Hygiene* **84(5)**, 792-800 (2011).

16. V. J. Theodorides, R.J. Gyurik, W. D. Kingsbury, R. C. Parish, Anthelmintic activity of albendazole against liver flukes, tapeworms, lung and gastrointestinal roundworms. *Cellular and Molecular Life Sciences* **32(6)**, 702-703 (1976).

17. Fred S Betz, Bruce G Hammond, Roy L Fuchs, Safety and Advantages of Bacillus thuringiensis-Protected Plants to Control Insect Pests. *Regulatory Toxicology and Pharmacology* **32(2)**, 156-173 (2000).

18. Joel P. Siegel, The Mammalian Safety of Bacillus thuringiensis- Based Insecticides. *Journal of Invertebrate Pathology* **77(1)**, 13-21 (2001).

19. Gary H Toenniessen, John C O'Toole, Joseph DeVries, Advances in plant biotechnology and its adoption in developing countries. *Current Opinion in Plant Biology* **6**, 191-198 (2003).

20. Rita Batista, Baltazar Nunes, Manuela Carmo,Carlos Cardoso, Helena Sao Jose, Antonia Bugalho de Almeida, Alda Manique, Leonor Bento, Candido Pinto Ricardo, Maria Margarida Oliveria, Lack of detectable allergenicity of transgenic maize and soya

samples. *Journal of Allergy and Clinical Immunology* **116**, 403-410 (2005).

21. Yan Hu, Bin Zhan Brian Keegan, Raffi V. Aroian, Mechanistic and Single-Dose In Vivo Therapeutic Studies of Cry5B Anthelmintic Action against Hookworms. *PLOS Neglected Tropical Diseases* **6-11**, e1900 (2012).

22. Geoffrey Edwards and Alasdair M Breckenridge, Clinical Pharmacokinetics of Antihelminthic Drugs. *Basic and Clinical pharmacology* **742**, 48-65 (1992).

23. Intestinal Worm – Strategy. World Health Organization. http://www.who.int/intestinal_worms/strategy/en/

24. Adoption of Genetically Engineered Crops in the U.S. United States Department of Agriculture. http://www.ers.usda.gov/Data/BiotechCrops/ (2015).

25. Pawel Grochulski, Luke Masson, Svetlana Borisova, Marianne Pusztai-Carey, Jean-Louis Schwartz, Roland Brousseau, Miroslaw Cygler, Bacillus thuringiensisCrylA(a) Insecticidal Toxin: Crystal Structure and Channel Formation. *Journal of Molecular Biology* **254**, 447-464 (2001).

26. Artem G. Evdokimov, Farhad Moshiri, Eric J. Sturman, Timothy J. Rydel,, J. W. S. Meiying Zheng, and Sonya Franklin, Structure of the full-length insecticidal protein Cry1Ac reveals intriguing details of toxin packaging into in vivo formed crystals. *The Protein Society* **231**, 1491-1497 (2014).

27. R.J. Morse, T. Yamamoto, R.M. Stroud, Structure of Cry2Aa Suggests an Unexpected Receptor Binding Epitope. *Cell*, 409-411 (2001).

28. Michael R. Sawayaa, Duilio Cascio, Mari Gingerya, Jose Rodrigueza Lukasz Goldschmidta,, M. M. M. Jacques-Philippe Colletierc, Sébastien Boutetf, Jason E. Koglinf, Garth J. Williams, Aaron S. Brewsterg, Karol Nassh, Johan Hattneg, Sabine Bothah, R. Bruce Doakh, Robert L. Shoemanh, Daniel P. DePonte, Hyun-Woo Park, Brian A. Federici, , Nicholas K. Sauterg, Ilme Schlichtingh, David S. Eisenberg, Protein crystal structure obtained at 2.9 Å resolution from injecting bacterial cells into an X-ray free-electron laser beam. *PNAS* **111(35)**, 12769-12774 (2014).

29. Nikolia Galitsky, Vivian Cody, Andrezej Wojtczak, Debashis Ghosh, Joseph R. Luft, Walter Pangborn, Leigh English, Structure of the insecticidal bacterial [delta]-endotoxin Cry3Bb1 of Bacillus thuringiensis. *Acta Crystalogr D Biol* **57-8**, 1101-1109 (2001).

30. Panadda Boonserm, Paul Davis, David J Ellar, Jade LI, Crystal Structure of the Mosquito-larvicidal Toxin Cry4Ba and Its Biological Implications. *Journal of Molecular Biology* **248**, 363-382 (2005).

31. Panadda Boonserm, Min Mo, Chanan Angsuthanasombat, Julien Lescar, Structure of

the Functional Form of the Mosquito Larvicidal Cry4Aa Toxin from Bacillus thuringiensis at a 2.8-Angstrom Resolution. *Journal of Bacteriology* **188-9**, 3391-3401 (2006).

32. Shuyuan Guoa, Sheng Ye, Yanfeng Liub, Lei Weic, Jing Xueb, Hongfu Wub, Fuping Songb, Jie Zhangb, Xiaoai Wuc, Dafang Huangd, Zihe Rao, Crystal structure of Bacillus thuringiensis Cry8Ea1: An insecticidal toxin toxic to underground pests, the larvae of Holotrichia parallela. *Journal of Structural Biology* **168-2**, 259-266 (2009).

33. Fan Hui, Ulrike Scheib, Yan Hu, Ralf J. Sommer, Raffi V. Aroian, Partho Ghosh, The structure and glycolipid-binding properties of the nematicidal protein Cry5B. *Biochemistry* **51(49)**, (2012).

34. Ruud A. de Maagd, Alejandra Bravo, Neil Crickmore, How Bacillus thuringiensis has evolved specific toxins to colonize the insect world. *Trends in Genetics* **17**, 1993-1999 (2001).

35. Sarah L Burton, David J Ellar, Jade Li, Dean J Drbyshire, N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a Bacillus thuringiensis insecticidal toxin. *Journal of Molecular Biology* **287**, 1001-1022 (1999).

36. Jennifer L. Meagher, Harry C. Winter, Porscha Ezell, Irwin J. Goldstein, Jeanne A. Stuckey, Crystal structure of banana lectin reveals a novel second sugar binding site. *Glycobiology* **15**, 1033-1042 (2005).

37. E. Schnepf, N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler, D.H. Dean, Bacillus thuringiensis and Its Pesticidal Crystal Proteins. *Microbiology* **62**, 775-806 (1998).

38. Xuebin Zhang, Mehmet Candas, Natalya B. Griko, Ronald Taussig, Lee A. Buella Jr., A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of Bacillus thuringiensis. *Proc. Natl. Acad. Sci* **103**, 9897-9902 (2006).

39. Yan Hu, Edward G. Platzer, Audrey Bellier, Raffi V. Aroian, Discovery of a highly synergistic anthelmintic combination that shows mutual hypersusceptibility. *PNAS* **107-13**, 5955-5960 (2010).

40. Francis Rajamohan, Oscar Alzate, Jeffrey A. Cotrill, April Curtiss, and Donald H. Dean, Protein engineering of Bacillus thuringiensis δ-endotoxin: Mutations at domain II of CryIAb enhance receptor affinity and toxicity toward gypsy moth larvae. *Proc Natl Acad Sci* **93**, 14338-14343 (1996).

41. Sheng-Jiun Wu, C.Noah Koller, Deborah L. Miller, Leah S. Bauer, D. H. Dean,

Enhanced toxicity of Bacillus thuringiensis Cry3A δ-endotoxin in coleopterans by mutagenesis in a receptor binding loop. *FEBS Letters* **473**, 227-232 (2000).

42. A Review of the Environmental Safety of the Cry3Bb1 Protein. *Center for Environmental Risk Assessment*, (2014).

43. Cheng Du, Phyllis A. W. Martin, Kenneth W. Nickerson, Comparison of Disulfide Contents and Solubility at Alkaline pH of Insecticidal and Noninsecticidal Bacillus thuringiensis Protein Crystals. *Applied and Environmental Microbiology* **60(10)**, 3847-3853 (1994).

44. Francis Rajamohan, Jeffrey A. Cotrill, Fred Gould, Donald H. Dean, Role of Domain II, Loop 2 Residues of Bacillus thuringiensis CryIAb δ-Endotoxin in Reversible and Irreversible Binding to Manduca sexta and Heliothis virescens. *The Journal of Biological Chemistry* **271**, 2390-2396 (1996).

45. Kariuki H. Njaanake, Birgitte J Vennervald, Paul E. Simonsen, Henry Madsen, Dunstan A. Mukoko, Gachuhi Kimani, Walter G. Jaoko and Benson B. Estambale, Schistosoma haematobium and soil-transmitted Helminths in Tana Delta District of Kenya: infection and morbidity patterns in primary schoolchildren from two isolated villages. *MBMC Infectious Diseases* **16:57**, (2016).

46. Soraya Gaze, Jeffrey Bethony, Maria Victoria Periago, Immunology of Experimental and Natural Human Hookworm Infection. *Parasite Immunology* **36**, 358-366 (2013).

47. Chengfang Liu, Renfu Luo, Hongmei Yi, Linxiu Zhang, Shaoping Li, Yunli Bai, Alexis Medina, Scott Rozelle, Scott Smith, Guofei Wang, Jujun Wang, Soil-Transmitted Helminths in Southwestern China: A Cross-Sectional Study of Links to Cognitive Ability, Nutrition, and School Performance among Children. *PLOS Neglected Tropical Diseases*, (2015).

48. Xiang-Qian Li, Anderson Tan, Michael Voegtline2, Senait Bekele3, Chang-Shi Chen, Raffi V. Aroian, Expression of Cry5B protein from Bacillus thuringiensis in plant roots confers resistance to root-knot nematode. *Biological Control* **47-1**, 97-102 (2008).

49. Serene A. Joseph, Martin Casapia, Brittany Blouin, Mathieu Maheu-Giroux, Elham Rahme, Theresa W. Gyorkos, Risk factors associated with malnutrition in one-year-old children living in the peruvian amazon. *PLOS Neglected Tropical Diseases* **8(12)**, e3369 (2014).

50. Lwanga Francis, Barbara Eva Kirunda, Christopher Garimoi Orach, Intestinal Helminth Infections and Nutritional Status of Children Attending Primary Schools in Wakiso District, Central Uganda. *Int J Environ Res Public Health* **9(8)**, 2910-2921 (2012).

51. Georgina Sanahuja, Raviraj Banakar, Richard M. Twyman, Teresa Capell, P. Christou, Bacillus thuringiensis: a century of research, development and commercial applications. *Plant Biotechnology Journal* (2011).

52. Leopoldo Palma, Delia Munoz, Colin Berry, Jesús Murillo, Primitivo Caballero Bacillus thuringiensis Toxins: An Overview of Their Biocidal Activity. *Toxins* **6(12)**, 3296-3325 (2014).

53. Arthur I. Aronson, Yechiel Shal. Why Bacillus thuringiensis insecticidal toxins are so effective: unique features of their mode of action. *FRMS Microbiology Letters*, (2001).

54. Jeremy L. Jenkins, Mi Kyong Lee, Algimantas P. Valaitis, April Curtiss, Donald H. Dean, Bivalent Sequential Binding Model of a Bacillus thuringiensis Toxin to Gypsy Moth Aminopeptidase N Receptor. *Journal of Biological Chemistry* **275**, 14423-14431 (2000).

CHAPTER 2

ALANINE SCANNING OF CRY5B

2.1 Summary

Cry5B has previously been shown to be toxic against *Caenorhabditis elegans*, and several intestinal helminths *in vivo*, including *Ancylostoma. ceylanicum* in hamsters, *Heligmosomoides polygyrus* in mice, and *Ascaris suum* in pigs (Urban 2013, Hu 2012, Hu 2010, Marroquin 2000). This Cry protein has great potential to be a future new antihelminthic to address the global problem of soil transmitted helminths. Due to the potential of this protein, I was interested in investigating if single amino acid changes could be introduced to increase the activity of this protein, potentially providing forms that would be more toxic than the wild-type protein against these worms. I took a large-scale approach to investigate this idea, choosing to attempt to mutate every amino acid in the toxin domain of Cry5B to an alanine (or glycine, if already an alanine), and subsequently test them for changes in activity on *C. elegans* as compared to the wild type. From this alanine scan, I found 34 amino acid changes that provisionally emerged

as showing increased activity to *C. elegans* as compared to the wild-type Cry5B, with the caveat that these were selected under conditions that did not control for uniform expression. These were selected to further pursue and investigate.

2.2 Introduction

Cry5B is a pore-forming toxin that attacks the intestines of invertebrates, causing death of the host. When *C. elegans* are exposed to Cry5B, they display a clear intoxicated phenotype – reduced coloration, reduced motility, smaller size, and reduced egg production (Marroquin 2000). The mechanism of action of Cry proteins in general has been determined, and it is assumed that particular amino acids play essential roles in governing these key steps. Therefore, single point mutations of these amino acids may alter toxicity by affecting, for example, the efficiency of these steps. While the majority of examples in the published literature show single amino acid changes leading to a complete loss in toxicity, there are a handful demonstrating that a change in a single amino acid can lead to an increase in activity against a Cry protein's invertebrate target. For example, variants H168R and N372A/G in Cry1A display a 3-fold and 8-fold increase in toxicity, respectively, against *Manduca sexta* larvae as compared to the wild type protein (Wu 1992, Rajamohan 1996). In Cry3A, a single point mutation led to a variant with approximately a 2.4 increase in toxicity as compared to the wild type (Wu 2000). Discussions in these and other papers correlate changes with particular steps in the mechanism of action as a potential explanation for the increase in activity. However, no approach has been reported that involved scanning all domains of the crystal protein.

In addition to broadening our knowledge about Cry5B, and potentially understanding the protein and its structure function relationship better, there are several additional reasons why identifying improved protein variant candidates would be beneficial to pursue. From the mindset of developing a drug to be realistically delivered in developing countries, a treatment of a single dose would be an ideal strategy. By striving to increase the toxicity of Cry5B, this could reduce the quantity of the drug needed, as well as reduce associated costs, which is also an important factor to consider. Additionally, as mentioned in chapter 1, current drugs have showed emerging reports of resistance, and it could be assumed that eventually Cry5B would suffer the same fate, as the STHs would start to develop resistance to the protein. Variant Cry5B proteins have the potential to combat and circumvent the potential of resistance (Deist 2004). Also, in addition to addressing the overall goal of finding a cure for intestinal parasitic worms in humans, these variants could also play a role in veterinary medicine as well.

When I started this project, random PCR mutagenesis in the Aroian lab had identified two amino acid changes in Cry5B that increase toxicity – Cry5B(D553N) and Cry5B(S407C). The screen that identified two variants assayed 1920 clones from a mutagenic PCR of Cry5B, testing the effect of feeding *E. coli* expressing each of these Cry variants to *C. elegans*. Quantitative LC-50 killing assays using purified protein against *C. elegans* were used to confirm the two hits. This information provided a basis to move forward with a large scale screen against Cry5B.

Amino acid scanning mutagenesis is a technique that has been employed to determine what the role of a specific amino acid residue plays in a protein. It does not target a few specific amino acids, but instead serves to attack the protein as a whole and

look systematically along the length of the protein. For example, a study to investigate

the anthrax protective antigen developed a protocol to mutate each of the 568 amino acids

in the protein to cysteine, and then characterize the results (Mourez 2003). I modeled my

methods based on the success of this study, choosing to use an alanine scanning approach

with Cry5B to scan for variants that show an increase in activity as compared to the wild-

type Cry5B in *E. coli* against *C. elegans*. Because the side chain of alanine is small, the

number of variants in which the overall structure of the protein is disrupted is expected to

be minimized. With this method, I sought to identify single amino acid variants of Cry5B

that had an increase in activity as compared to the wild-type by carrying out an alanine

scan along the length of the 698 amino acid toxin domain. No mutagenesis was planned

or conducted for the 547 amino acids outside the toxin domain in the full-length protoxin;

all activity resides in the toxin domain following cleavage of the protoxin by invertebrate

midgut proteases.

2.3 Optimization of assay/Initial testing

To adapt the assays in the lab to this specific study, I started with a basic protocol

to test feeding *C. elegans* with *E. coli* expressing Cry5B in the context of the alanine scan

(Bischof 2006). Specifically, I examined whether induced expression of the clone was

necessary, as well as how the parameters of number of worms seeded per plate, dilution

percentage of Cry5B, and plate size affected the assay procedure and outcome.

Mode of expression of recombinant Cry5B gene. These studies were carried out

with the OP50 bacteria transformed with a pQE80 derived plasmid that encoded wild-

type Cry5B. The bacteria were either uninduced or induced with 0.5mM IPTG for 3hr at

37°C to drive Cry5B expression and then spread on ENG-IA plates in triplicate. I then seeded the sets of three plates with 20, 40, or 60 L1-stage *C. elegans* and monitored worm survival. While it was observed that the induced cultures displayed higher toxicity on the worms as compared to the un-induced, it was not determined to be necessary step to actively detect the toxicity phenotype on *C. elegans*. This detail was important as I established the conditions for the screen, looking to balance quality of the assay with the need to have a streamlined system to scan all 698 amino acids of the toxin domain of Cry5B.

Worm number. For worm number, 20 L1/plate was too low of a number for an accurate assay, as many of the worms were lost on the plate, while 60 L1 was unnecessarily high. Therefore I decided to seed 40 L1 *C. elegans* on each plate.

Cry5B concentration. To determine what concentration of Cry5B to use, I set up a blind experiment with one of the Cry5B hyperactive variants (S407C) that was already known in the lab. Wild-type and S407C Cry5B were expressed in pQE80/OP50 after no induction or induction with 0.5mM IPTG for 3 hr at 37°C. Bacteria transformed with the empty pQE80 vector served as a negative control. Assays were carried out with concentrations relative to the starting preparation of 12.5%, 25%, 50%, and 100%. Because a difference between the wild-type Cry5B and S407C could most readily be discerned at the lower doses, and that difference could be seen clearly without induction, I reaffirmed my decision not to include an induction step in the protocol.

Duration of assay. The most significant factors for detecting S407C as hyperactive compared to wild-type Cry5B were worm coloration, size, movement, and

the presence of eggs/progeny, all of which were best detected at a time-point of 60 hrs or longer.

Toxin plate size type. I tried 12-well plates, 6-well plates, and 60mm plates, and adjusted the amount of bacteria spread and worms seeded accordingly. Although the 12-well and 6-well formats increased the ease of spreading the bacteria and reduced the time in scoring, the reduced surface area led to a large percentage of worms crawling off the plates (even when the bacteria was not spread to the edges). This created situations where there were not enough worms present on the plate to accurately score the assay successfully. After observing the same results in repeat assays, I adopted use of the more reliable and consistent 60 mm plates for the remainder of the assays.

Pilot Study

With the overall goal being to scan the toxin domain, I started with a small-scale approach, working with just the first 12 amino acids in domain III of the protein (aa545-556). The choice of these residues for the pilot was based on the availability of the necessary oligonucleotides in the lab library. Initially, I had planned to transform the variants into homemade electrocompetent OP50 cells and perform the assay in OP50 *E. coli*. However, I was unable to obtain the necessary transformants using the OP50 cells. I therefore switched to the XL10-gold *E. coli* cells provided with the commercial mutagenesis kit. Following the protocol described in the anthrax scanning study (Mourez, 2003), I picked two colonies for each variant from the transformation and sequenced in the region of interest to confirm the presence of the mutation. I did not obtain any transformants for one variant. Of the remaining eleven, ten had the correct sequence in

the mutagenized region. As a further control, I randomly selected six of these clones for full sequencing. For none of the six were any additional mutations detected.

The general protocol and time course of the alanine scan is outlined in Figure 1.1. Owing to the simplicity of the Cry5B-*C. elegans* system, I was able to carry out the mutagenesis and assay at a rate of 45 amino acids per week.
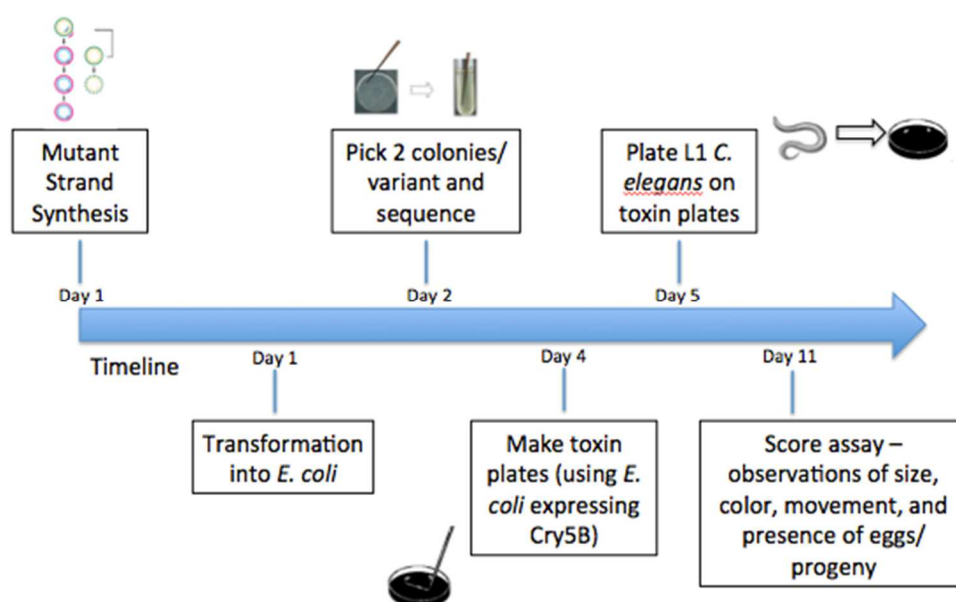
Figure 2.1: Alanine Scanning of Cry5B and *C. elegans* standard toxicity assay

Timeline of the process from generation of the variant Cry5B to testing against *C. elegans* for changes in toxicity.

In the course of these experiments I found that the Cry5B preparation from uninduced XL-10 gold cells was less toxic to worms than the Cry5B expressed in OP50,. I therefore set out to redetermine a useful working concentration for the assay procedure. In particular, I sought conditions under which the mutants could be sorted into 4 categories: 0 – nontoxic (pQE80), + - slightly toxic (pQE80 < mutant < wtCry5B), ++ - toxic (wtCry5B), and +++ - hyperactive (S407C). Toxin plates were made following the protocol with wt Cry5B and S407C in XL10-gold cells induced and un-induced (0.5mM IPTG 3hr 37C) at varying concentrations (25%, 30%, 33%, 37.5%, 42%, 45%, and 50%) to find the ideal concentration at which to screen. Toxin plates were also set up at a 25% concentration with the first 12 amino acids from domain III of Cry5B. (Figure 2.2). At the 25% concentration, I observed hyperactivity of D553A compared to the wild-type. Because one of the previously known variants is D553N, I concluded that this result was meaningful. Nevertheless, I chose to go forward with the 37.5% concentration, as it provided a clearer difference in toxicity between wtCry5B and S407C and between wtCry5B and pQE80 in the uninduced XL-10-gold cells.

During this pilot assay, the plates were monitored daily, and day 6 was chosen as the best time-point to score the assay. In particular, I noted that at this time point 1) Cry5B showed good active against the *C. elegans*, 2) a loss of toxicity variant could be identified (N545A), and 3) there was a clear difference from the wild-type for both a variant with reduced toxicity (V456A) and one with increased toxicity (D553A). Furthermore, the worms were running out of food, as those feeding on bacteria transformed with the empty pQE80 vector were starved or close to starvation. Data supporting these conclusions is provided in Figure 2.3.
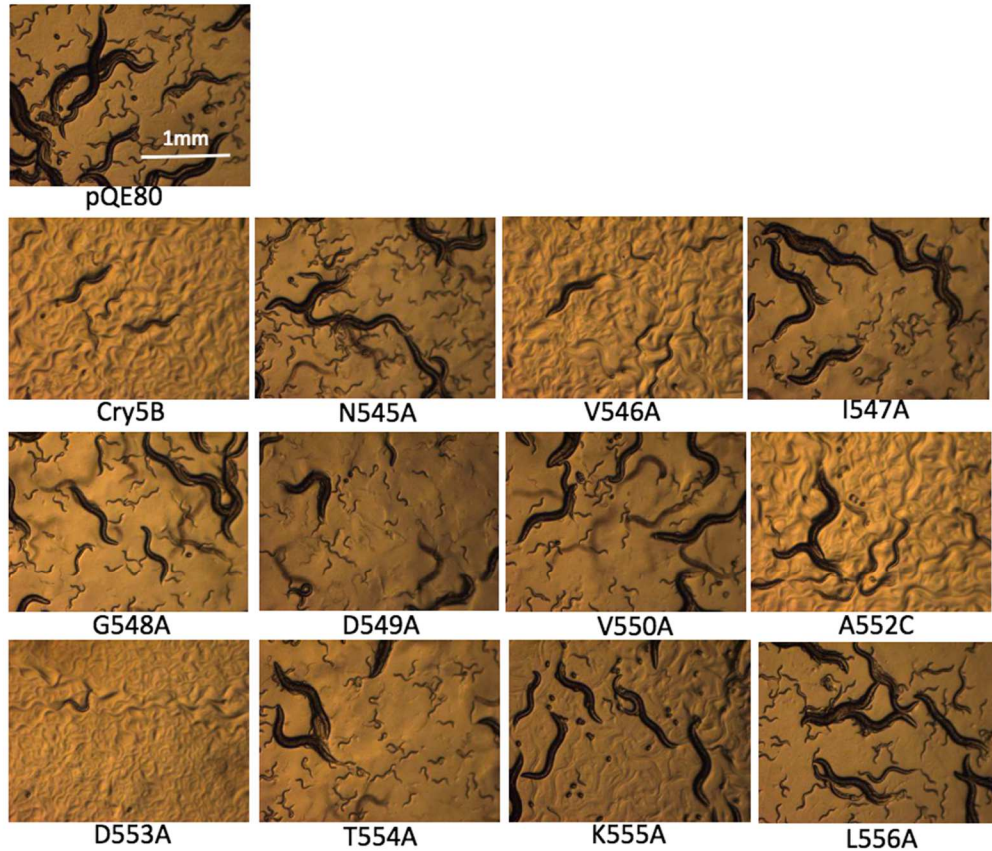
Figure 2.2: Initial testing of assay optimization conditions

The first 12 amino acids of domain III were mutated and tested against *C. elegans* as an initial test to verify the assay parameters and determine protocol efficiency. It can be observed that toxicity differs for the wtCry5B, empty vector, and these 11 variants (one variant did not produce a product).
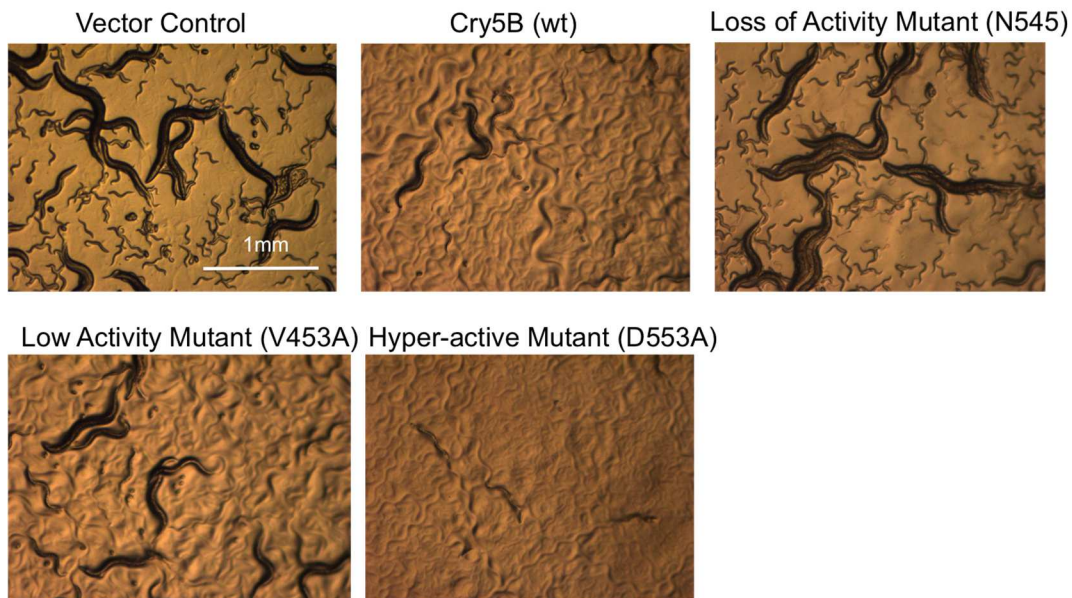
Figure 2.3: Breakdown of the different levels of toxicity observed from the alanine scan of Cry5B

Alanine substitution varies in effect– from complete loss of activity, to reduced activity, to an increase in activity. Considerations for classification include worm size, coloration, motility, presence of eggs/progeny.

To set-up a protocol for confirmation, I chose to sequence two colonies per transformation plate in the region of the mutation, as well as create toxin plates with these two separate colonies. If the sequence of both colonies was identical in the region of the mutation, but they displayed different phenotypes in the plate assay, I fully sequenced the gene to screen for any additional mutations.

In some cases neither colony represented a plasmid with the correct sequence. I found that by shortening the primers slightly and ensuring that the 3' and the 5' ends included one or more G's or C's usually solved this problem. However, for the large

scale screen, I abandoned those residues for which the correct mutagenesis product was not produced.

Results from the pilot screen provided the basis for the large-scale alanine scan of Cry5B. The mutagenesis product was produced, and 2 colonies were picked per variant. Both these colonies were then used to make toxin plates. Duplicate 60 mm plates were spread with un-induced XL10-gold *E. coli* expressing Cry5B diluted out to 37.5% with empty pQE80 vector. These plates were then seeded with 40 L1 worms, and incubated for 6 days. D553A was used as a positive control for each assay.

Once these conditions were established and confirmed, I was able to scan through the toxin domain of Cry5B at a rate of ~ 45 amino acids per week. Worms were scored based on coloration, size, movement, presence of eggs/progeny as compared to wild-type (with the main discerning factors being size and numbers of progeny) on the following scale: 0-loss of toxicity; + - reduced activity; ++ - like wt Cry5B; +++ - slightly hyperactive; ++++ very hyperactive. The categories of +++ and ++++ were originally one category, but were separated once I determined that I most interested in variants that showed a dramatic increase in activity. Variants of interest were sequenced in the region of the mutation and those that were further investigated were fully sequenced in both directions.

2.4 Results of alanine scan

Overall, 551 amino acids were successfully mutated out of the 698 amino acids in the Cry5B toxin domain. (Figure 2.4A). Successfully tested was defined by correct sequencing of the mutation in the region of interest and being tested/scored against *C.*

elegans on toxicity plates. It is interesting to note that in Figure 2.4A, some residues where I did not obtain the desired substitution grouped together, perhaps indicating regions on the protein where changes make it too toxic to the *E. coli* for it to grow.

As shown in Figure 2.4B, Tables 2.1 and S1, 336 alanine substitutions eliminated toxicity (61.0%), 70 retained some activity (12.7%), 54 had activity comparable to wild type Cry5B (9.8%), 57 showed a mild increase in toxicity (10.3%), and 34 appeared to have a substantial increase in toxicity (6.2%). Four additional mutants showed increased toxicity in an initial assay, but not in repeat experiments. Photographs of the 34 plates for which worm number and size was well below wild-type are shown in Figure 2.5. These 34 putative hyperactive variants were each sequenced across the entire gene in both directions to confirm that there were no additional mutations and thus that the observed phenotype was due only to the single amino acid change.
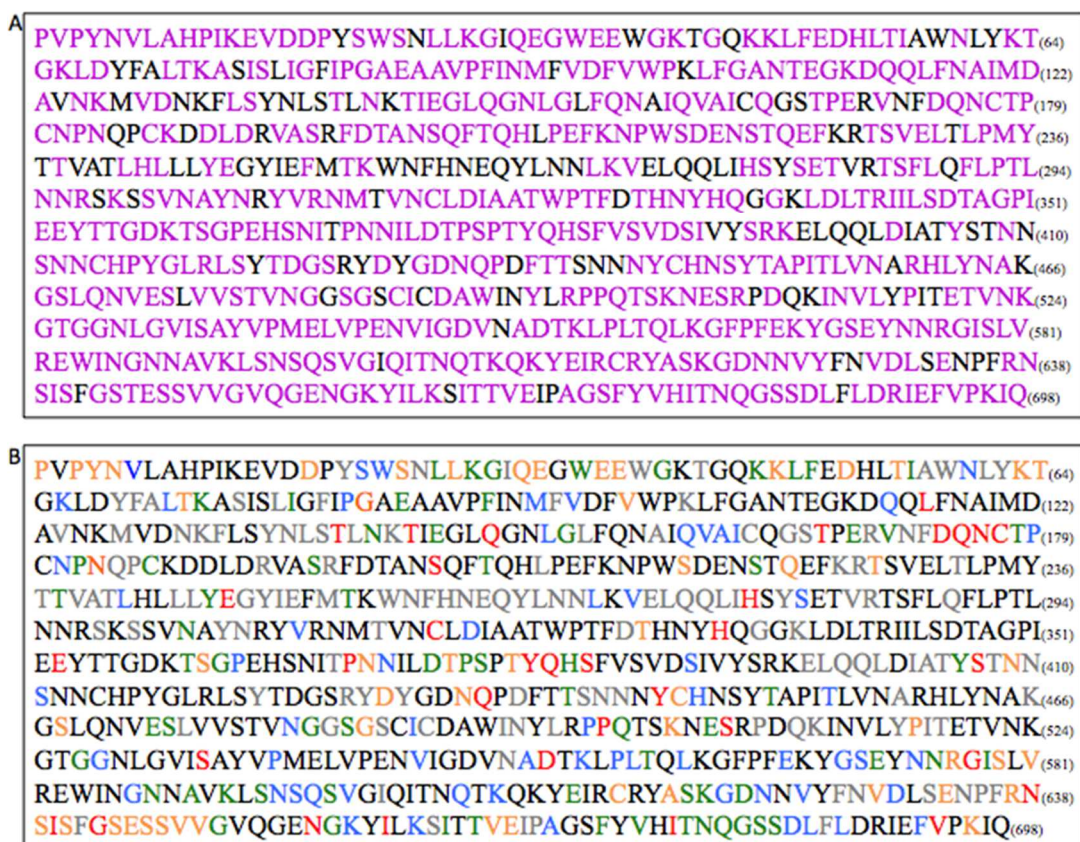
Figure 2.4: Cry5B mutation analysis

A. Of the 698 residues in the toxin domain, I had success in mutating 551, shown in purple; the 147 not successfully mutated are shown in black. B. Breakdown of the variants and how they scored in the toxicity plate assay. Red: ++++ Orange: +++ Green: ++ Blue: + Black: 0 Gray: n/a

Table 2.1: General results of the Cry5B alanine scan

The breakdown of the 551 amino acids successfully tested and verified categorized by level of activity as compared to wild-type Cry5B.

Activity	Number	%	Scored as:
Lost of activity	336	61.0%	0 (pQE80)
Retained some activity	70	12.7%	+ (< Cry5B)
Like wt Cry5B	54	9.8%	++ (Cry5B)
Hyper-active	57	10.3%	+++ (Cry5B <)
Very hyper-active	34	6.2%	++++
Total	551		
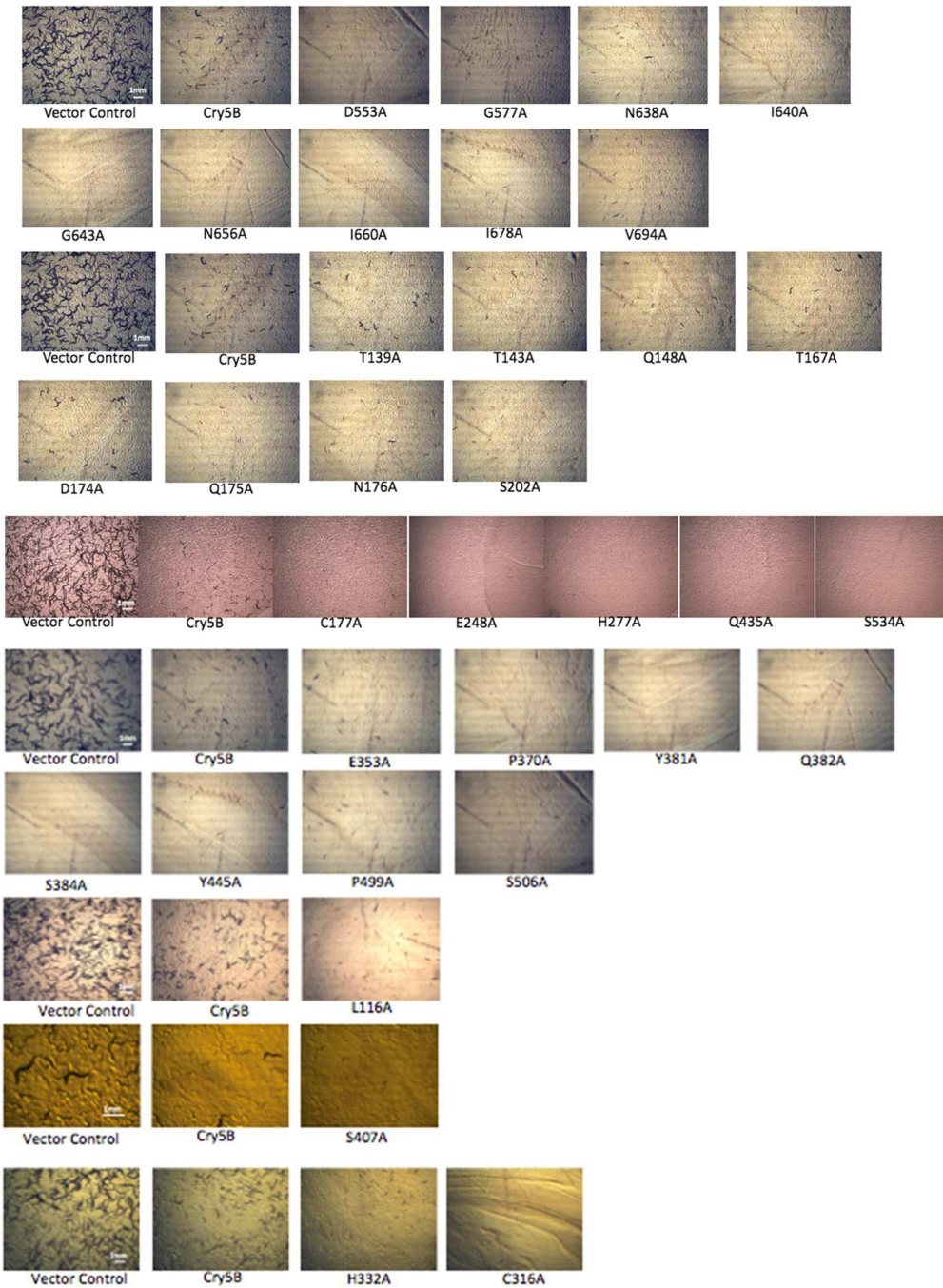
Figure 2.5: Thirty-four hyperactive variants

The 34 variants that stood out from the screen, along with S407C, with their respective controls (pQE80 and wtCry5B). Hyperactive variants display an exaggerated toxicity phenotype compared to the wild-type Cry5B with respect to coloration, size, motility, and presence of eggs/progeny.

2.5 Additional confirmation with nine variants from domain III

Domain III of the alanine scan mutagenesis was completed first, and nine hyperactive variants were identified to be of interest. I used these nine variants to do additional testing to confirm their hyperactivity. In particular, I wanted verify that the observed phenotype was solely due to this single amino acid mutation. To address this, I mutated these single mutants back to the wild-type sequence, and tested them in the *E. coli* plate assay against *C. elegans,* with the goal being to restore the wild-type Cry5B level of activity. The results confirmed that all nine hyperactive variants now displayed the wild-type Cry5B level of activity.

While these nine hyperactive variants in domain III all appeared to be more active than the wild-type in the *E. coli* plate assay, this is not a highly quantitative assay. In order to begin to distinguish between the different variants, I set up a dilution series with two variants from domain III – G577A and I640A (Figure 2.6). As the *E. coli* was further diluted out, the differences between the variants and the wild-type Cry5B became less pronounced, but still detectable. Additionally, while at the 37.5% concentration, G577A and I640A appear to be similar in their level of toxicity, at a lower concentration G577A appears to be more active than I640A.
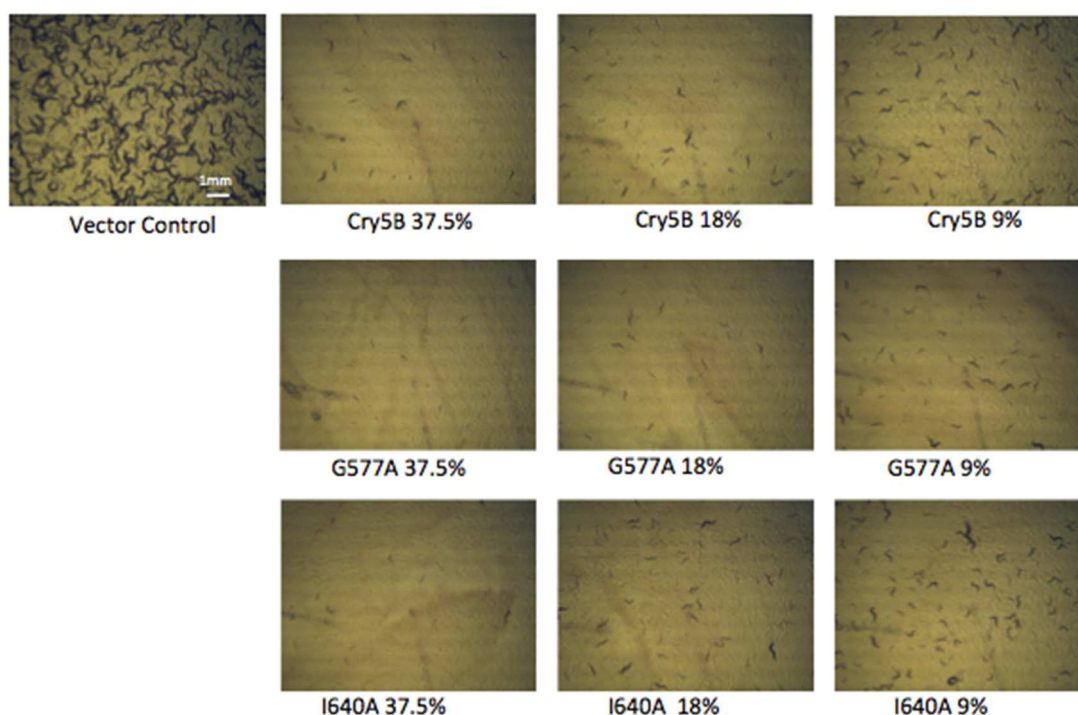
Figure 2.6: Dilution series of G577A and I640A

Cry5B and variants were diluted down to 18% and 9% to start to further understand how these variants compared to one another in *E. coli*.

To further validate the authenticity of these variants, I also wanted to demonstrate that the hyperactivity observed was plasmid-associated, while still keeping the variants in *E. coli*. I took these nine variants and transformed them into the *E. coli* strain JM103. Since I knew that this bacteria expressed Cry5B at a higher level, I dropped the concentration in the *C. elegans* toxicity plate assay down to 18% (Figure 2.7). Although the JM103 strain of bacteria were sometimes distributed asymmetrically on the plates, the worms overall were exposed to the same amount of bacteria on each plate. It was

observed that these nine variants were also more toxic than the wild-type Cry5B in the

JM103 bacteria. Although similar confirmation tests were not carried out for the other

mutants, the nine mutants studied above (roughly ¼ of all the hyperactive mutants) were

taken as representative for the entire batch.
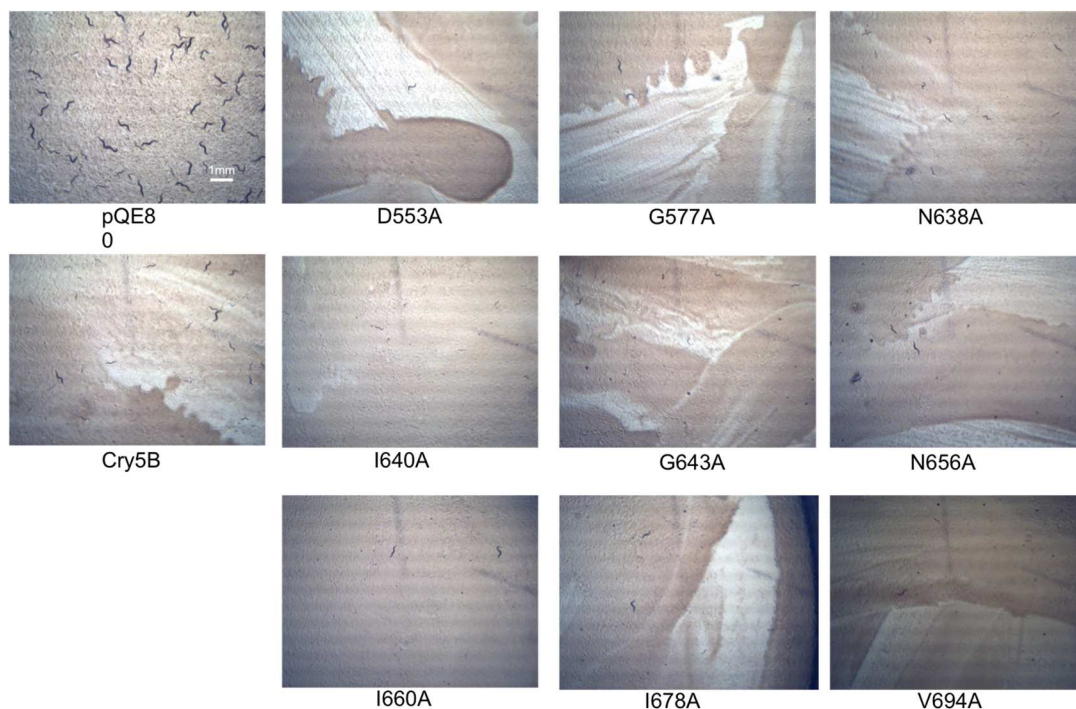
Figure 2.7: The nine hyperactive variants in domain III tested against *C. elegans* in JM103

The nine identified hyperactive variants in domain III of Cry5B were tested in JM103 *E. coli* to show that this observed hyperactivity is plasmid specific.

2.6 Expression levels

One variable of importance that the plate assays do not control for is expression

levels. I did not assay during to screen to determine if the increase in activity that is being

observed is due to a change on a protein function level, or simply due to an increase in expression levels. It is also possible some important hyperactive mutants would have been missed if their expression levels were lower in the E. coli and thus came through as low-normal toxicity. Therefore I performed a few western blot analyses to determine if any dramatic changes in expression levels were being observed, and were of concern with regards to moving forward with the 34 identified variants. *E. coli* cultures expressing the wt Cry5B and the nine hyperactive variants from domain III of Cry5B were grown up overnight, induced, and then blotted with an anti-Cry5B antibody in both XL10-gold cells and JM103 cells (Figure 2.8). For the Cry5B expressed in XL10-gold, variant I678A shows a potential increase in expression, while for the Cry5B expressed in JM103, the same variant (I678A) and three additional one - D553A, I660A, and V694A - appeared to increase expression. Since I planned to use a more quantifiable version of the protein from *Bacillus thuringiensis* going forward, investigation into the expression levels on the plates were not pursued further. An important caveat of these experiments is that they were not carried out under the same conditions used for screening and confirmation (i.e., not induced, on plates…). Nonetheless, I feel they are representative of how well these variants are expressed in *E. coli* in general.
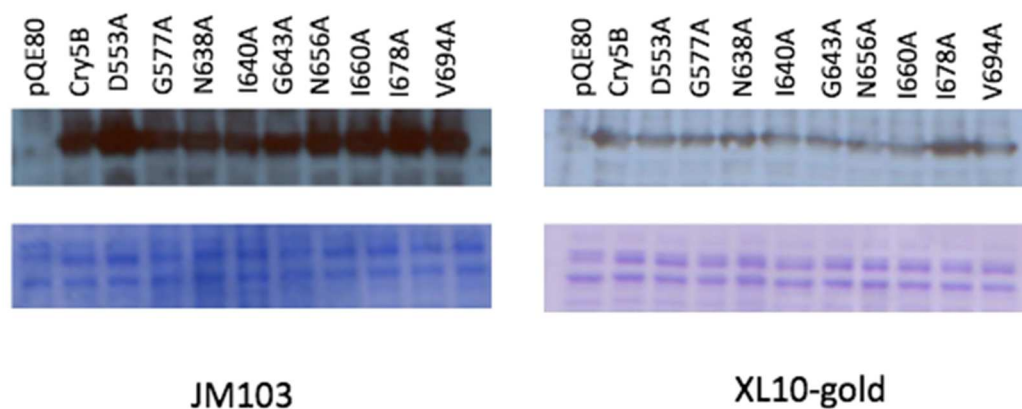
Figure 2.8: Expression levels of nine hyperactive variants in domain III in JM103 and XL10-gold *E. coli*

Western blots and associated coomassie stains of Cry5B and nine hyperactive variants in two separate *E. coli* strains – JM103 and XL10-gold. Commassie stain of background bands provides a normalization for loading.

2.7 Discussion

These results demonstrate that a crystal protein can be approached in a large scale

systematic way to identify single amino acid changes that potentially lead to changes in

activity against an invertebrate target. I was able to attempt to mutate and test 551 out of

698 amino acids in the toxin domain of Cry5B and assess for changes in toxicity against

C. elegans as compared to the wild type protein in a streamlined, efficient manner. The

importance of determining the right parameters for this screen was crucial for being able

to take advantage of the *E*.coli-Cry5B-*C. elegans* system. Through assay optimization, I

was fully able to benefit from the fact that we can feed *C. elegans E. coli* expressing

Cry5B, and observe the worms for signs of intoxication. The simplicity and beauty of this system has allowed me to attempt to screen many amino acids in a rapid and precise manner.

The results show that 61% of these variants lost all toxicity when one amino acid was changed. I would hypothesize that a majority of these have lost toxicity due to interrupting the protein's ability to fold, but I speculate that there are a large number of these loss of toxicity mutants that play important roles in function, and whose protein confirmation is not altered or compromised. Without a quick and easy way to separate them out, it would be a large undertaking to determine which variants from this category are useful to investigate in further detail. The potential importance of these mutants, however, must not be overlooked, for it would be predicted that the site of receptor binding on this protein is contained in this category. Although not investigated in this study, this category of variants provides a considerable resource for future investigation of structure-function relationships for the Cry5B protein.

There were 34 variants that showed an increase in toxicity as compared to the wild-type Cry5B in my assay. This is category that I have the most interest in, and have pursued these 34 variants further in my thesis. It is not clear what role these amino acids play in the function of the protein from this study, only that changing them to an alanine, or disrupted the 'normal' function of the amino acid and appeared to increase their toxicity against *C. elegans*. Although a qualitative assay, steps were then taken in order to attempt to verify and confirm these results while staying in the *E. coli* system. There is a balance between being able to do a large-scale screen in a timely manner, and controlling for every variable in this case. Full sequencing confirmed no additional alterations in

sequence were present, and this was also verified by reverting nine variants to the wild-type and re-observing wild-type Cry5B activity. This confirmed the importance of this single amino acid change for the change detected in my assay, regardless of what role the specific amino acid plays. Testing several dilutions of the variants indicate that these 34 variants have different levels of toxicity. Additionally, preliminary western blot analysis indicates the possibility that for several of these amino acid changes, the increase in toxicity may reflect an increase in protein stability and thus level.

The weakness in this assay is the use of an *E. coli* system without a way to control for equal levels of protein. A single Western blot at a single dilution can not rule out for example, that there is a 30% change in protein expression that could account for the observed change in activity. Additionally, it is also possible that one of the variants classified as 'like wild-type' are hyperactive, but have reduced protein expression and were therefore not identified in this screen. Ideally, one would also quantify the levels of protein directly from the plate assay since this is how the variants were selected that I further pursued (as opposed to quantification from a liquid culture).

Additionally, in looking at the future potential of Cry5B protein variants, it would be important to note that these variants were all found through screening for a putative increase in toxicity against *C. elegans*. At this point, it was not known whether any hyperactive variants would be observed in any of the parasite systems, nor whether any of the variants from the other categories of toxicity might show a different effect on one of the parasites as opposed to *C. elegans*. Although not explored in this study, this is also something that would be of interest to examine.

2.8 Materials and Methods

Site-directed Mutagenesis

Mutations were made using a whole plasmid mutagenesis approach in the Cry5B-pQE80 system using the QuikChange II LX-Site-Directed Mutagenesis kit by Agilant/Stratagene. Two primers were designed for each variant containing the specific mutation of interest with assistance from the primer design program on the Agilant website and produced by Bioneer. Primers were then manually altered if necessary. Following the site-directed mutagenesis, the template strand was digested by Dpn1, and the product was transformed into *E. coli* XL10-gold cells, and grown overnight on LB ampicillin plates at 37°C. Two colonies were picked the following day per variant, plasmid prepped, and sent for sequencing (Genewiz) only in the region of the mutation for confirmation. Full sequencing of the gene in both directions was performed for those variants identified from the screen to be of interest. To test the efficiency of making the mutation (with no extra changes to the sequence), I fully sequenced in both directions six variants irrespective of whether they were hyperactive or not. In this case, five of them were good, and the sixth had an extra nucleotide deletion resulting in a frameshift mutation and an observed loss of toxicity (the kit claims an 85% success rate). When sequencing only in the region of interest, I found that for the first 12 amino acids (two colonies for each) that I changed (aa545-556), there was a success rate of 92%.

C. elegans Plate Toxicity Assays

Qualitative toxicity assays were performed to analyze *C. elegans* intoxication between Cry5B and the variants. Overnight bacterial cultures of XL10-gold *E. coli*

expressing the Cry5B variants were diluted with the pQE80 vector control to 37.5% at OD=2.0 . The point variants grew up approximately +/- 0.2 OD as compared to the wild-type, and was observed to be consistent as the mutations were made along the protein. For each sample, 30ul of bacterial culture was spread on a 60 mm enriched nematode growth plate supplemented with IPTG and ampicillin (ENG-IA – 100ug/mL Amp and 0.1mM IPTG) in duplicate and grown overnight at 25°C. The following day, 40 freshly bleached L1 *C. elegans* were seeded on the plates with *E. coli* expressing the Cry5B variants, the wild-type Cry5B, or the pQE80 vector control (no Cry5B expression). The plates were incubated for 6 days at 20°C, and then scored visually as compared to the wild-type Cry5B on the basis of size, motility, coloration, plate starvation, and the presence of eggs/progeny. For testing in JM103 *E. coli*, the assay was set-up in the same manner, except the Cry5B was diluted out to 18% using the empty vector and still normalized to OD=2.0.

Western Blots with *E. coli*

Overnight *E. coli* cultures were made in either XL10-gold cells or JM103 from single colonies. XL10-gold cells were diluted 1:10 with ampicillin shaking for 1hr at 37°C, and then induced with 0.1mM IPTG for 3 hr shaking at 37°C. The cultures were normalized to OD=3.0 run on a 6% gel, and then transferred to a PVDF membrane via a semi-dry method for 45min at 60mA in transfer buffer (50mM Tris base, 384 mM glycine, 10% methanol). The membrane was then blocked in blocking buffer - 5% non-fat milk in TBST - (10mM Tris pH=7.5, 100mM NaCl, 0.1% Tween 20) for 1 hr at room temperature. The membrane was washed 3x with wash buffer (TBST) 5min/wash, and

then incubated with an anti-Cry5B antibody at 1:1000 overnight in blocking buffer. The

following day, the membrane was washed 4X with wash buffer (5min/wash), and

incubated 1 hr at hoom tempearature with an anti-rabbit secondary antibody (1:10,000) in

blocking buffer. Followng four wash steps in wash buffer, the membrane was exposed.

JM103 cells, were diluted 1:10 with ampicillin shaking for 1 hr at 37°C, and then induced

with 0.5mM IPTG for 6 hr shaking at 30°C. The cultures were normalized to OD=3.2,

run on a 6% gel, and then blotted with an anti-Cry5B antibody at 1:1000. For both cells, a

second gel was run in parallel and stained with coomassie as a loading control.

2.9 References

1. Michael Mourez, Ming Yan, D. Borden Lacy, Lisa Dillon, Lori Bentsen, Amy Marpoe, Clémence Maurin, Eileen Hotze, Darran Wigelsworth, Ruth-Anne Pimental, Jimmy D. Ballard, R. John Collier, and Rodney K. Tweten, Mapping dominant-negative mutations of anthrax protective antigen by scanning mutagenesis. *PNAS* **100,24**, 13803-13808 (2003).

2. Dong Wu and Arthur I. Aronson, Localized Mutagenesis Defines Regions of the Bacillus thuringiensis Endotoxin Involved in Toxicity and Specificity. *Journal of Biological Chemistry* **267**, 2311-2321 (1992).

3. Francis Rajamohan, Oscar Alzate, Jeffrey A. Cotrill, April Curtiss, and Donald H. Dean, Protein engineering of Bacillus thuringiensis δ-endotoxin: Mutations at domain II of CryIAb enhance receptor affinity and toxicity toward gypsy moth larvae. *Proc Natl Acad Sci* **93-25**, 14338-14343 (1996).

4. Sheng-Jiun Wu, C.Noah Koller, Deborah L. Miller, Leah S. Bauer, D. H. Dean, Enhanced toxicity of Bacillus thuringiensis Cry3A δ-endotoxin in coleopterans by mutagenesis in a receptor binding loop. *FEBS Letters* **473**, 227-232 (2000).

5. Joseph F. Urban Jr , Yan Hu, Melanie M. Miller, Ulrike Scheib, Ying Y. Yiu, Raffi V. Aroian Bacillus thuringiensis-derived Cry5B Has Potent Anthelmintic Activity against Ascaris suum. *PLOS Neglected Tropical Diseases* **7(6)**, 2263 (2013).

6. Yan Hu, Bin Zhan, Brian Keegan, Raffi V. Aroian, Mechanistic and Single-Dose In Vivo Therapeutic Studies of Cry5B Anthelmintic Action against Hookworms. *PLOS Neglected Tropical Diseases* **6-11**, e1900 (2012).

7. Yan Hu, Sophia B. Georghiou, Alan J. Kelleher, Raffi V. Aroian, *Bacillus thuringiensis* Cry5B Protein is Highly Efficacious as a Single Dose Therapy against an Intestinal Roundworm Infection in Mice. *PLOS Neglected Tropical Diseases* **4**, (2010).

8. Lisa D. Marroquin, Dino Elyassnia, J. S. F. Joel S. Griffitts, Raffi V. Aroian, *Bacillus thuringiensis* (Bt) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans. Genetics* **155**, 1693-1699 (2000).

9. Benjamin R. Deist, Michael A. Rausch, Maria Teresa Fernandez-Luna, Michael J. Adang, Bryony C. Bonning, Bt Toxin Modification for Enhanced Efficacy. *Toxins* **6**, 30005-30027 (2014).

10. Larry J. Bischof, Daniella L. HuffmanRaffi V. Aroian, Larry J. Bischof, Danielle L. Huffman, Raffi V. Aroian. *Methods in Molecular Biology* **351**, 139-154 (2006).

CHAPTER 3

INDEPENDENT TEST OF HYPERACTIVITY

3.1 Summary

From the previous chapter, 34 Cry5B variants were identified within the specific

conditions of the plate assay as potentially having greater activity than the wild-type

Cry5B against *C. elegans*. The challenge with the plate assay is the lack of control of the

expression on the plates over the course of six days. Hence, it is not possible to be

certain of the actual dose of Cry5B during the duration of the selection. It is possible, for

example, that small changes in expression (e.g., a 20% increase) might result in apparent

hyperactivity over the duration of the six day assay.

To get around these issues, I decided to introduce the mutations into *Bacillus*

thuringiensis, where protein quantitation is easier and the assays are easier to control. I

successfully subcloned and transformed 19 of the mutant Cry5B genes into *Bacillus*

thuringiensis, and harvested protein spore crystal lysates for quantitative killing assays

against *C. elegans*. Once assay conditions were established, all 19 of these variants were

tested in triplicate repeats against *C. elegans* in a LC-50 killing assay. Of the 19 variants tested in this assay against *C. elegans*, I found that the majority (nine) were more active than the wild-type Cry5B as measured with LC-50 values with non-overlapping 95% confidence intervals.

3.2 Introduction

As described in the previous chapter, I identified 34 variants from the screen as having potentially increased toxicity as compared to the wild-type Cry5B. However, because of uncertainties in *E. coli* expression, it was unclear whether these variants have higher specific activity (e.g., activity on a µg/mL basis). Previous studies in insects have addressed this by harvesting protein from either *Bacillus thuringiensis* or *E. coli*, quantitating it through standard methods (Bradford or Coomassie standards), and then testing it in a dose-dependent quantitative manner against the appropriate insect through different feeding methods and recording mortality. From here, the lethal dose ratio can be determined to identify any significant differences between the wild type and the variants being tested (Wu, 2000, Chandra, 1999). In the case of Cry5B, a single variant - Cry5B (N586) – shown to be more active against L1-stage *C. elegans* in 1) a feeding assay that used different doses of purified protein and that measured worm size, and 2) a brood size assay in which L4-stage *C. elegans* were fed purified protein and their progeny counted. In these assays the production of purified protein involved transforming the mutant of interest into *Bacillus thuringiensis*, allowing sporulation and lysis to occur and collecting crystal proteins, which I quantitated using BSA standards on a gel.

Having this basic standard from which to work, I planned to move forward to quantitatively test these variants against *C. elegans* as pure protein from *Bacillus thuringiensis* to determine which variants were statistically more active than the wild-type Cry5B. From previous experience in working with Cry5B in *Bacillus thuringiensis* following the established lab protocols, we would transform the variants into *Bacillus thuringiensis*, grow them up in media, and purify Cry5B away from spores using solubilization/filtration/precipitation. However, I had preliminary data from working with a Cry5B variant that this protocol might not be able to be readily applied to all variants. In particular, a Cry5B mutant with multiple mutations (mutant A – (note: sequencing data)) once transformed into *Bacillus thuringiensis*, sporulated and expressed protein, but produced crystals that could not be isolated in the same manner and with the success of the wild-type Cry5B. I therefore decided to not purify the protein away from the spores but use the entire Bt spore crystal lysate (SCL) for assays. The protein in SCL can be accurately quantitated and can be used in liquid assays and where the level of protein in the well is better controlled than in the plate assay. Using SCL would thus allow me to overcome the main limitations of working with *E. coli.*

3.3 Subcloning into *Bacillus thuringiensis*

To test my potential hyperactive variants on a quantitative comparative basis to see if they are actually more active than the wild-type Cry5B, I decided to put these modified toxins back into their host strain of *Bacillus thuringiensis*. This would enable me to test these variants side by side as well as against the wild type in a quantitative fashion, while eliminating the potential issue of altered expression levels. Using a

protocol established in our lab (detailed below in 3.10), a simple cut and paste strategy

was employed to cut out the Cry5B mutant within the gene at two internal restriction sites

with using the enzymes NheI and BglII which are located at 42bp and 2052bp

respectively (Fig. 3.1). The shuttle vector, pCry5B (Chandra 1999) into which the insert

was placed contains the Cry5B gene as well as both *E. Coli* and *B. thuringiensis* origins

of replication, and can be selected for with either ampicillin or erythromycin. I

successfully subcloned 19 of the 34 variants using this method into the pCry5B shuttle

vector. This vector was then transformed into *Bacillus thuringiensis* via passage through

GM2163 cells, a dam- strain of *E. coli*.
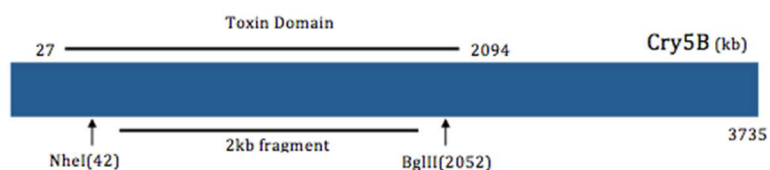
Figure 3.1: Digestion of Cry5b

The cry5B fragment is digested with enzymes NheI and BglII
to release a 2kb fragment to be ligated into the pcry5B vector.

Though a simple copy and paste subcloning method was applied, it is worth

nothing the challenges presented by the size (21kb) of the pCry5B shuttle vector.

Alternative methods were investigated to streamline this procedure. One was to use the

site-directed mutagenesis kit (for which the spec sheet supplied places an upper size limit

of 14kb) directly to introduce the mutation in the pCry5B vector. Through alteration of

the thermocycler protocol to accommodate a longer extension time, (25min/cycle) a

single clone was obtained and verified during an initial test with the D553A primers. Additional primer pairs were then attempted for other variants of interest, but no colonies were obtained on the transformation plates after multiple attempts, and this method was subsequently dropped.

Time was also spent to optimize and workout the details for the digestion and ligation of the vector. The restriction enzyme MluI was explored as an alternative digestion site to BglII, but was found to often trim off the end of the Cry5B gene. NheI and BglII have incompatible restriction buffers, and several different avenues were explored for how to best approach this issue. Sequential digestions led to the loss of too much product, most likely due to the size of the vector and the commercial kit restrictions of how much can be recovered during purification. Buffer 2 from New England Biolabs (NEB) was then used for which both enzymes had reduced activity, and I therefore increased the incubation time to 120min for the digestion to accommodate this. This proved to be a useful choice and increased the success rate of the protocol, although caution was required since too long of an incubation yielded no bands on a gel.

A robust and efficient protocol to transform the vector into *Bacillus thuringiensis* had previously been established, and I had no problems transforming the unmethylated DNA into Bt. From here, all 18 of the variants that were transformed into *B. thuringiensis* were initially confirmed with colony PCR, and then verified through sequencing of the entire Cry5B gene.

3.4 Level 1 protein purification attempts

Following confirmation of the variants in *Bacillus thuringiensis*, I set out to purify protein using laboratory standard protocol (Cappello, 2006). Flasks with PGSM media were inoculated with a seed culture of Cry5B, and incubated with shaking at 30°C for 5 days. The culture then underwent two salt and two water washes. The resulting mixture was then dissolved in acid solubilization medium, and precipitated out with tripotassium citrate to remove and isolate the crystals from the spores. I initially harvested wild-type Cry5B, S407C, and D553N (Figure 3.2a), followed by a harvest of G577A and G643A (Figure 3.2b). From the coomassie stained gels, the variants and the wild type Cry5B appear to be very similar in intensity. Furthermore, the banding patterns running faster than full-length are similar, suggesting a similar pattern of degradation. I planned on testing these proteins side by side, and since we don't necessary know whether the degradation products are active, the similarity in pattern was important for a side-by-side comparison.

Once I confirmed that I had successfully produced spore protein for the variants S407C and D553N, I began growing up the rest of the 19 variants that I had transformed into Bt following the same protocol. However, a large number of these variants did not produce a collectable precipitate. To address this, I tried growing up the variants in a second sporulation media, M03. M03 is a glucose rich media, and the sporulation process occurs much faster than in PGSM. In addition, I decided to investigate the timeline of the harvest, which is five days for the wild-type Cry5B from inoculation to harvesting, based on the idea that these variants may be expressing their protein at different points than the wild-type Cry5B,, as previously suggested for the Cry5B variant N586A (Wang, 2012). Using Cry5B, S407C, D553N, G577A, and G643A, I took

aliquots from the flasks at different time points, checking for crystals under the microscope and running the samples on a SDS-PAGE gel to check for protein expression (Figure 3.2). Five aliquots were taken in M03 media starting at 48 hr; nine aliquots were taken in PGSM media starting at 24 hr.
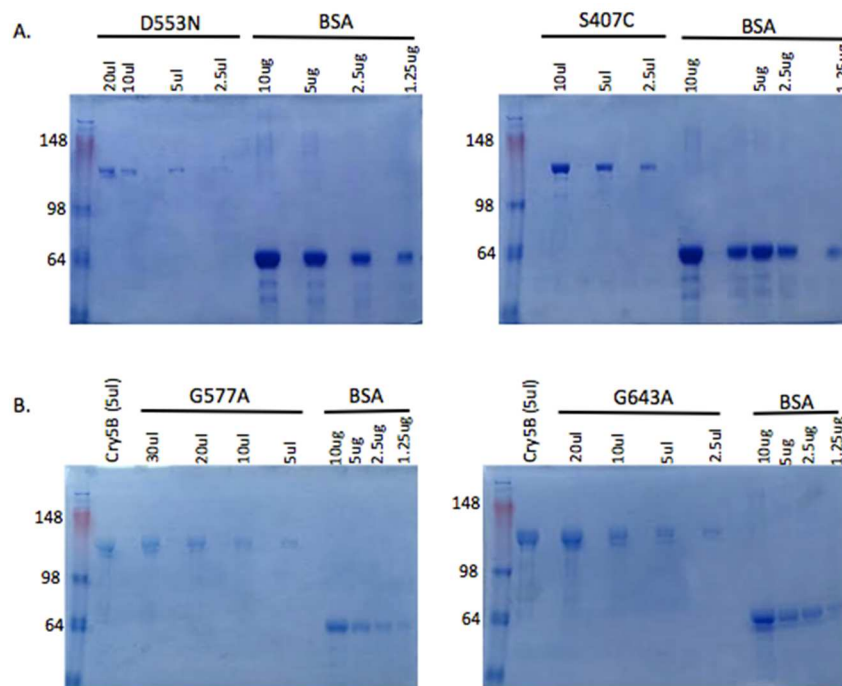
Figure 3.2: Cry5B variant level 1 protein harvest – D553N, S407C, G577A, G643A

Coomassie stained SDS-PAGE gel Level 1 protein harvest for the Cry5B variants D553N, S407C (a), G577A, G643A(b) and BSA standards.

In the M03 media I observed the formation of spores and crystals from the wild type after 48 hr, although there were many vegetative cells present. At 52 hr the cells began to lyse to release the spore/crystal mixture, and at 67 hr, there were almost no vegetative cells in the mixture. The variant S407C does not seem to either move through this process at the same rate, as vegetative cells were present at all three time points. The

variant G577A had a similar pattern to the wild-type, but at a slightly slower rate. However at the 67 hr time point, almost all of the cells had lysed. Lastly, G643A was the most similar to wild-type, perhaps even progressing through the sporulation process at a faster rate, as no vegetative cells were present at the 52 hr time point.

In the PGSM media, the time course of the wild-type Cry5B was as expected – at 24 hr, vegetative cells are observed, and at 48 hr, sporulation is observed, with a mixture of cells and the beginning of cell lysis. At the 80 hr time point, the majority of the mixture is spores and crystals, with some vegetative cells, and at 96 hr, almost all the cells have lysed. At the 24 hr time point, S407C, G577A, and G643A all showed vegetative cells, while sporulation and lysis was observed in D553A. At 48 hr, S407C and G643A appeared similar to wild type, with a mix of vegetative cells and the beginning of lysis starting to occur, while D553A and G577A already showed spore/crystals present in the mixture. By the 80 hr and 96 hr time points, all four variants appeared to have released their spores and crystals and the majority of the vegetative cells have lysed. However, it appeared that the peak protein expression occurred closer to the 96 hr time point in the PGSM media, and so the harvest time point was adjusted accordingly.
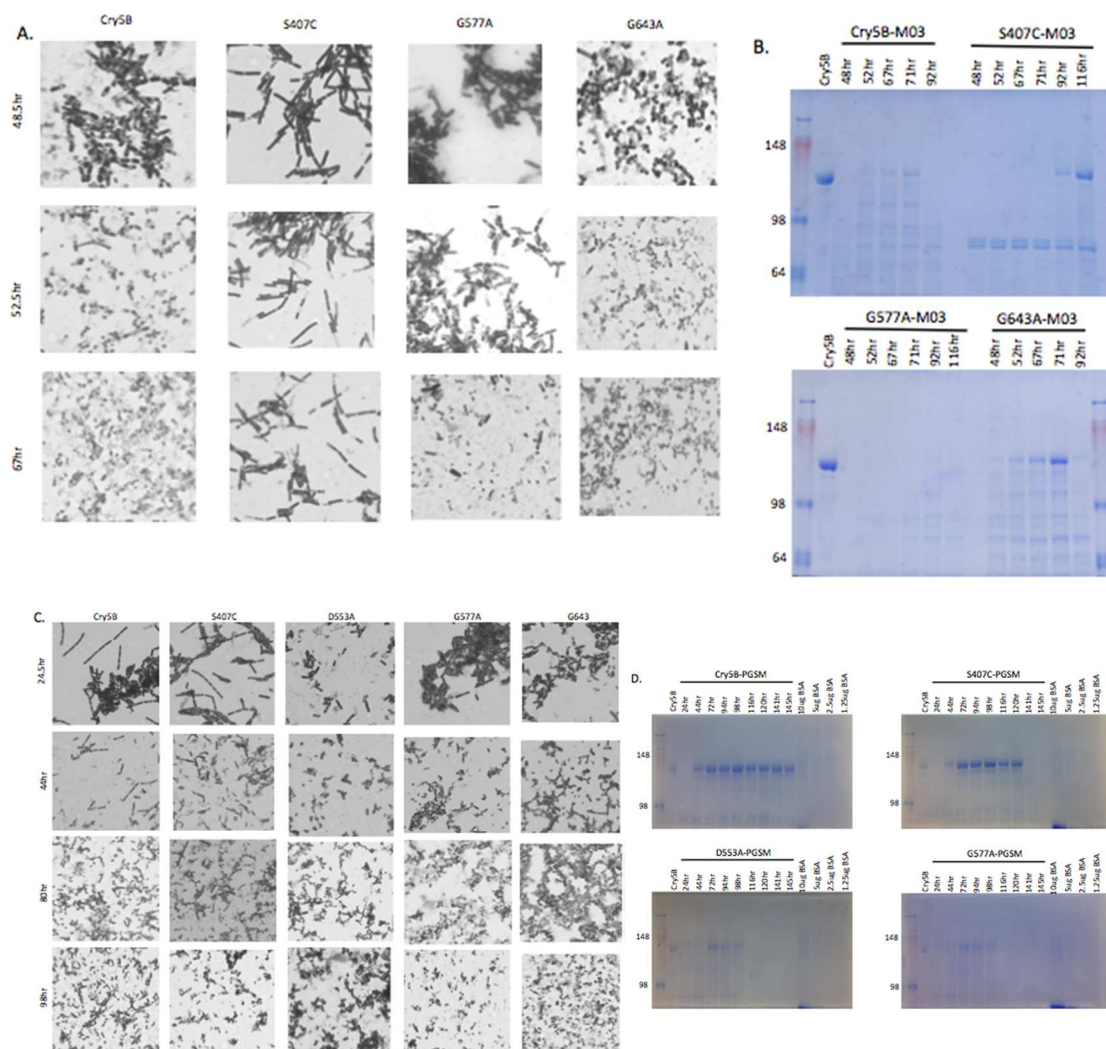
Figure 3.3: Time-course of Cry5B + select mutants

Following the natural progression Cry5B + variants S407C, G577A, and G643A in M03 media at selected time-points to look at sporulation progress (a) and expression (b). Following the natural progression of Cry5B + variants S407C, D553A, G577A, and G643A in PGSM media at selected time-points to look at both sporulation progress (c) and expression (d).

A widespread contamination issue in the lab impacted these studies. Initially, all nine variants from domain III were transformed, harvested, and purified out of *Bacillus thuringiesis*. It was later determined that these were not the point variants, but all wild type Cry5B. Several additional control steps were added to this harvest protocol to address this issue: the use of mock flasks in parallel with any cultures that are being grown up, and sequencing directly off the resulting harvest with colony PCR to ensure that what was growing in the flasks is the variant or wild type of interest.

Other issues that arose in the growing up and harvesting of the protein included mock flasks contaminated with wtCry5B, hardware/equipment issues (foil ripping on flasks; flasks cracking), issues producing a colony PCR product, and accurate quantification of the protein. All of these issues were individually addressed, and resolved. In particular, glass flasks with commercial caps were substituted for the plastic flasks, solving the mock contamination and the issues with the flasks cracking. A parallel experiment was conducted in these glass flasks to confirm similar yields between glass and plastic.

3.5 Other ways to harvest out level 1 protein

Due to the issues encountered with harvesting Cry5B level 1 protein (detailed above in section 3.4), I sought alternatives to the level 1 harvest protocol. One alternative method was to use the truncated version of Cry5B (aa1-772) to purify the protein from *E. coli* (Hui 2012). Cry5B is expressed in the pQE9 vector containing an N-term His-tag that is used to purify the protein out by use of a Ni-NTA agarose column. The protein was successfully purified, but when run on an SDS-PAGE gel and stained with

coomassie, was found to be largely degraded (figure 3.3). Because of the uncertainty with

regard to which bands were active, I did not pursue this or attempt to refine this technique

any further.
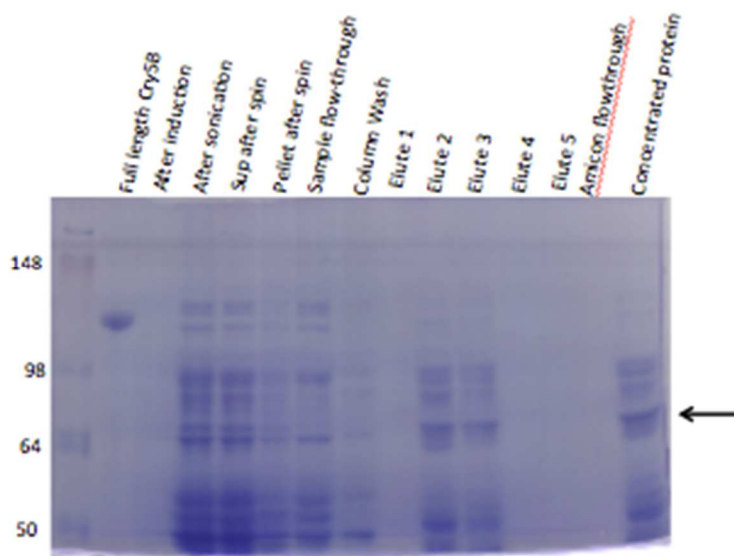
Figure 3.4: Purification of Cry5B from *E. coli*

Wild-type truncated Cry5B (aa1-772) was purified out from *E. coli* using an NTA agarose column, run on a gel, and stained with coomassiee. The purified truncated Cry5B is seen at 72kDa.

A second method I looked into was collaborating with the Felgner laboratory at

UCI and utilizing their high throughput protein expression system (Liang, 2001). Due to

the constraints of their system (max 3kb), I made a series of truncations to Cry5B (aa720,

aa772, aa800, aa900, aa1000) and tested for changes in toxicity in the pQE9 vector in *E.*

coli plate assays. It was found that the full length (aa1245) and the aa772 versions had

similar levels of toxicity, while the aa1000 version had only slightly less toxicity. The

aa720 and aa800 versions lost a fair amount of toxicity, and the aa900 version was never tested (Figure 3.5a). Because the full length Cry5B appeared to be the best option in terms of toxicity, I decided to see if the 3.7kb full length version could be expressed in their system. The Felgner lab was able to express 4 clones in duplicate, which I confirmed via western blot (Figure 3.5b,c). The reaction mixture was determined to be at a concentration of approximately 1.7ug/ml in a 20ul reaction volume. To test this against *C. elegans*, I chose to utilize a 384-well plate format, and did a pre-screen with *C. elegans* and Cry5B to test the worms in this system by both picking and pipetting L4 *C. elegans* into the wells (Figure 3.5d). I tested 2ul of the Cry5B reaction mixture in 384-well plates against *C. elegans* (controls – Cry5B level 1, the negative reaction mixture, buffer only), and found that only the worms in the *buffer only* control well were healthy. Because there was evidently something in their procedure that remained in the reaction mixture and was toxic to the worms, this approach was not pursued further.
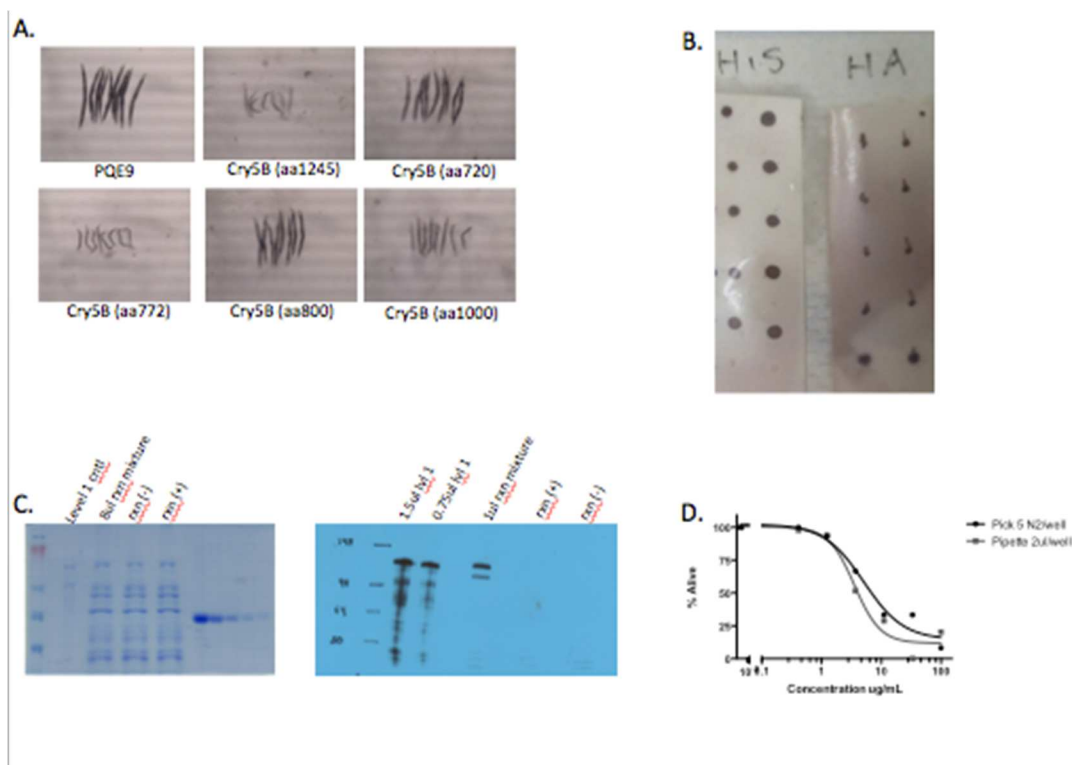
Figure 3.5: Purification of Cry5B using a high throughput protein expression system

(a) *C.elegans* was exposed to different truncated versions of Cry5B and assayed for changes in toxicity. (b) The full length Cry5B was expressed in the expression system – shown four clones in duplicate, and a positive control on the bottom line. (c) The reaction mixture was run on a SDS-PAGE gel and stained with coomassie as well as probed with a Cry5B antibody. (d) *C. elegans* were exposed to level 1 Cry5B in 384-well plates to assay for their reaction in this smaller system set-ups.

3.6 Assays with level 1 protein

Moving forward, I produced enough level 1 protein of Cry5B, D553N, S407C, G577A, and G643A to test against *C. elegans* in quantitative LC-50 killing assays. These assays were first set-up with just D553N and S407C so as to gain an initial read and optimize assay conditions. These two variants were isolated prior to my arrival in the

laboratory and had been shown using purified protein to be hyperactive against C.

elegans in quantitative killing assays. Thus, they served as a positive control. The initial

parameters I used were: pH 7.3 S-media, an incubation of six days at 25°C with level 1

protein dissolved in 20 mM HEPES, pH 8.0, at the concentrations of 48, 16, 8, 4, and 1

ug/ml. The protein was quantified with a nanodrop, and after the assay was set-up, the

left over protein was run on a gel to ensure the same amount of wild-type and variant

protein was being used (Figure 3.6a).

I did not observe the hyperactivity that had been previously been found S407C.

One explanation may be that previous assays used CaCl2. Additionally, I wanted to

investigate the effect of incubating the assay at 20°C for eight days. Based on this

information, I changed a few of the assay parameters as follows: pH 6.0 S-media, with

$CaCl_2$, with level 1 protein dissolved in 20 mM citrate buffer, pH 3.0. Assays were set up

with D553N and S407C level 1 protein in quadruplicate repeats at both 20°C and 25°C,

Both these variants were more active than the wild type (Figure 3.6b,c). The LC-50

values of Cry5B, D553N, and S407C were 3.15, 2.21, and 1.12, respectively, with non-

overlapping confidence intervals. Thus, I was able to confirm the results previously

obtained in the lab.
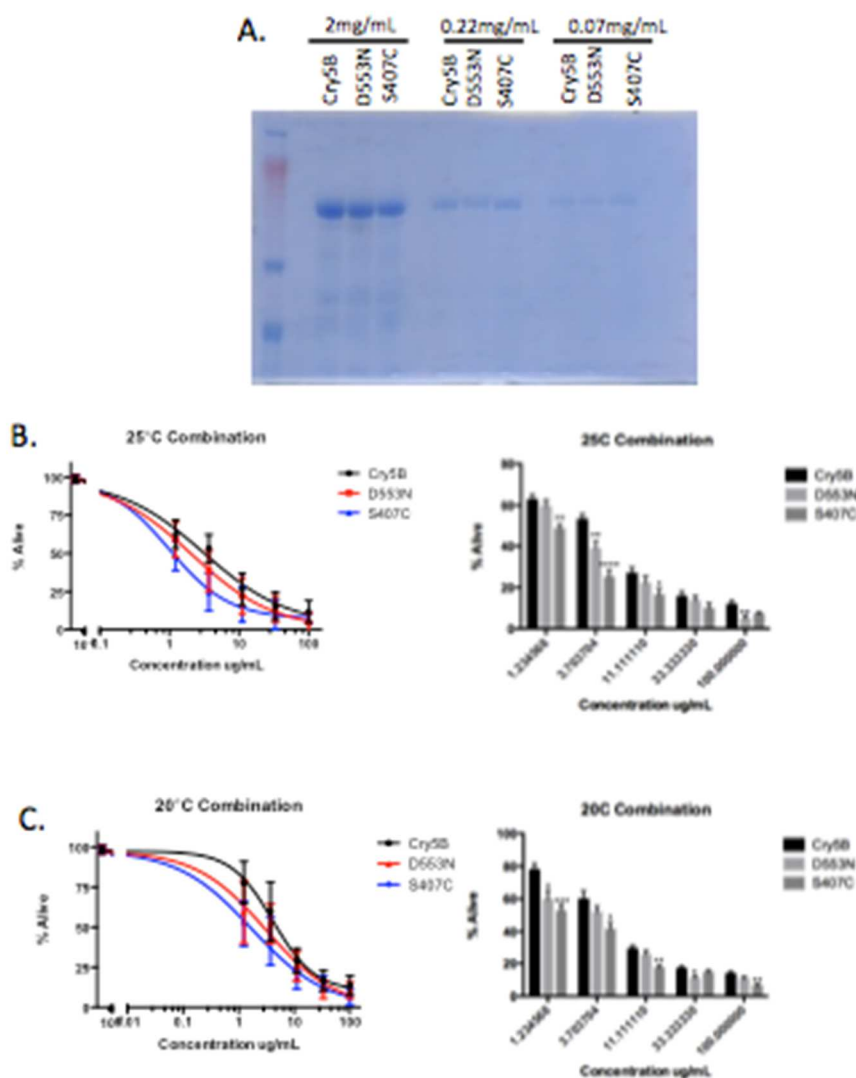
Figure 3.6: *C. elegans* **displays a dose response to pure level 1 protein of Cry5B(D553N) and Cry5B(S407C) in a LC-50 killing assay**

(a) Gels were run to ensure that the worms were being exposed to similar amounts of Cry5B and the two variants being tested. (b) Four individual trials combined at 6 days at 25C. LC-50 values with 95% confidence intervals as follows: Cry5B - 3.15(2.46-3.89), D553N - 2.21(1.83-2.61), S407C - 1.12(0.80-1.47) (c) Four individual trials combined at 8 days at 20°C. LC-50 values with 95% confidence intervals as follows: Cry5B – 3.07(2.39-3.81), D553N – 2.80(2.17-3.47), S407C – 1.54(1.12-1.99).

I then tested two variants from my screen from domain III, G577A and G643A, utilizing S407C as a positive control. S407C continued to be more active than the wild-type, while both G577A and G643A actually displayed a reduction in toxicity as compared to wtCry5B (Figure 3.7). I considered several possible explanations. First, the purified protein is pre-solubilized before it is added into the assay and alterations in solubility can alter toxicity (Wang 2012, Du 1994). I could potentially address this by using spore crystal lysates or testing for solubility changes in media with different pH values. A second hypothesis would be that the worms are developmentally different in the plate assay vs. the LC-50 assay. Essentially, I am looking at static non-reproducing L4 worms vs. the L1 worms on a plate. A third possibility is differences in expression levels in *E. coli*, in which the point mutations may increase expression levels in a way not accounted for in the plate assays. I could investigate this by potentially running western blots off the plate assays and see if expression levels are changing in these variants.
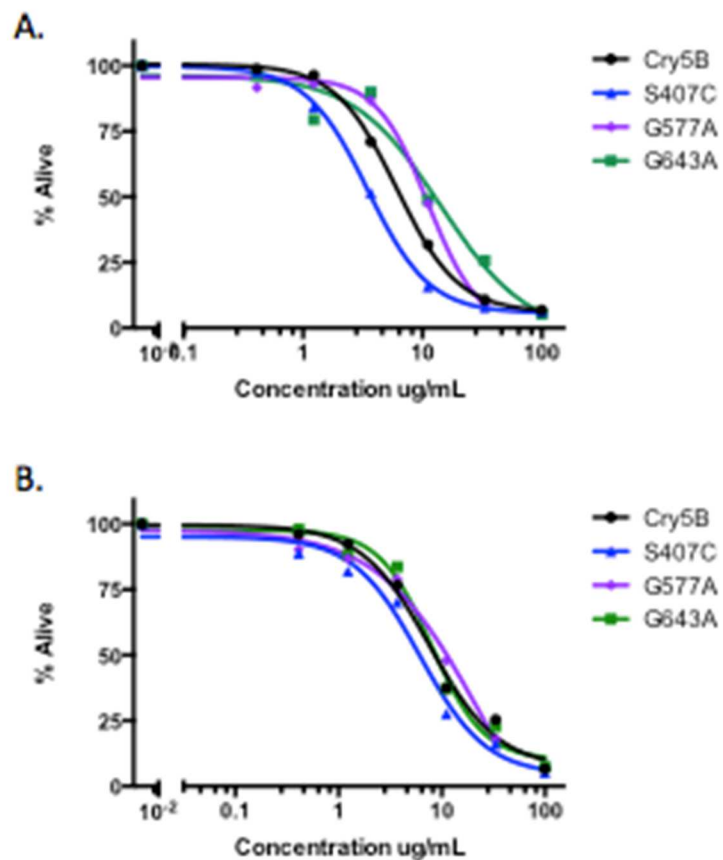
Figure 3.7: Cry5B variants G577A and G643A do not appear to be more toxic than the wild-type Cry5B against *C. elegans* in LC-50 killing assays as purified level 1 protein

(a) Combined duplicate repeats of Cry5, S407C, G577A, and G643A as level 1 protein for 6 days at 25C (b) A single trial of Cry5B, S407C, G577A, and G643A as level 1 protein for 8 days 20C.

3.7 Harvesting of spore crystal lysates

To address the issues with the level 1 protein, I decided to test the same variants instead as spore crystal lysates (SCL). This spore/crystal mixture is very different from level 1 purification since no purification is involved--- merely washing of the bacterial

culture pellets. Since the variant SCL on a gel looked similar to the wild type Cry5B

(Figure 3.8), I felt confident using the SCL to test against *C. elegans* in the dose-

dependent mortality assay. In addition, only the same Cry5B wild-type that was

harvested at the same time as the variants was used in the same LC-50 assay to compare.,

thus controlling for small variances in LC-50 assays that have been observed between

batches of Cry5B assayed as spore crystal lysates. The final parameters were as follows

– 200 mL of PGSM media (supplemented with a salt solution, CaCl$_2$, and ampicillin) was

inoculated with a 1:20 dilution of an overnight culture of Cry5B. Flasks were incubated

shaking at 30°C for 96 hr, and then the SCL was harvested (full details in materials and

methods).
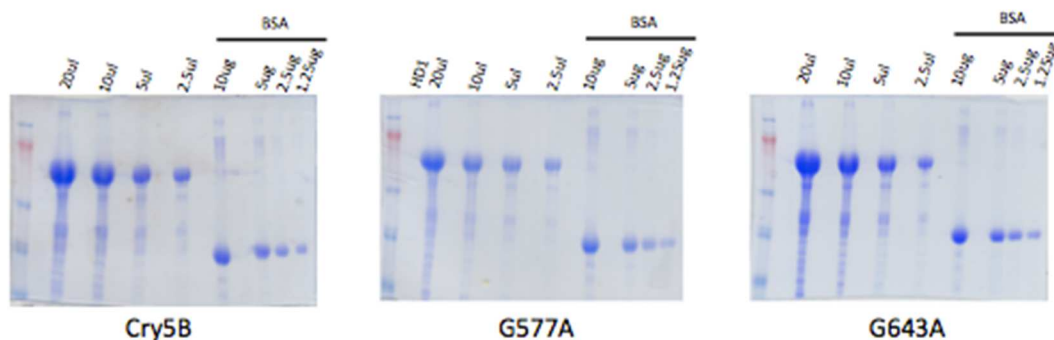
Figure 3.8: Spore Crystal Lysate harvests of wtCry5B, G577A, and G643A with BSA standards

Harvested SCL of Cry5B, G577A, and G643A was run on an 8% SDS-PAGE gel and stained with coomassie. The banding/degradation bands were compared for similarity, and the protein was then quantified using the BSA standards run on the gel.

3.8 LC-50 assays with spore crystal lysates

When the spore crystal lysates were tested against *C. elegans* in triplicate repeats,

G577A and G643A both increased activity as compared to the wild-type (figure 3.9).

Both curves are left-shifted, with the individual data points located below and to the left

of the wtCry5B. The LC-50 values of Cry5B, G577A, and G643A were 13.09, 7.78, and

6.05 respectively, with non-overlapping confidence intervals. Additionally, spore counts

were comparable at 10^{-8} and 10^{-10} dilutions (Table 3.1) I then decided to move forward

to test all 18 variants that had been transformed into Bt in the LC-50 assay as SCL using

a final protocol detailed out in section 3.10 below.

Table 3.1: CryB, G577A, G643A spore counts

Spore counts for wild-type Cry5B, G577A, and G643A were similar at both the 10^{-8} and
10^{-12} dilutions. Cry5B and G643A were almost identical, and the counts of G577A were
slightly lower. Average of two plates/dilution.

	10^{-8} dilution	10^{-10} dilution
Cry5B	22	11
G577A	18	8.5
G643A	22.5	10
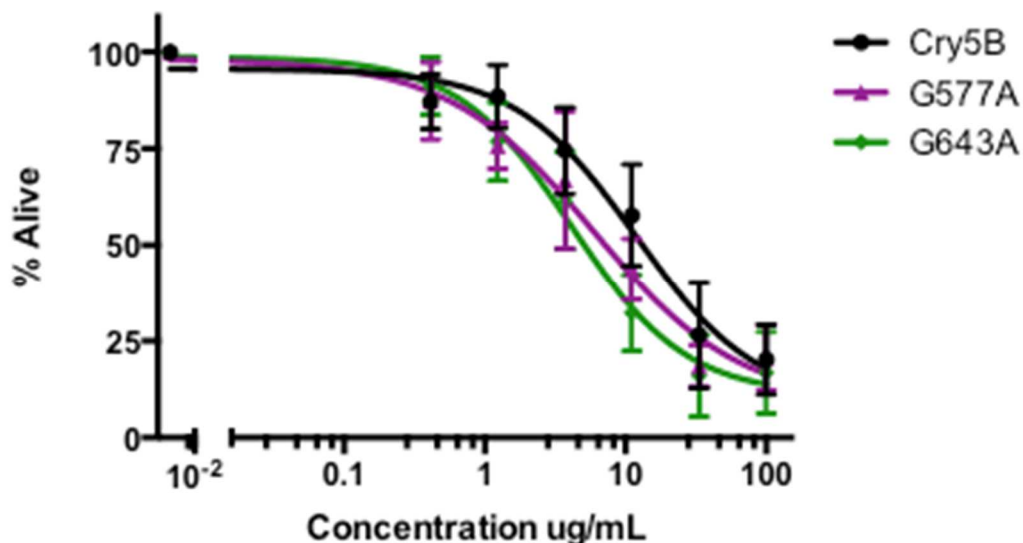
Figure 3.9: Cry5B variants G577A and G643A are more toxic than the wtCry5B when tested as Spore Crystal Lysates in triplicate combined repeats in LC-50 killing assays against *C. elegans*.

G577A and G643A are more toxic than the wtCry5B. LC-50 values with 95% confidence intervals as follows: Cry5B – 13.09(10.88-15.90), G577A – 7.78(6.35-9.46), G643A – 6.05(4.98-7.29).

In the established protocol for testing the SCL in the LC-50 assay, the protein is diluted in water and then added to the wells, not pre-dissolved as with the level 1 protein. However, I did a few preliminary tests diluting the SCL in parallel in water and 20mM citrate pH=3.0 (figure 3.9). I found that when comparing these results side by side, the SCL of G577A and G643A that was diluted in citrate buffer was not as toxic as compared to the SCL of G577A and G643A diluted in water. Besides this difference in appearance, there was no noticeable difference between the two. I speculate these point mutants may

have had an effect on the solubility of the protein, however, this was not further pursued

and remains an interesting avenue to explore in the future.
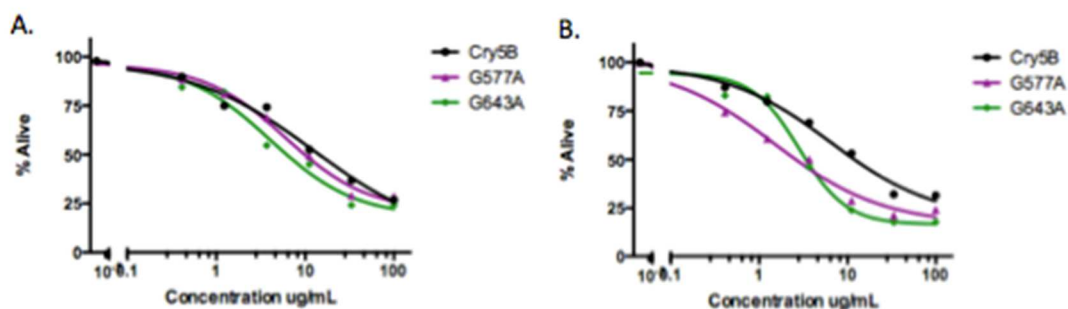
Figure 3.10: SCL of Cry5B, G577A, and G643A were tested in parallel in the LC-50 assay diluted in water and citrate buffer

When diluted in (a) 20mM citrate buffer pH=3.0, the SCL of variants G577A and G643A was found to be less toxic to the wild-type as compared to the SCL being diluted purely in (b) water.

I ran all 18 of these variants that were harvested as SCL through the LC-50 killing

assay against *C. elegans* in triplicate repeats (Figure 3.10). Each of these graphs shows

the combined data for all three trials, in which the wild-type Cry5B was grown up and

harvested in parallel with the variant of interest. Each graph represents two separate

harvests. For the variants that are shown to be hyperactive, this increase in activity is

observed in the individual trials in additioned to the combined data. Although there is

some variance seen in the level of toxicity of the protein between harvests, I found that

the curves of the wild-type Cry5B and the variant always shift together, indicating

perhaps that the protein varies in its activity from batch to batch, but with parallel effects

for the variants and the wild-type.
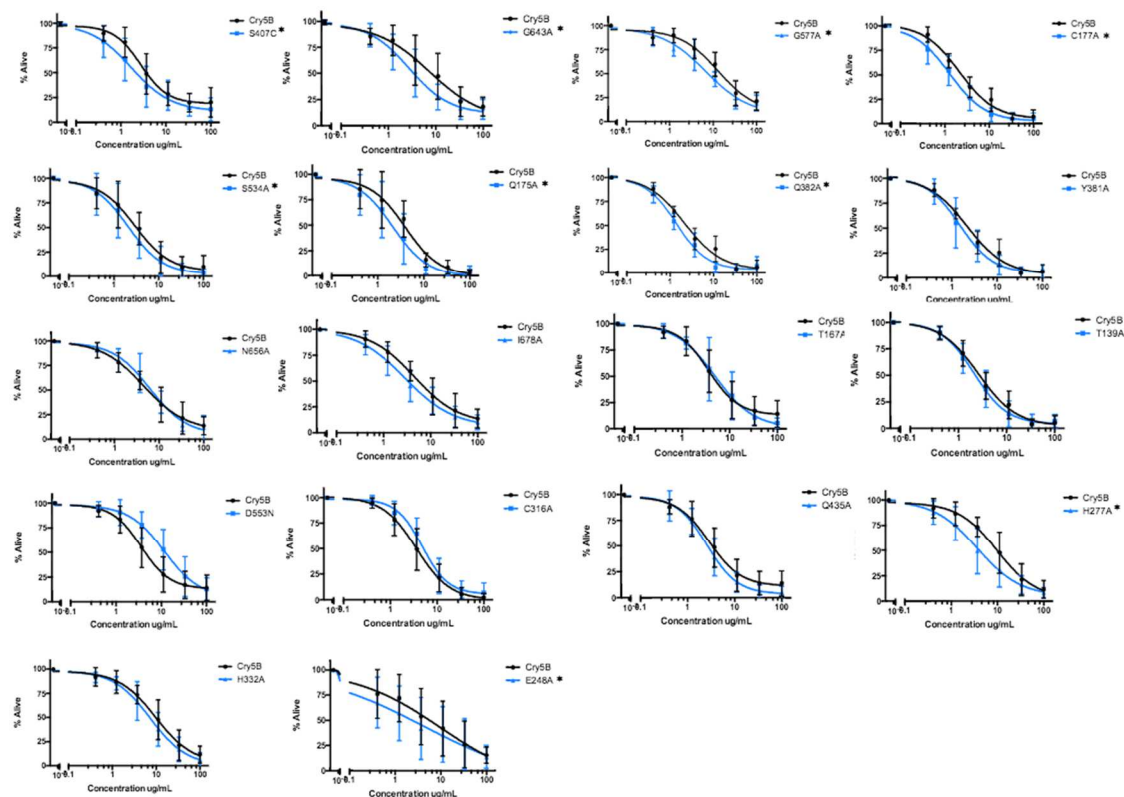
Figure 3.11: Triplicate combined repeats of 18 Cry5B variants tested against *C. elegans* as Spore Crystal Lysates
18 Cry5B variants tested against *C. elegans* in triplicate repeats in LC-50 killing assays. * designates a variant with an LC-50 value that is lower than the wild-type Cry5B with non-overlapping 95% confidence intervals.

LC-50 values for the variants can be seen in table 3.2. Also included are the 95%

confidence intervals and the amount of times more active the variant is than the wild-

type. I have observed that nine out of the eighteen tested have significant hyperactivity

as compared to the wild-type, while nine do not display an increase (or in some cases

show a loss of toxicity) in activity as compared to the wtCry5B. For those considered to

be significantly hyperactive, it was observed that the 95% confidence intervals were non-

overlapping as calculated by probit.

Table 3.2: LC-50 values for the 18 variants tested

LC-50 values with their associated 95% confidence intervals as calculated by probit
for the 18 variants tested. Those that have non-overlapping intervals are designated
by a * and those variants are considered to be significantly more active than the wild-
type Cry5B.

Variant	Variant LC-50	Cry5B LC-50	x More Active
S407C	4.5 (3.5-5.8)	11.0 (8.5-14.7)	2.4*
G643A	6.1 (5.0-7.2)	13.1 (10.9-15.9)	2.1*
G577A	7.8 (6.4-9.5)	13.1 (10.9-15.9)	1.7*
C177A	1.3 (1.0-1.6)	3.0 (2.5-3.6)	2.3*
S534A	1.9 (1.6-2.2)	3.3 (2.8-3.8)	1.7*
Q175A	1.9 (1.7-2.0)	3.3 (3.1-3.6)	1.7*
Q382A	1.6 (1.4-1.7)	2.6 (2.4-2.9)	1.6*
E248A	0.5 (0.3-0.6)	1.5 (1.3-1.8)	3*
H277A	3.6 (2.2-5.9)	9.7 (5.9-16)	2.7*
I678A	2.9 (1.8-4.5)	4.5 (2.9-7.1)	1.6
Y381A	1.5 (1.1-2.1)	2.2 (1.7-2.8)	1.5
T167A	4.9 (3.1-7.6)	3.6 (2.5-5.0)	0.7
T139A	2.1 (1.7-2.6)	2.6 (2.1-3.2)	1.2
D553N	11.6 (6.7-20.1)	3.6 (2.5-5.0)	0.3
Q435A	2.6 (2.0-3.3)	2.8 (2.0-4.0)	1.1
H332A	7.3 (4.8-11.0)	9.7 (5.9-16)	1.3
N656A	6.2 (4.1-9.2)	4.5 (2.9-7.1)	0.7
C316A	4.9 (4.0-5.9)	3.6 (2.8-4.6)	0.7

One issue with these experiments is that each set of Bt was harvested at different times. As seen, the variability in LC50 of wild-type Cry5B from batch to batch is significant. To get around this and independently confirm my results, six of these putatively hyperactive mutants were simultaneously grown and harvested by another member of the laboratory, along with two batches of wild-type Cry5B. Dose-dependent mortality assays were then carried out. Hyper-activity was confirmed for only two of the six variants. One was the S407 positive control; the other was C177A.
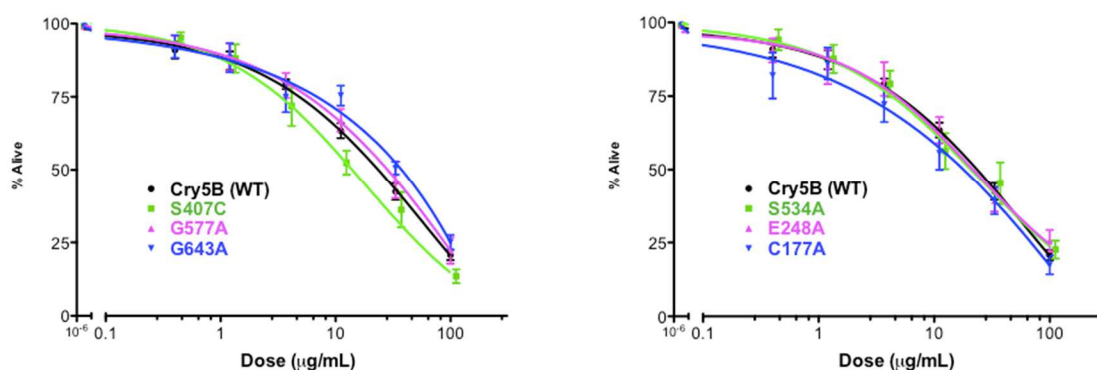
Figure 3.12: Independent test of six amino acid variants

Assays carried out with a six amino acid variants all harvested simultaneously and tested at the same time. S407C and C177A are the only mutants that appear hypersensitive in these assays.

3.9 Discussion

From the results of the initial screen in the plate assays, it was observed that there were 34 variants that displayed a phenotype that may indicate more activity than the wild-type Cry5B. With regards to the LC-50 values, however, these results do not appear to repeat once harvested spore crystal lysates are used under these specified assay

conditions. Specifically, only nine out of the 18 (50%) tested in triplicated repeats were hyperactive in one set of experiments. Furthermore, when five out of nine were tested as SCLs independently in the laboratory in a dose-dependent mortality assay, four did not appear to be hypersensitive. This brings up the question of whether the initial screen was a good way to search for hyperactive variants, especially since I did not test for any false negatives. With regards to the initial screen, the thought process was that it would reveal enough variants to pursue further that showed an increase as compared to the wild-type, so if any variants were not identified (ex. if the mutation was made, but additional mutations occurred unknowingly in the sequence and therefore the variant was scored as a 0), there wouldn't be a lack variants to investigate further.

There are several hypotheses that might explain only 9/18 variants appearing hyperactive in the SCL test. The first one is that expression levels of the variants were increased relative to wild-type Cry5B upon cloning and expression in *E. coli*. Therefore, once this variable was controlled for by use of SCL in the LC-50 assays, these variants no longer were more toxic compared to the wtCry5B. A second reason could be strictly structure/function related. This could include a change in activity due to the specifics of production or assay conditions, e.g., use of solid medium vs. liquid assay conditions (which would affect how the worms respond), pH of the assay environment, lack of stability from production in Bt vs. E. coli, or the nature of the assay itself—reproduction on a plate over 6 days versus mortality assays). As previously mentioned, there are studies showing that changes in solubility of the protein or the conditions can affect the activity of crystal proteins, as well as crystal protein variants. Additionally, the single amino acid change in these variants might affect the stability of the protein, leading to a

case in which the protein is more active, but in a spore crystal lysate form from Bt, it is not as stable (not crystal proteins are often packaged as crystals in E. coli as well). Therefore, it degrades faster in the well, and the worms are not exposed to the active, toxic form as long. This could always be investigated further by testing the state of the SCL on day 0 of the assay, and then again 48 hours later when the assay is scored by running the protein on an SDS-PAGE gel (I did this for the SCL against the larval AC assay, not the LC-50 assay).

From the LC-50 values, it is seen that there is a 2-3-fold increase in toxicity as compared to the wild-type. While this does not appear to be as large an increase as suggested by the plate assays, it is actually in line with what is observed in the insect studies with single amino acid changes (Wu 1992, Rajamohan 1996,). At the same time, it is most likely too small an increase in toxicity to be useful in an applied context. It is worth noting that multi-variant combinations could still offer the hope of finding a >10X fold increase in toxicity, which would be a major step toward the overall goal of finding a cure for intestinal parasitic helminths.

The lack of repeat by independent tests in the laboratory could be indicative of the difficult nature of working with single mutants that have only small effects on the activity of the protein. Further testing of all mutants, with the exception of S407C, is warranted to test their hypersensitivity. One important item of note is that the initial screen on plates looks for something very different phenotypically than an LC50 assay in wells. The plate assay compares relative intoxication, but not killing, between the wild-type Cry5B and the variants. The LC50 killing assay is looking only at alive vs. dead, and does not take into consideration of how sick the alive worms look. Indeed, in the LC-50

assay, there were times where the worms looked sicker in one variant, but were still alive, a finding not accounted for in the strict output of this assay. On the other hand, the plate assay does not necessarily predict that the variants are more effective at killing the worms, just that it makes them sicker under those conditions. Looking down the line, ideally the overall goal is to use a variant to increase the efficiency of Cry5B in killing the parasites, so I would think the emphasis would be on killing the worms.

3.10 Materials and Methods

Subcloning into *B. thuringiensis*

Cry5B variants were subcloned into the pcry5B mycogen vector using restriction sites located inside the Cry5B gene with the enzymes NheI and BglII. Both the vector and the Cry5B variant of interest were incubated with the enzymes at 37°C for 90 min, and then run on a 0.8% agarose gel. Digested DNA was isolated from the gel, and the vector was dephosphorylated. Ligation was performed at a 3:1 ratio at 4°C overnight, and the ligation product was transformed into E. coli XL10-gold cells. Positive confirmation of ligation was confirmed by sequencing off positive colony PCR clones. The positive colony was then plasmid prepped, and transformed into *Bacillus thuringiensis* 4D8-HD1 (non-crystal producing) via the *E. coli* GM2163 (Dam-) strain. Success was confirmed by full sequencing of the Cry5B gene in both directions.

Level 1 Cry5B Protein Purification

200mL of PGSM (peptone-glucose-salt-media) + erythromycin was inoculated with a fresh overnight culture of Cry5B (or variants) at a 1:50 dilution and incubated at

30°C shaking for 5 days. The resulting culture was washed twice with 1M NaCl and twice with ddH20. The mixture is then solubilized in 30mL of 50mM acid solubilization medium (tripotassium citrate and citric acid monohydrate titrated to pH=3.0)/flask, incubated with mild agitation for 5 min, and then spun down for 10 min at 10,000rpm. The supernatant is filtered through a 0.22um filter, and 1mL/flask of 1M tripotassium citrate is added to the supernatant to precipitate the protein. The supernatant is left on ice at 4°C over night, and the precipitate is harvested (via a clinical centrifuge at 4C) and suspended in ddH20 the following day.

Harvesting of Spore Crystal Lysates

200mL of PGSM (peptone-glucose-salt-media) + erythromycin was inoculated with a fresh overnight culture of Cry5B (or variants) at a 1:50 dilution and incubated at 30°C shaking for 4 days. The resulting culture was washed once with 1M NaCl and twice with ddH20 and resuspended in 4mL of ddH20 to yield the spore crystal lysates (SCL). All centrifugation steps are performed at 8000rpm for 8 min at 4°C. The resulting SCL is confirmed by sequencing off colony PCR from the harvested culture. The resulting SCL is run on an 8% SDS-PAGE gel with BSA standards for quantification

Truncated Cry5B *E. coli* Purification

Truncated Cry5B(aa1-772)-pQE9 in M15 *E. coli* was grown overnight at 37°C to OD=0.6-0.8. The culture was induced with 0.15mM IPTG for 10 hours at 25°C. The bacteria were harvested by centrifugation, and then lysed by sonication in PBS. The supernatant was applied to a NTA agarose column, and washed with 3 volumes of wash

buffer (0.5M NaCl, 20mM HEPES). Cry5B was eluted off the column with wash buffer supplemented with 0.5M imidazole. The Cry5B elution was dialyzed into 50mM NaCl, 20mM HEPES and concentrated using an Amicon ultra centrifugal filter.

Coomassie Staining and imaging of Crystals

30ul of agitated culture is pipetted onto a glass slide and fixed with heat. Coomassie stain (0.133% coomassie blue in 50% acetic acid) is added in abundance, and incubated for 10 min. Excess stain is washed off with water, and the slides are allowed to air dry for 10 min. Slides are imaged at 500x using a compound microscope.

***C. elegans* LC-50 assays**

For level 1 protein, LC-50 killing assays were set-up with synchronized L4 stage *C. elegans* with varying concentrations of protein (48ug/ml diluted 3-fold to 1ug/ml) in a 48-well plate in S-media (S-basil pH=6.0, trace metals, 10mM citrate buffer pH=3.0, 3mM $MgSO_4$, 5ug/ml cholesterol, 10mM $CaCl_2$) with added 8mM 5-fluorodeoxyuridine and OP50 OD=3.0. Level 1 protein was quantified by nanodrop in 20mM HEPES pH=8.0 at 3x and 5x dilutions. The protein was dissolved in 20mM citrate buffer pH=3.0, and serially diluted in 20mM citrate buffer pH=3.0. The plate was wrapped in a wet paper towel and incubated for 6 days at 25C. Worms were scored alive vs. dead using motility as the indicator when stimulated with an eyelash pick three times. Leftover level 1 protein was prepped for a gel by reducing the sample with gas and boiling with 2X SDS-sample buffer. The prepared sample was then run on an 8% SDS-PAGE gel and stained with coommassie.

For spore crystal lysates, quantitative LC-50 killing assays were set up with

synchronized L4 stage *C. elegans* with varying concentrations of SCL (100ug/ml diluted

3-fold to 0.3ug/ml) in a 48-well plate in S-media with added 8mM 5-fluorodeoxyuridine

and OP50 OD=3.0. SCL protein was quantified by imageJ after running it on an 8%

SDS-PAGE gel with BSA standards (10ug, 5ug, 2.5ug, 1.25ug). The SCL was serially

diluted in ddH20. The plate was wrapped in a wet paper towel and incubated for 48 hr

days at 25°C. Worms were scored alive vs. dead using motility as the indicator when

stimulated with an eyelash pick three times. Leftover SCL was prepped for a gel by

boiling with 2X SDS-sample buffer. The prepared sample was then run on an 8% SDS-

PAGE gel and stained with coommassie.

Statistical Analysis

LC-50 analysis and 95% confidence intervals were determined using the program

XL-stat./probit.

3.11 References

1. Fenshan Wang, Yingying Liu, Fengjuan Zhang, Lujun Chai, Lifang Ruan, Donghai Peng, Ming Sun, Improvement of Crystal Solubility and Increasing Toxicity against *Caenorhabditis elegans* by Asparagine Substitution in Block 3 of *Bacillus thuringiensis* Crystal Protein Cry5Ba. *Applied and Environmental Microbiology* **78(20)**, 7197-7204 (2012).

2. Sheng-Jiun Wu, C. Noah Koller, Deborah L. Millerc, Leah S. Bauerb, Donald H. Dean, Enhanced toxicity of *Bacillus thuringiensis* Cry3A δ-endotoxin in coleopterans by mutagenesis in a receptor binding loop. *FEBS Letters* **473**, 227-232 (2000).

3. Arti Chandra, Paramita Ghosh, Ajin D. Mandaokara, Amal K. Berab, Rameshwar P. Sharmaa, Sudipto Dasb, Polumetla A. Kumar, Amino acid substitution in α-helix 7 of

Cry1Ac δ-endotoxin of *Bacillus thuringiensis* leads to enhanced toxicity to *Helicoverpa armigera* Hubner. *FEBS Letters* **458(2)**, 175-179 (1999).

4. Fan Hui, Ulrike Scheib, Yan Hu, Ralf J. Sommer, Raffi V. Aroian, Partho Ghosh, The structure and glycolipid-binding properties of the nematicidal protein Cry5B. *Biochemistry* **51(49)**, (2012).

5. Cheng Du, Phyllis A. W. Martin, Kenneth W. Nickerson, Comparison of Disulfide Contents and Solubility at Alkaline pH of Insecticidal and Noninsecticidal *Bacillus thuringiensis* Protein Crystals. *Applied and Environmental Microbiology* **60(10)**, 3847-3853 (1994).

6. Dong Wu and Arthur I. Aronson, Localized Mutagenesis Defines Regions of the *Bacillus thuringiensis* δ-Endotoxin Involved in Toxicity and Specificity. *The Journal of Biological Chemistry* **267(4)**, 2311-2321 (1992).

7. Francis Rajamohan, Oscar Alzate, Jeffrey A. Cotrill, April Curtiss, Donald H. Dean, Protein engineering of *Bacillus thuringiensis* δ-endotoxin: Mutations at domain II of CryIAb enhance receptor affinity and toxicity toward gypsy moth larvae. *PNAS* **93(25)**, 14338-14343 (1996).

8. Xiaowu Liang, Andy Teng, Dawn M. Braun, Jiin Felgner, Yan Wang, Scott I. Baker, Shizong Chen, Olivier Zelphati, Philip L. Felgner, Transcriptionally Active Polymerase Chain Reaction (TAP): HIGH THROUGHPUT GENE EXPRESSION USING GENOME SEQUENCE DATA. *Journal of Biological Chemistry* **277(5)**, 3593-3598 (2001).

9. Michael Cappello, Richard D. Bungiro, Lisa M. Harrison, Larry J. Bischof, Joel S. Griffitts, Brad D. Barrows, Raffi V. Aroian, A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicum*. *PNAS* **103(41),** 15154-15159 (2006).

CHAPTER 4

ANCYLOSTOMA CEYLANICUM EGG TO LARVAL *E. COLI* ASSAYS

4.1 Summary

Of the 34 candidate single amino acid variants of Cry5B were identified through the initial screen, nine were significantly more active than the wild-type in the LC-50 killing assay. I decided to test all 34 variants against the hookworm parasite *Ancylostoma cyelanicum* to investigate how well the results of the initial screen correlated with activity against parasites, as well as simply to target parasites, the threat that we were ultimately interested in addressing. I tested all 34 Cry5B variants expressed in *E. coli* across the egg to larval stages of the hookworm *A. ceylanicum*, asaying for changes in activity as compared to the wild-type Cry5B. Once the appropriate assay conditions were determined, triplicate repeats were set-up and imaged for all 34 variants. Additionally, I quantitated expression levels assaying both the protein that went into the well with the worms (day 0) and the protein on the day the assay was scored (day 4). Of the 34

candidate variants, 13 displayed an increase in toxicity as compared to the wild-type. Of these, nine had expression comparable to or lower than the wild-type.

4.2 Introduction

Purified Cry5B protein is active against the hookworm *A. ceylanicum* in the egg to larval stage (Cappello, 2006). However, there was little information regarding the response of worms to *E. coli* expressing Cry5B. Since the 34 variants of interest were in *E. coli*, and *A. ceylanicum* eggs are readily available, I tested all 34 in *E. coli* on the egg to larval stage of this nematode. Because the LC-50 assays did not use a parasitic system, and I included all 34 variants to ensure that something of interest would not be missed. I also hypothesized that these variants might have displayed a hyperactive phenotype on the *C. elegans* in the *E. coli* plate assay purely due to increased expression levels. By investigating expression levels on the *E. coli* cultures being directly used in this assay, I hoped to eliminate variants that showed an increase in expression as compared to the wild-type, and only move forward with the variants that displayed both an increase in activity and comparable (or lower) expression as compared to the wild-type Cry5B.

4.3 *A. ceylanicum* larvae are sensitive to Cry5B in *E. coli*

To test these variants on a parasite, I first worked with the mouse intestinal roundworm, *Heligosmoides polygyrus*, previously shown to be sensitive to Cry5B *in vivo* (Hu, 2010). I made toxin plates following the same parameters as established in chapter 2, but left the percentage of *E. coli* expressing Cry5B at 100%. Forty newly hatched *H. polygyrus* nematodes were seeded per plate, and images were taken on day 11 (Figure

4.1). No difference was seen between Cry5B and empty vector, or Cry5B and either of the two variants tested, G643A and N656A. In looking at the worms on the plate, it was inconclusive if the worms were ingesting the bacteria and growing up. Rather than altering the assay parameters, I chose to use a different parasite to assay these variants.
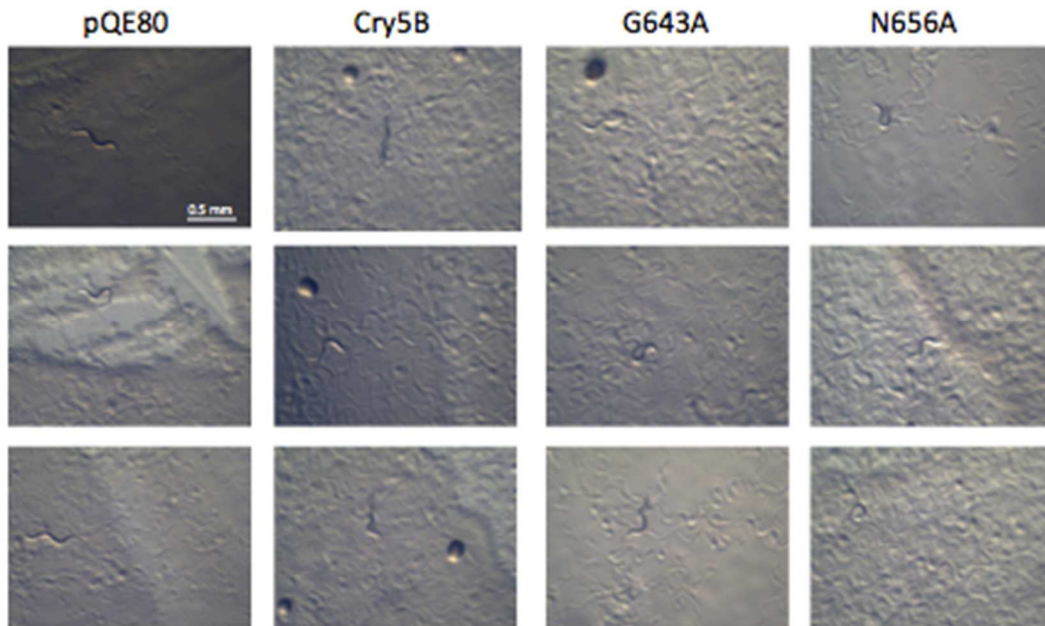
Figure 4.1: *E. coli* expressing Cry5B and variants against larval *Heligosmoides polygyrus*

Larval *H. polygyrus* L1 were seeded on plates containing *E. coli* expressing the empty vector, wild-type Cry5B, or two Cry5B variants, G643A and N656A at 100%. No difference was observed between any of the experimental conditions, indicating a different system or a change in assay conditions was necessary.

I switched to a well-based assay against the hookworm *A. ceylanicum*, and initially tested only two Cry5B variants to see if this system could be optimized to test all 34 variants of interest. I tested against the egg to larval stage of the hookworm *A. ceylanicum* because previous data supported the activity of Cry5B against this worm (Capello 2006), and the eggs from this worm are readily available in large quantities to test in the lab. It was known that the larval stage of *A. ceylanicum* was sensitive to the Cry5B protein, but the specific details of the assay needed to be worked out.

I initially worked to establish a dose response with the wild-type Cry5B against the egg to larval *A. ceylanicum*. An assay was set-up using Cry5B in the pQE80 vector in XL10 gold cells at 100%, 50% and 25% while varying the IPTG concentrations (0.05 mM, 0.1 mM, and 0.5 mM), and induction times (3 hr and 6 hr at 30°C) to find a condition that would show a dose response in the worms as a result of toxin exposure. No difference was observed between the wild type Cry5B and the vector only control in any of the conditions. I next tested Cry5B in the pQE9 vector in OP50 *E. coli* at the same IPTG concentration and toxin percentages as tested above. In this assay, a dose response was observed in the worms at the 0.5 mM IPTG with 6hr induction (Figure 4.2). I used these parameters in moving forward with this assay.
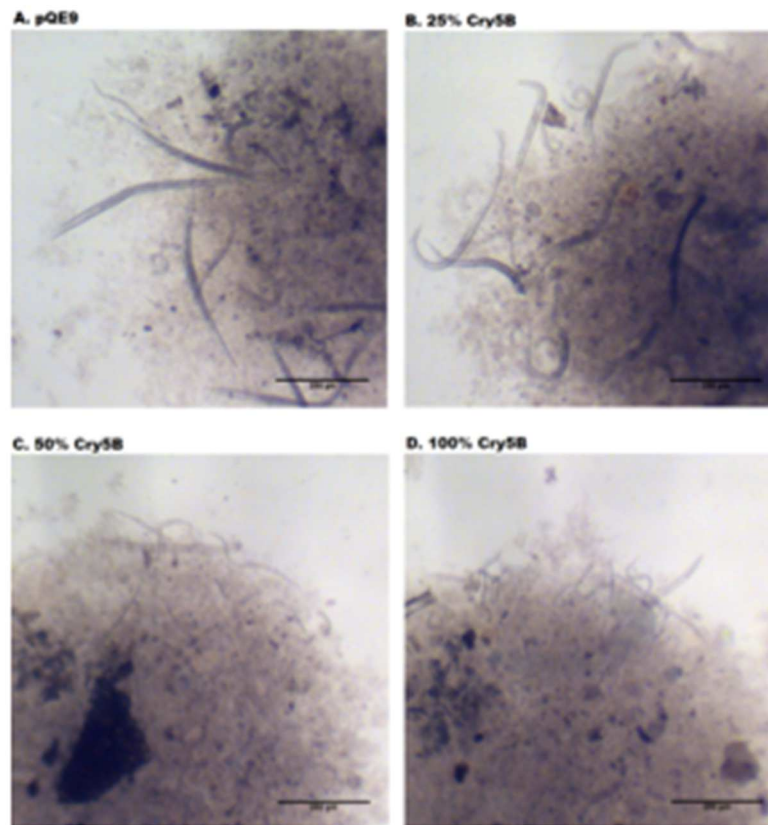
Figure 4.2: Dose response of *A. ceylanicum* to Cry5B expressing OP50 in pQE9 vector

The hookworm *A. ceylanicum* displays a dose response to Cry5B expressed in the pQE9 vector in OP50 bacteria. As the percentage of Cry5B in the well increases, so does the observed toxicity – the worms are smaller, paler, and have reduced motility.

The conditions established at this point were as follows: Approximately 10 *A. ceylanicum* eggs were placed in a 96-well plate with Cry5B in pQE9/OP50 at OD=3.0 induced with 0.5 mM IPTG for 6 hr at 30°C. Once these conditions were established, I tested the two Cry5B variants already known in the lab for hyperactivity against *A. ceylanicum*. At the 25% concentration, both D553N and S407C showed greater toxicity than the wild-type Cry5B. The worms had a reduction in size, color, and motility. The wild-type Cry5B was in turn distinguishable from the vector control. The vector control worms were robust and healthy and displayed a normal, predictive sinusoidal movement (figure 4.3).
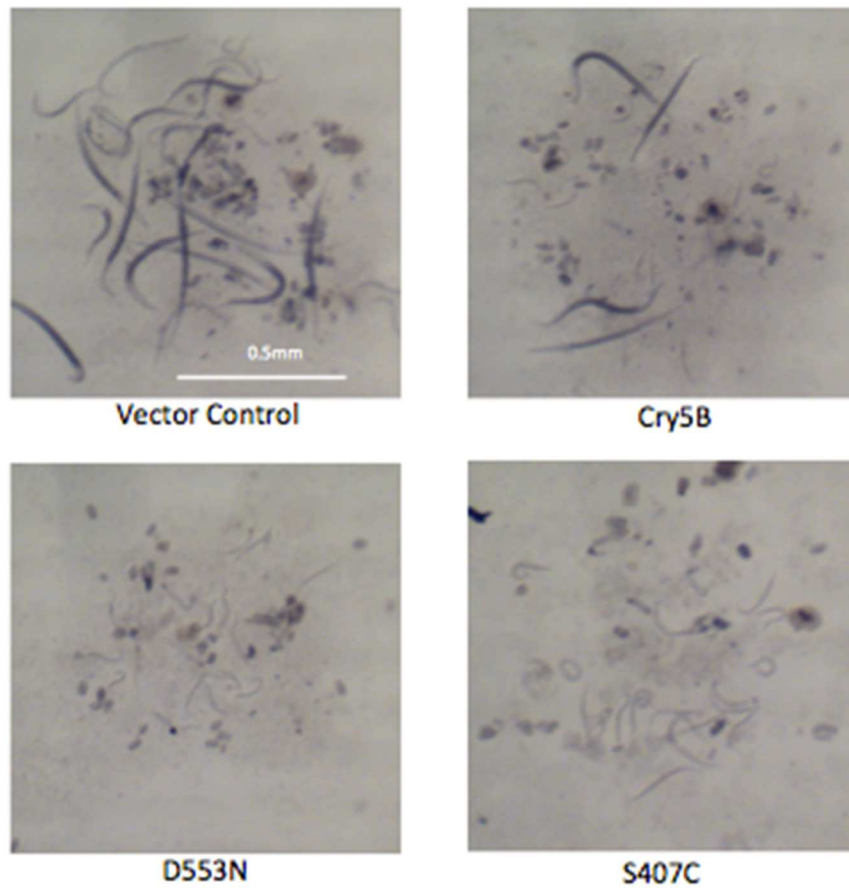
Figure 4.3: Cry5B variants D553N and S407C are more toxic than the wild-type Cry5B against *A. ceylanicum* when expressed in *E. coli*

At a 25% concentration of Cry5B variant diluted out with the pQE9 vector control in OP50, both D553N and S407C were more active against *A. ceylanicum* as compared to the wild-type.

4.4 Optimization of the egg to larval *A. ceylanicum* assay

Based on these results, I next wanted to assay all 34 hyperactive variants in the *A. ceylanicum* egg to larval assay within the *E. coli* system. However, since all the variants were currently stored in *E. coli* XL-10 gold cells in the pQE80 vector, I went back to see

if I could optimize the assay to test the variants in this state. The first set of conditions I tested an induction of 6 hr with 0.5 mM IPTG at 30°C at 100%, 50%, and 25% concentrations of toxin. No difference was observed between the bacteria control, vector control, the wtCry5B control, or the two variants of S407C and G643A (figure 4.4), and all worms were observed to be healthy and developing as predicted if no toxin were present.
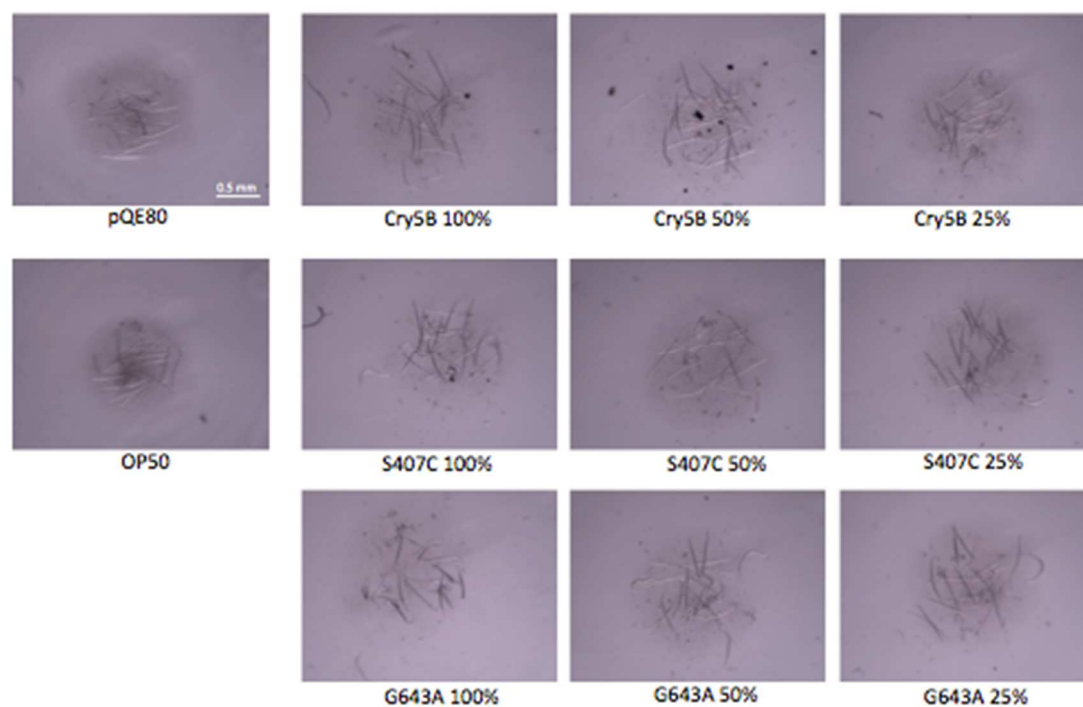
Figure 4.4: Testing of various conditions in the pQE80 vector in XL10-gold *E. coli*

No difference was observed between any of the experimental conditions or any of the controls in these assay conditions (6 hr induction at 30°C with 0.5mM IPTG in the pQE80 vector in XL10-gold cells).

I identified two parameters – bacteria volume, and addition of OP50 – that I could alter in seeking experimental conditions that would allow this system to be of use. I tested both 7.5 ul and 15 ul of bacteria expressing wild-type Cry5B (at 100% induced with 0.5 mM IPTG for 6 hr at 30°C). I hypothesized that increasing the number of bacteria in the well would also increase the amount of Cry5B to which the worms had access and thus potential compensate for suboptimal expression. I used pQE9/OP50-Cry5B as a positive control to ensure that the worms were sensitive to Cry5B in these assay conditions, and that the particular batch of *A. ceylanicum* eggs was in fact sensitive to Cry5B. Once again, no difference was observed between the pQE80 control and the Cry5B-pQE80 in the XL-10 gold cells with either varying amount of bacteria added, or when small amounts of OP50 were added (Figure 4.5). A small amount of OP50 was added to the wells, hypothesizing that since these worms are more prone to eating OP50 over the XL10-gold cells, a small amount of OP50 could be introduced to each well to increase the feeding, and potentially increase the uptake of the Cry5B expressing XL10-gold cells. The positive control displayed activity against the worms.
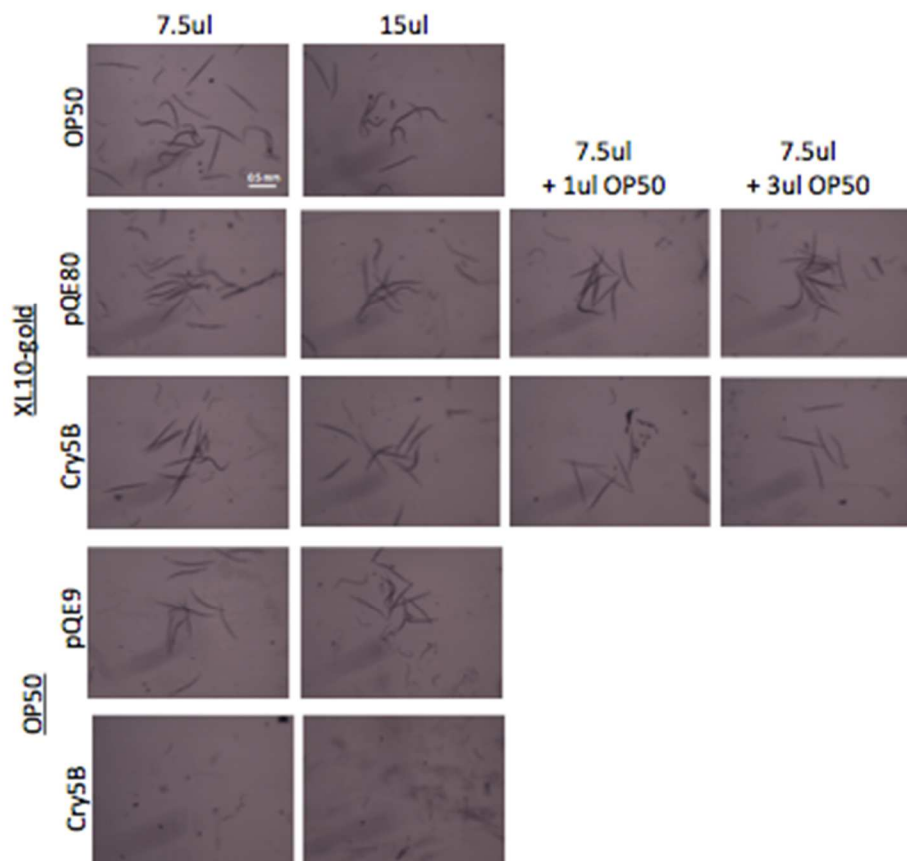
Figure 4.5: Testing of various conditions in the pQE80 vector in XL10-gold *E. coli* – alteration of bacteria conditions and volumes

None of the changes introduced into the pQE80-XL10-gold Cry5B system produced a set of conditions that showed activity against the egg to larval *A. ceylanicum*.

The next set of parameters investigated was induction conditions. I altered induction time (3 hr and 6 hr), induction temperature (30°C and 37°C), and IPTG concentration (0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 5 mM). This assay was set up with one well/per condition, with 100% of the bacteria being Cry5B-pQE80/XL10-gold, and

the same conditions were run on Cry5B-pQE9/OP50 in parallel (figure 4.6). While all of the Cry5B/pQE9-OP50 conditions proved to be toxic to the worms, none of the induction conditions assayed for the Cry5B-pQE80/XL10-gold system were observed to be toxic. At this point, I concluded that it was either the pQE80 vector or the XL10-gold cells that were the limiting factor in the success of this assay.
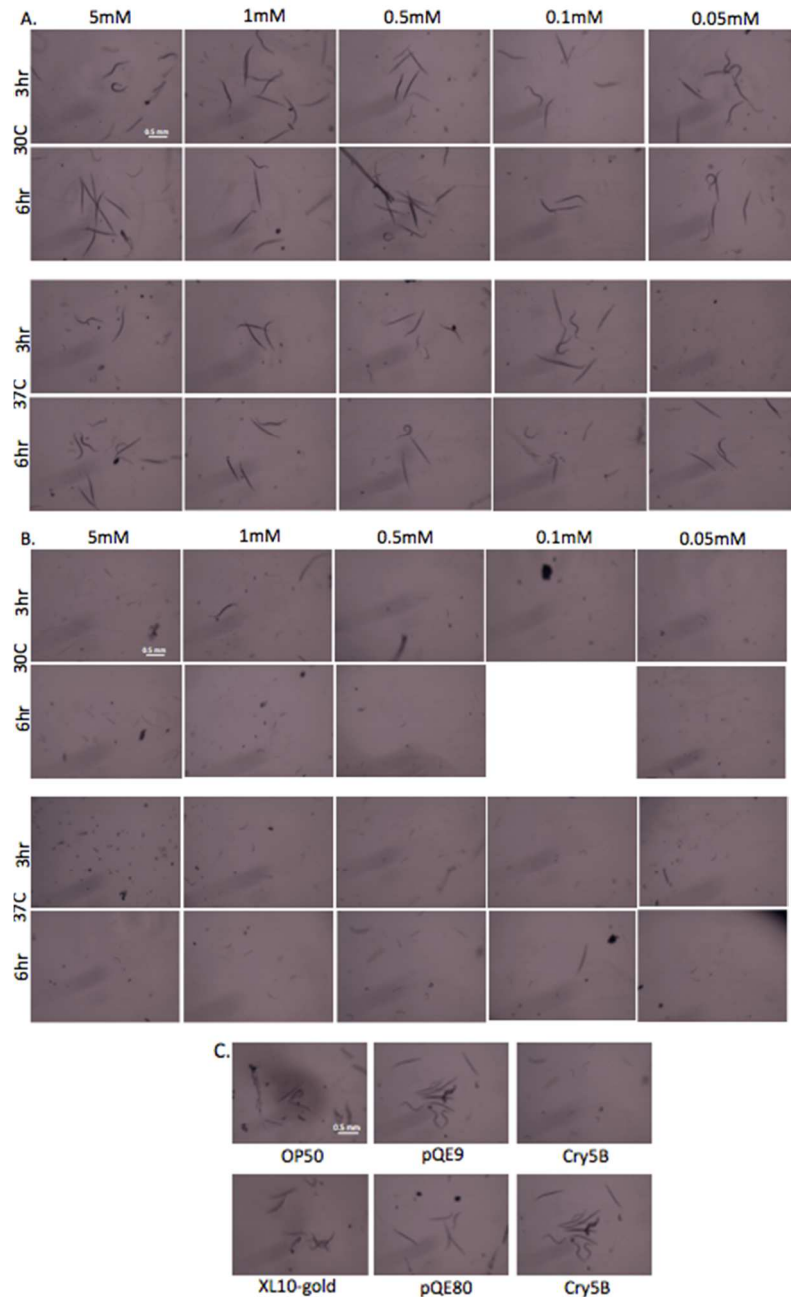
Figure 4.6: Testing of various conditions in the pQE80 vector in XL10-gold *E. coli* – alteration of induction conditions (time, temperature, and IPTG concentration)

(a) Varying the IPTG concentration, the temperature, and the induction time in the Cry5B-pQE80/XL10-gold cells did nothing to show activity against the egg to larval *A. ceylanicum* assay. (b) All conditions in the Cry5B-pQE9/OP50 cells showed activity in the egg to larval *A. ceylanicum* assay. (c) Vector only controls – OP50, pQE9/OP5, XL-10-gold, pQE80/XL10-gold

I tested Cry5B expression in the four separate Cry5B vector/E. coli systems –
pQE80/XL10-gold, pQE80/OP50, pQE9/XL10-gold, and pQE9/OP50 (figure 4.7). Not
surprisingly, toxicity correlated with Cry5B expression, which is dependent upon both
the vector used and the *E. coli* strain. Specifically, both the Cry5B in pQE9/OP50 and
pQE80/OP50 showed toxic effects on the worms, while having the highest expression out
of the four different combinations respectively. Cry5B in pQE9/XL10-gold showed
reduced expression, and reduced toxicity, and Cry5B in pQE80/XL10-gold showed very
low levels of expression, and no toxicity. pQE9 displays higher expression of Cry5B than
the pQE80 vector system, which would be expected since the pQE9 vector system is not
as tightly regulated. Also, XL10-gold cells express lower levels than OP50, which is also
expected given that they are a cell type designed for difficult subcloning, not expression.
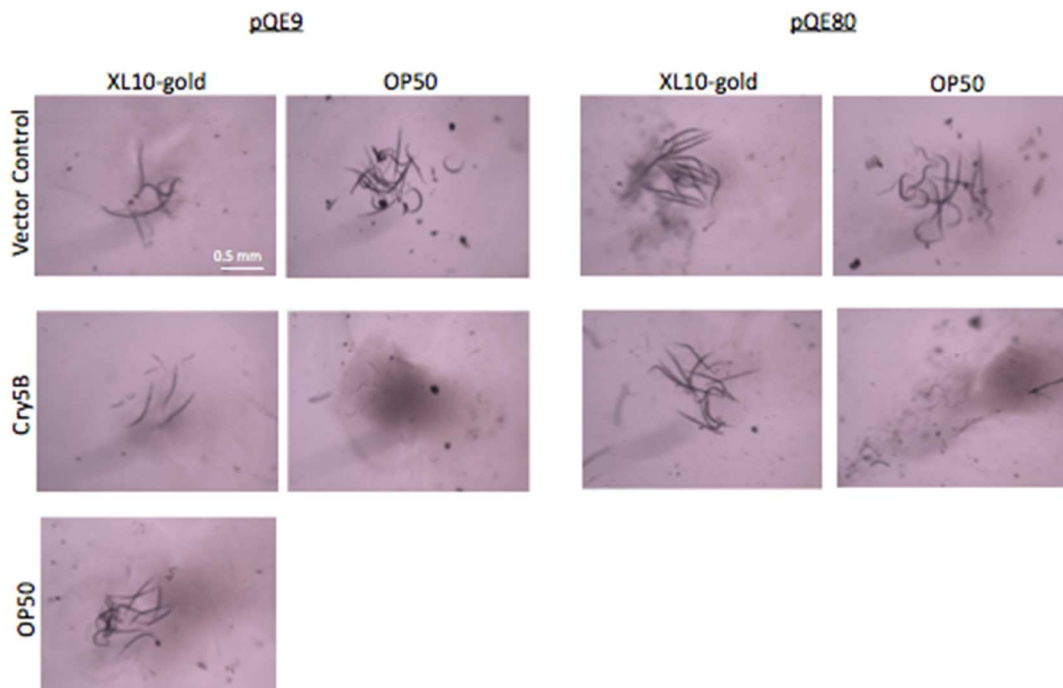
Figure 4.7: Cry5B expression in the four separate bacteria/vector system combinations with OP50, XL10-gold, pQE80, and pQE9

The toxicity observed in the worms in these four systems correlate back to the expression levels observed. While pQE90/OP50 was observed to be the highest expressing and most toxic, the system of pQE80/OP50 was also determined to show high enough activity against the egg to larval *A. ceylanicum,* thus making this system the most adaptable and useful for testing all 34 variants of interest.

I chose to move forward with the Cry5B-pQE80/OP50 system. The level of toxicity observed was sufficient to detect clear toxic effects on the worms. Furthermore, transforming all 34 variants into these bacteria was efficient and simple. This system was subsequently confirmed using dilutions at 100%, 50%, and 25% (figure 4.8) to show that the larval *A. ceylanicum* display a dose response to the decreasing amounts of Cry5B

present in the well. The final, detailed assay parameters are described below in section 3.7.
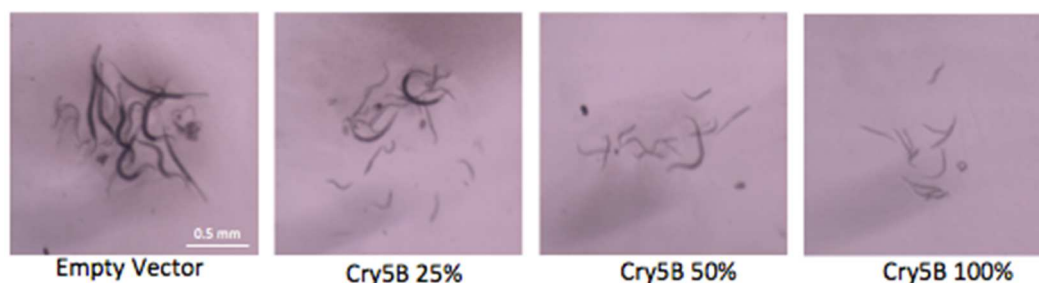
Figure 4.8: Dose response of larval *A. ceylanicum* to Cry5B expressed in pQE80/OP50

A clear toxic dose response was observed on the larval *A. ceylanicum* to Cry5B expressed in pQE80/OP50 with varying percentages of Cry5B in each well. The wells consisted of 100ul volume of media with 7.5ul of *E. coli* epxressing Cry5B. The assay was incubated at 25°C for 96hr.

4.5 Expression levels

Unlike the initial Cry5B screen, I assessed expression levels in this assay to see if any of the point variants had a change in expression as compared to the wild-type. I established the conditions for the western blot using wild-type Cry5B and an internal *E. coli* loading control, sigma70 (Hu 2010). I assayed for changes in Cry5B expression by varying IPTG concentrations (un-induced, 0.05 mM, 0.1 mM, 0.5 mM). Changes in Cry5B expression were observed in conjunction with the increase in IPTG, but no changes were observed in expression of the sigma70 control (figure 4.9).
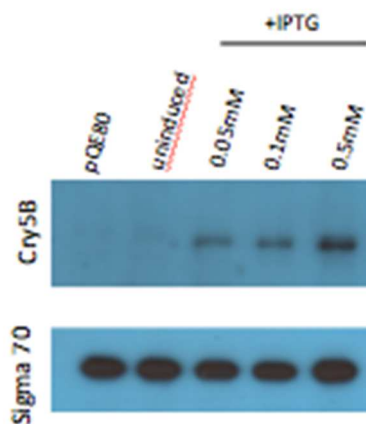
Figure 4.9: Establishing conditions for expression levels in the egg to larval *A. ceylanicum* assay

Changes in Cry5B expression were observed in conjunction with the increase in IPTG, but no changes were observed in sigma70 expression.

4.6 Testing of the 34 hyperactive variants

Of the 34 hyperactive variants from the screen, 30 were tested in the egg to larval

A. ceylanicum assay in triplicate repeat with associated western blots (figure 4.10).

Disregarding the western blot results, of the 30 variants tested, 13 display a phenotype

that indicates that they may be more active than the wild-type Cry5B.

For the 12 that did not show an increase in activity, five had no detectable protein

expression, and therefore no conclusion can be drawn. For seven variants, there was no

obvious increase in activity on the worms as compared to wild-type, or the potential

increase in activity was so subtle that no conclusion could be drawn with regards to

hyperactivity. Such a result may be simply an artifact of the system used (pQE80/OP50), or these variants may not have the same effects on these worms as they do on *C. elegans*. Of the 13 variants that showed a potential increase in activity (S407C, H435A, H332A, H277A, C177A, T167A, Q382A, N656A, N638A, I640A, C316A, S534A, E248A), four had a clear increase in expression as compared to the wild-type Cry5B (H435A, H332A, S534A, E248A), while the other nine had expression that was comparable to the wild-type Cry5B. However, I could not conclude that the increased activity of any particular variant reflected increased expression, no variants were discarded simply due to an observed increase in expression levels.

Overall, the results of this assay and the LC-50 assay was used to narrow down from 34 variants to eleven to pursue in further testing (figure 4.11). Of these eleven, four did not show increased activity against *C. elegans* on a dose basis (Table 3.1). Close examination of the figure, however, reveals that perhaps four of the mutants, N656, C177, H277, and S407 have potential to be hyperactive given relative expression patterns of the protein in *E. coli* and efficacy against the egg-to-larval stages. Of these, all but N656 were hyperactive in *C. elegans* on a dose basis. A number of these hyperactives are present at higher levels (higher expression or stability), at least to some extent (S534A, E248A, I678A, H332A, Q435A).
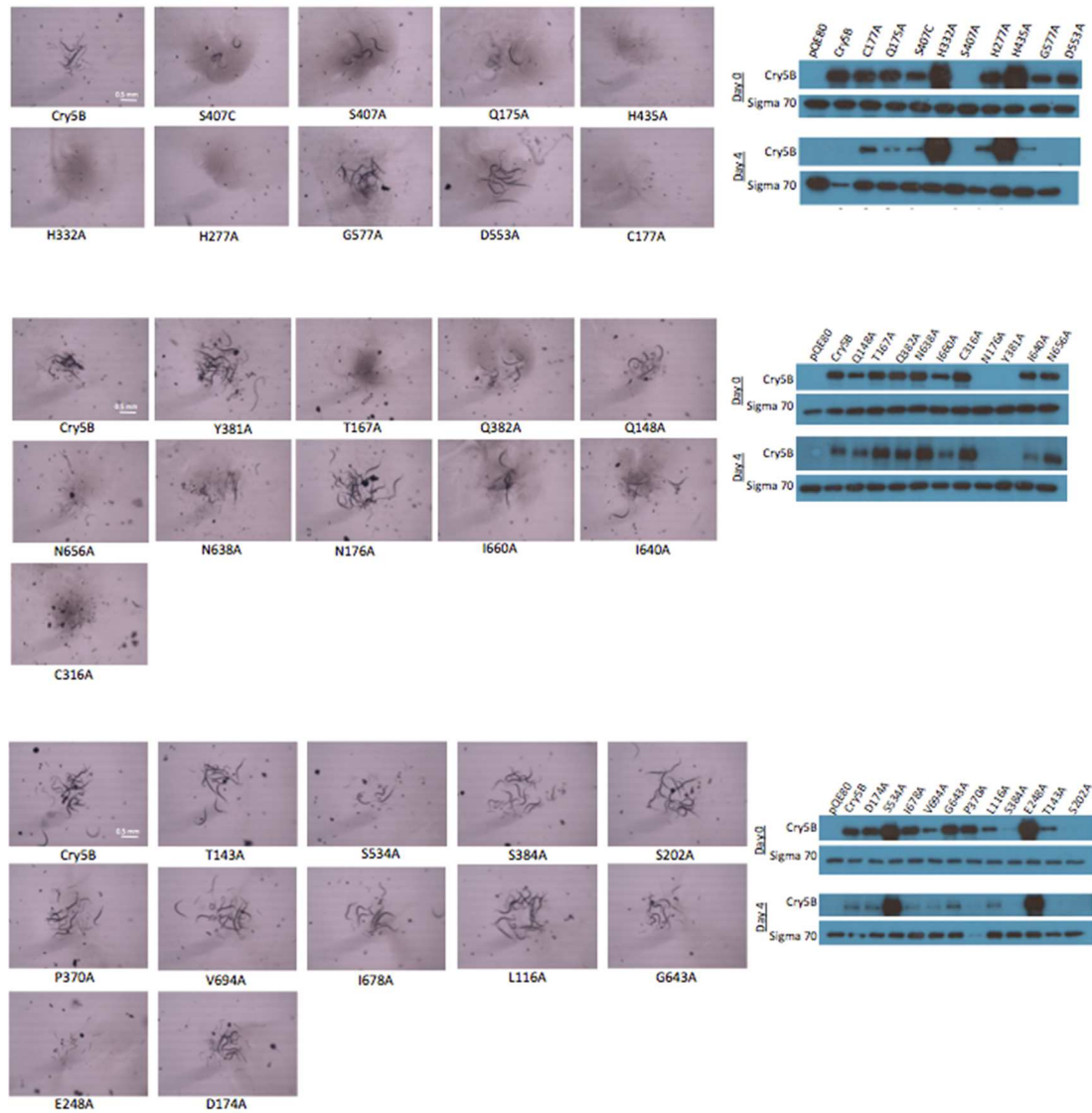
Figure 4.10: Thirty variants were tested against egg to larval *A. ceylanicum* in triplicate repeats

Each assay is a representation of triplicate repeats of 30 Cry5B single point variants tested against the *A. ceylanicum* egg to larval stage worms. Western blots were assayed for at day 0 and day 4 to investigate changes in protein expression.
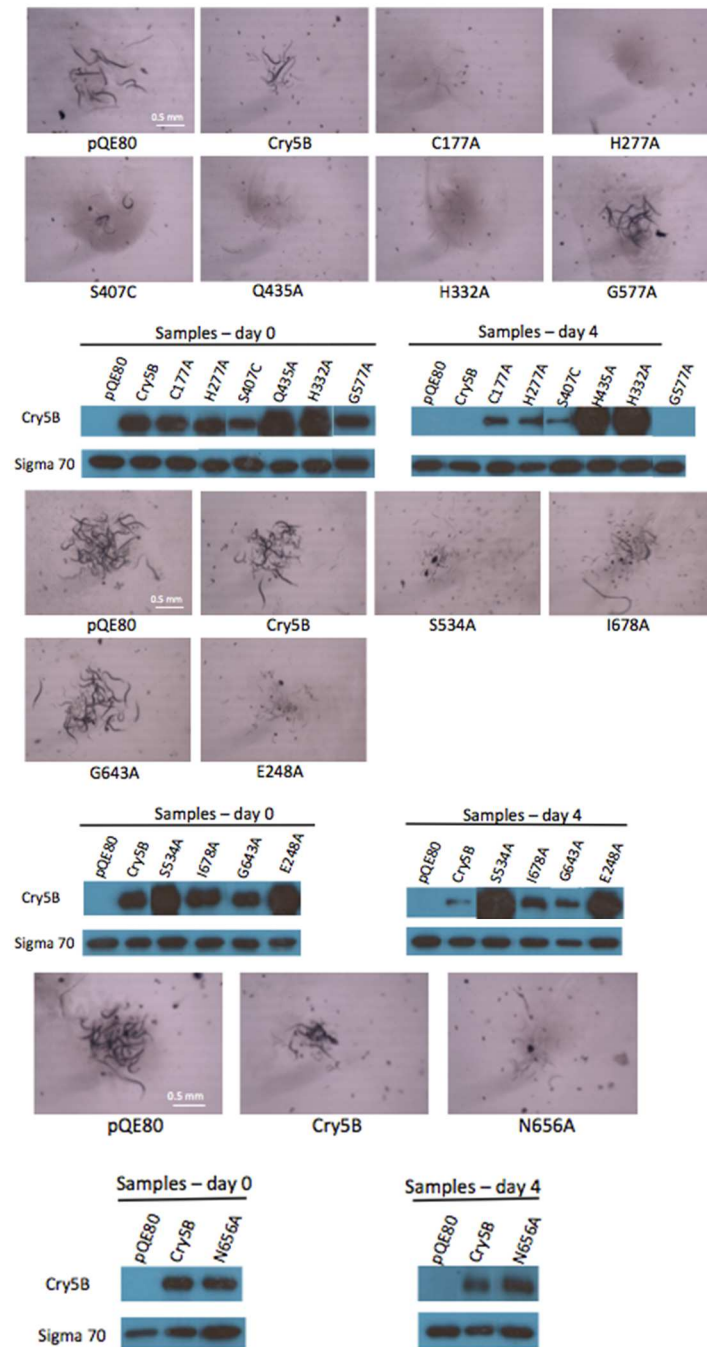
Figure 4.11: The 11 variants of interest that will be used for future testing

These 11 variants of interest were narrowed down due to a combination of looking at the results from the LC-50 assays and the egg to larval *A. ceylanicum* assays. Additionally, expression levels were assayed for in the samples on the day of the assay set-up, as well as 4 days later when the pictures were taken.

4.7 Discussion

While all four different vector/*E. coli* combinations tested express Cry5B at some level, there is clearly a minimum threshold of expression that needs to be reached in order for the worms to become visibly intoxicated. The expression is not solely dependent on either the vector or the *E. coli* strain used, but instead on a combination of both. It was observed that OP50 bacteria tend to have the highest expression overall with both vectors, and the pQE9 vector's lack of strict control as opposed to the pQE80 vector allows for a higher expression in the XL10-gold *E. coli* to observe a visible intoxication effect on the larval hookworms. Because I had all 34 variants in the pQE80 vector in XL10-gold cells, this information was useful in ultimately deciding in which system to test these variants. It is also worth noting that expression levels in *E. coli* are not consistent between the mutants. It is clear based on my work that: 1) some form of quantitation of the egg-to-larval assay including multiple doses is needed; and 2) some form of quantitation of the bacterial expression is needed.

This was the first round of testing of the hyperactive variants from the screen against a parasitic roundworm. From these results, a few variants were identified that were more active than the wild-type Cry5B against the larval *A. ceylanicum* when expressed in *E. coli.* Even after setting aside those that displayed no expression, many variants did not show an increase in activity compared to wild-type Cry5B. This may reflect biological differences between *A. ceylanicum* and *C. elegans* in the way they interact with the protein. I would have predicted that not all variants screened in the *C. elegans* system would display the same hyperactive phenotype on the hookworms, and this observation is not surprising. Although both worms are in clade V, there are

differences between these two worms that could contribute to how they are affected by the different Cry variants. In these assays, I did not test any of the variants that did not appear hyperactive in the initial screen, so there is the possibility that something of interest in the larval *A. ceylanicum* assays was missed due to initially screening on *C. elegans*. Additionally, this assay targets the larval stage of the *A. ceylanicum* hookworms. This developmental state is easy to assay in a laboratory due to the easy of obtaining eggs in large quantities, but ultimately it does not target the stage of the worms that would be actively treaded in an infection. Therefore, this assay does not show if the larval and adult worms have different sensitivities to the Cry5B variants. The outstanding question lingers of whether these variants are more active than the wild-type in a setting that has potential future clinical application.

I used the results from this assay in conjunction with the LC-50 data to narrow down the variants to a smaller sub pool to carry on with further testing against the hookworms as spore crystal lysates. The criteria that were considered initially was to identify the variants that stood out dramatically from either one of the assays individually, even if not observed in both assays. From there, variants that displayed a lesser degree of hyperactivity, but observed in both assay, were selected to move forward. Based on this criteria, I narrowed down my initial list of 34 variants to 11 variants to continue to investigate. Within these 11, four of them stood out from the rest, with three of those statistically more active in the *C. elegans* killing assays.

4.8 Materials and Methods

Isolation of *A. ceylanicum* eggs

Adult Golden Syrian hamsters were gavaged with 150 third-stage (L3i) *A. ceylanicum* infectious larvae. Feces was collected between days 18-30 post-infection (pi) for egg isolation. 30mL of 15% 1 M NaCl was added to 5g of feces, incubated for 30min, strained through a mesh strainer, and repeated. The flow-through was centrifuged at 3000g for 5min, and the supernatant was collected. This was then split into two 50 mL conical tubes, brought up to 50 mL with H20, and spun at 3000g for 5 min. The resulting pellets were resuspended in 5 mL 30% sucrose, and centrifuged 3000g 5 min. The supernatant was transferred to a new 15 mL conical tube, brought up to 15 mL volume with H_2O, and centrifuged 3000g for 5 min. The resulting pellet was resuspended in 4.5 mL H_2O. 500mL bleach was added for 1 min with mild agitation. Following the bleach step, five water washes at 2200g for 2 min were performed, and the final egg mixture was suspended in S-media.

Egg to larval *A. ceylanicum* Assays with *E. coli* expressing Cry5B

E. coli OP50 expressing Cry5B cultures in the pQE80 vector were diluted from a fresh overnight culture at a 1:10 dilution in LB with ampicillin and allowed to shake at 250 rpm for an hour at 37°C. Cultures were induced with 0.5 mM IPTG for six hours shaking at 250 rpm at 30°C. Twenty *A. ceylanicum* eggs were added to a 96-well plate containing 7.5 ul of the induced *E. coli* expressing Cry5B at OD=3.0 (tested at 100%, 50%, and 25%), and S-media (S-basil pH=6.0, trace metals, 10 mM citrate buffer pH=3.0, 3 mM MgSO$_4$, 5ug/ml cholesterol, 10 M CaCl$_2$). Plates were incubated at 25°C for four days, scored visually, and imaged to compared the variants to the wild-type Cry5B on the basis of size, motility, and coloration.

Western Blots on *E. coli* expressing Cry5B

  E. coli culture expressing Cry5B on days 0 and 4 was isolated, normalized to OD=3.0, and resuspended in 1X SDS loading buffer. Samples were run on an 8% gel, and blotted with a Cry5B antibody 1:1000. Following exposure primary exposure, blots were stripped and then probed with sigma70 1:5000 (*E. coli* internal loading control).

4.9 References

1. Michael, Cappello, Richard D Bungiro, Lisa M. Harrison, Larry J. Bischof, Joel S. Griffitts, Brad D. Barrows, Raffi V. Aroian, A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicum*. *PNAS*. **103(41)**15154-9**. (2006)

2. Yan Hu, Sophia B. Georghiou, Alan J. Kelleher, Raffi V. Aroian, *Bacillus thuringiensis* Cry5B protein is highly efficacious as a single-dose therapy against an intestinal roundworm in mice. *PLOS NTD*. (2010).

3. Lisa D. Marroquin, Dino Elyassnia, Joel S. Griffitts, Jerald S. Feitelson, Raffi V. Aroian, . *Bacillus thuringiensis* (Bt) Toxin Susceptibility and Isolation of Resistance Mutants in the Nematode *Caenorhabditis elegans*. *Genetics*. **155:** 1693-1699**. (2000).

CHAPTER 5

ELEVEN CRY5B VARIANTS HYPERACTIVE AS SPORE CRYSTAL LYSATES AGAINST *A. CEYLANICUM*

5.1 Summary

Thirty-four candidate hyperactive variants were identified from the initial screen, and narrowed down to 11 putative candidates through LC-50 testing against *C. elegans* and assays in *E. coli* against *A. ceylanicum* larvae. These 11 variants were then run through two assays against hookworms as spore crystal lysates: the egg to larval *A. ceylanicum* assay and an adult *A. ceylanicum* assay. From here, I selected six of the most promising single variant candidates from the data to test *in vivo* against *A. ceylanicum* at a single dose of 6 mg/kg. This not only provided insight into which single variants potentially are more active against a parasite in a host system, but also provided

additional information on how we can best predict *in vivo* activity of Cry5B based on the current assays we have in the lab.

5.2 Introduction

From the initial alanine scan, I identified 34 candidate hyperactive variants that I investigated further in quantitative LC-50 assays against *C. elegans* as SCL for 18 of them and qualitative *A. ceylanicum* larval assays in *E. coli* with 30 of them. My goal was to test the variants against the parasites in a quantitative, comparable way. Thirty-four variants was a larger number to test against the parasites, so I narrowed it down based on the information from the previous assays, as previously mentioned. From these results, 11 variants were chosen to investigate further.

Several assays were already worked out in the lab to test the spore crystal lysates of these 11 variants against *A. ceylanicum*, each having both positive aspects and drawbacks (Cappello 2006). Spore crystal lysates can be used as the drug component in the established egg to larval assay. Appealing aspects of this assay include that *A. ceylanicum* eggs are easy to access and isolate in large quantities. However, testing occurs in an artificial system with worms that are free-living rather than in a host. Additional drawbacks are that the assay is not quantitative as executed here (quantitative egg to larval assays with parasites can be accomplished by reporting the percentage that develop to the L3i stage), and that we don't know how testing against larval stages correlates to treating the adult stage worms in a human or animal.

The second assay is testing spore crystal lysates against adult hookworms *in vitro*. Advantages of this assay include testing against the actual stage of the worm that would

be treated. Also, it is a quantitative assay that we can analyze statistically (assaying for motility on an ordinal scale). Drawbacks are the lack of availability of the adult parasite, the fact that the number of healthy, robust worms isolated from a single hamster is variable, and the lack of information on how the artificial *in vitro* system affects the worm-drug interaction in a way that could potentially correlate with *in vivo* efficacy.

The third assay would be to test spore crystal lysates in the *in vivo* hamster-*A. ceylanicum* system. Advantages of this are testing the protein in an animal model system with all associated variables in a quantitative manner. However, larger scale hamster experiments are tedious, there are vital variables that we are unable to control (ensuring robust infections), and there is the ethical issue of testing many variants of uncertain efficacy on animals (Cappello 2006).

The ultimate goal would be to identify a variant that would have a significant increase in toxicity *in vivo*. One of the large challenges in approaching this was that we do not, at this time, have a predictive *in vitro* assay for *in vivo* efficacy. Based off this information, I decided to test these 11 variants in the egg to larval assay and the adult assay as spore crystal lysates, and then use the resulting data to narrow down the 11 to a smaller handful of variants to test *in vivo*.

5.3 Egg to larval *A. ceylanicum* assays with Cry5B spore crystal lysates

The basic parameters of the *A. ceylanicum* larval assay have been previously worked out (chapter 4), but the specific doses to use in this assay were not determined. Initially, I tested a wide range of concentrations, which I then narrowed down to a smaller range of three-fold dilutions from 11 ug/mL down to 0. 1ug/mL. These doses

were then tested on the wild-type Cry5B, S407C, and G643A (figure 5.1a). From these results, the two highest doses were found to be too toxic for use in this assay – the worms were highly intoxicated, and no difference was observed between the wild-type and the hyperactive variants. For the lower range of doses, the 1 ug/mL dose did not produce a strong toxicity effect of Cry5B. I then fleshed out the doses in this region between 1 ug/mL and 3 ug/mL, and tested in the range of 3 ug/mL to 0.6 ug/mL, diluting 1.5x (Figure 5.1b). Here, it was observed that both 3u g/mL and 2 ug/mL were revealing concentrations, in that Cry5B, G643A, and S407C showed toxicity to the worm, with both G643A and S407C displaying an increase over the wild-type Cry5B. To finalize the assay conditions, I included doses on the high and low ends of this concentration range that would allow for potential changes and small fluctuations within the assay and between the variants. Additionally, these high/low doses would be controls for this system, confirming both the activity of the Cry5B and the health of the isolated worms. HD1, a strain of *Bacillus thuringiensis* that produces spores with no crystals (Cry-), was used as a negative control.

A repeat confirmation of these parameters was conducted, adding in two additional variants (C177A and E248A), and making small adjustments to the doses tested (figure 5.1c). In this confirmation repeat, increases in activity were observed with all four variants of S407C, G643A, E248A, and C177A, specifically at the doses of 0.57 ug/mL and 0.23 ug/mL. While this increase was observed, this preliminary conclusion is the result of a descriptive observation, as opposed to definitive quantifiable data, within these parameters of this assay. In this single trial, C177A appeared to be dramatically more active at all doses as compared to the wild-type. As with the LC-50 *C. elegans*

assays, protein gels were run with the leftover SCL that went into the wells, and stained with coomassie to confirm that equal quantities of protein were being used for each variant.
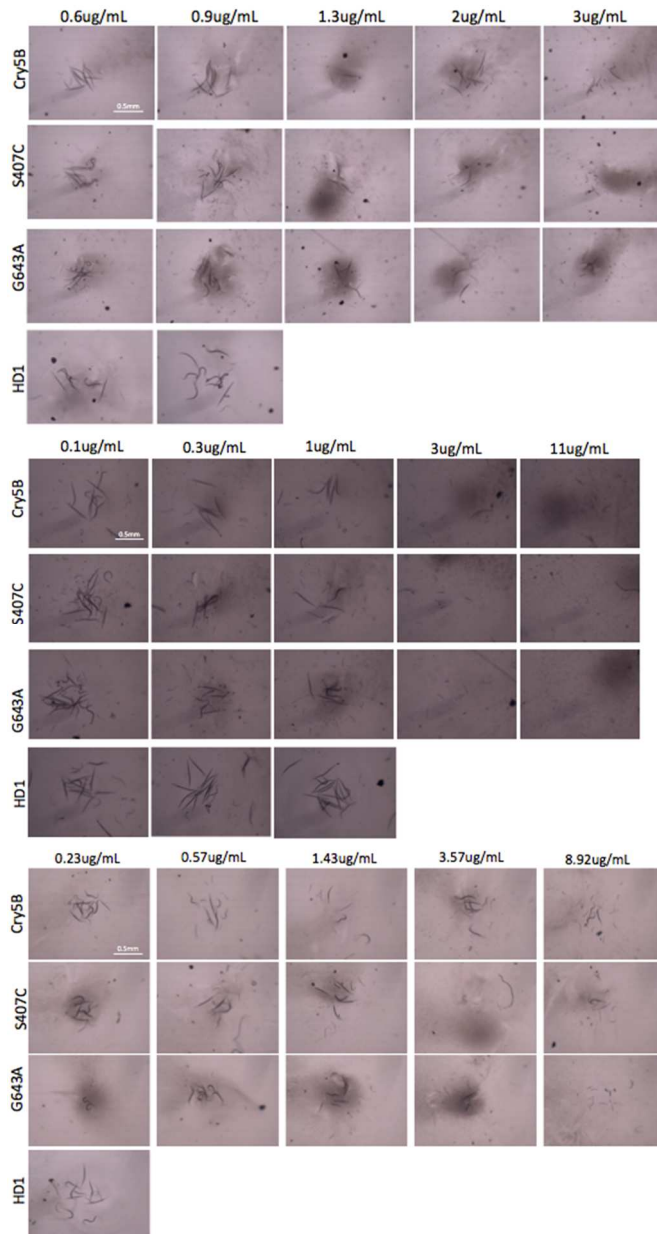
Figure 5.1: Egg to larval *A. ceylanicum* on Cry5B variant spore crystal lysates

(a) *A. ceylanicum* hookworms are sensitive to spore crystal lyastes from Cry5B, S407C, and G643A, but the doses do not seem to be optimized to see a potential difference between these variants. (b) The doses of both 3ug/ml and 2ug/ml seemed ideal to observe sensitivity of the larval *A. ceylanicum* to the wild-type Cry5B, and increased sensitivity of the hookworms to both S407C and G643A. (c) Dose concentrations were finalized in the SCL larval *A. ceylanicum* assay, with increases in activity observed as opposed to wtCry5B in S407C, G643A, C177A, and E248A, specifically at the doses of 0.57ug/mL and 0.23ug/mL.

I next tested the 11 variants in triplicate repeats in this SCL egg to larval *A. ceylanicum* assay. Figure 5.2 shows images of a single trial that is representative of what was observed in the repeats. In this assay, all 11 variants behaved slightly different from each other. The variants that stood out across all repeats as being consistently and noticeably hyperactive at one or more doses were S407C, G643A (although observed to be less active than wild-type at some doses as well), H332A, Q435A, and C177A. Among these five variants, there were different levels of activity at the different doses. Specifically, C177A was observed to be more active than the wild type at all five doses tested, and was clearly the most active of the 11 variants tested here. For some variants, like G643A, hyperactivity was observed at only one or two doses, while for others, like S407C, hyperactivity was observed across two or three doses. Variants G577A, N656A, and S534A appeared to be more active in only two out of the three repeats. In order to help draw more conclusive results on these variants, the assay was set-up and imaged a fourth time. Although the assay appears to have a level of inherent variability that will be addressed in section 5, this fourth repeat confirmed and provided confidence in the hyperactivity in this assay of the variants S407C, G643A, C177A, E248A, G577A, and S534A.
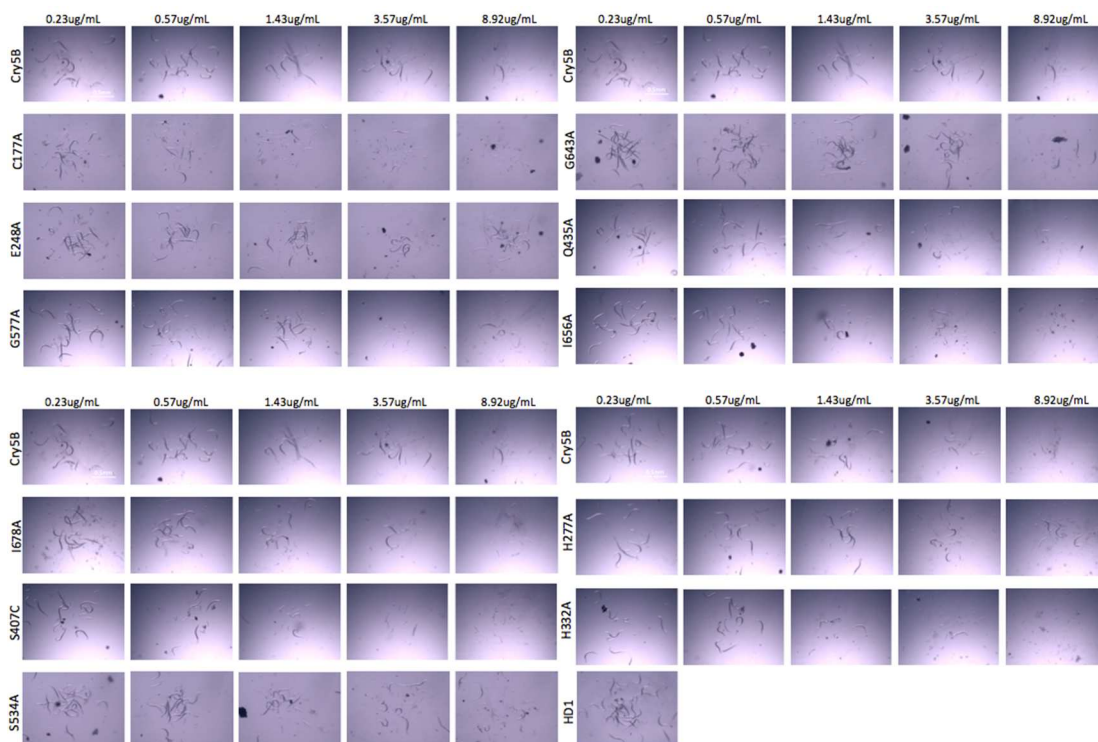
Figure 5.2: Eleven Cry5B variants tested as SCL in the egg to larval *A. ceylanicum* assay

11 of the Cry5B variants were tested against the egg to larval *A. ceylanicum* in a dose dependent manner.

For the final variants, I678A looked convincingly hyperactive in a single assay, inconclusive in a second, and not in a third, making it hard to draw definite conclusions about the behavior of this specific variant against the larval stage of this hookworm. H277A was not observed to be hyperactive in any of the repeats. One limitation of this assay is that it is not a quantitative assay. Although using spore crystal lysates allows a

side-by-side comparison of the variants, it is still a comparison of the observed phenotypes, and does not provide a way to statistically conclude that one variant is more active than the wild-type.

5.4 Adult *A. ceylanicum* assays with Cry5B spore crystal lysates

The basic parameters of the adult *A. ceylanicum* assays have been established (Cappello 2006), but as with the previous assay, the appropriate concentration of Cry5B spore crystal lysates to be used needed to be determined and optimized. For this assay, I wanted to test a single discriminating dose because adult worms are not as readily available as eggs, and therefore a dose response was not a practical choice under these conditions. Based on the previous studies in the lab, I hypothesized that 1 ug/mL dose would enable me to see a moderate Cry5B effect while leaving room for any potential increase in activity from the variants . Because this dose was a prediction, I also went up and down 3-fold to include 0.3 ug/mL and 3 ug/mL to widen the scope. Initial tests were conducted using G577A and G643A against adult *A. ceylanicum* worms (figure 5.3) in triplicate repeats. From these combined results, there is no significant difference between the wild-type Cry5B and G643A or G577A at the 0.3 ug/mL dose. At the higher 1 ug/mL dose, both G577A and G643A show an increase in activity at all time points tested as compared to the wt Cry5B (p=0.019 and p=0.018, respectively, with a one way ANOVA with repeated measures). At the highest dose of 3 ug/ml, only G643A (p=0.048) displays an increase in activity as compared to the wtCry5B, while G577A is not significant. I believe the 3 ug/ml does to be too toxic to discern between the wild-type and any variants with a potential increase in activity. I did not pursue a dose up to identify a max out

toxicity point, since my goal was to identify a single dose at which to test the 11 different variants.

I concluded that the 1 ug/ml dose was the best single concentration with which to move forward with the eleven variants of interest in the adult *A. ceylanicum* assays. My previous data supports hypersensitivity being different at different doses for different variants, but I believe a single dose to test at was best in this case. Due to the limitations of adult *A. ceylanicum*, there was not the opportunity to do a dose curve to investigate multiple doses for 11 different variants. Additionally, since this dose was observed to be low enough to not kill all of the wild type, but high enough to observe some killing, I hypothesized that anything with a significant effect on toxicity would be detectable in this assay at this one dose.
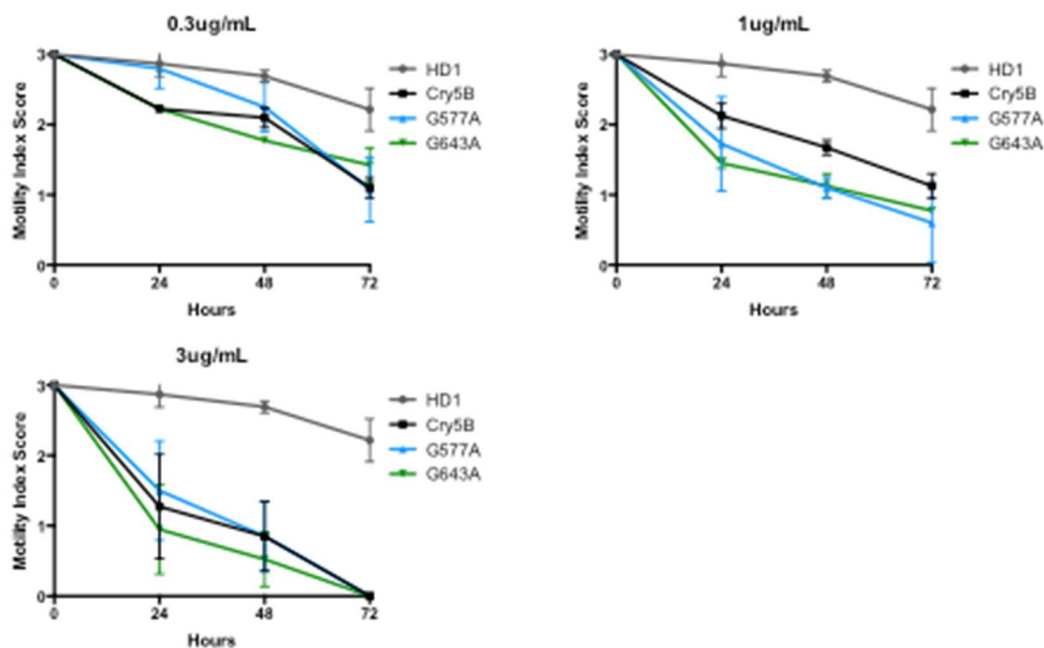
Figure 5.3: Cry5B, G557A and G643A were tested as spore crystal lysates against adult *A. ceylanicum* at 0.3 ug/ml, 1 ug/ml and 3 ug/ml

From this set of preliminary assays, it was determined that the if only testing a single dose against adult *A. ceylanium*, that the dose of 1 ug/mL was the single best dose to use, due to the wild-type having a moderate effect on the worms, while still leaving room for hyperactive variants to potentially have an increase in toxicity effect, as seen here with G577A and G643A.

I tested the 11 variants of interest in this assay in triplicate repeats at the 1ug/mL dose (figure 5.4). From these results, six out of the 11 are significantly more toxic at a single dose of 1ug/ml in triplicate combined repeats as compared to the wild type Cry5B against the adult stage *A. ceylanicum* when analyzing with one way ANOVA with repeated measures. As with previous assays, SDS-PAGE gels were run and coomassie

stained with the protein used in that specific assay to verify and demonstrate equal

protein being added to the wells, and to confirm that the quantification efforts were

correct and precise. S534A (p=0.0004) was the only variant with a p value < 0.001.

S407C (p=0.0021), E248A (p=0.0015), and I678A (p=0.0011) all had p values < 0.01,

while G643A (p=0.0331) and G577A (p=0.0337) have a p value < 0.5. Of the other five

variants tested, C177A and H277A appear to be very similar to the wild-type, while

N656A, Q435A, and H332A might have some small hyperactive effect, but not one of

statistical significance.
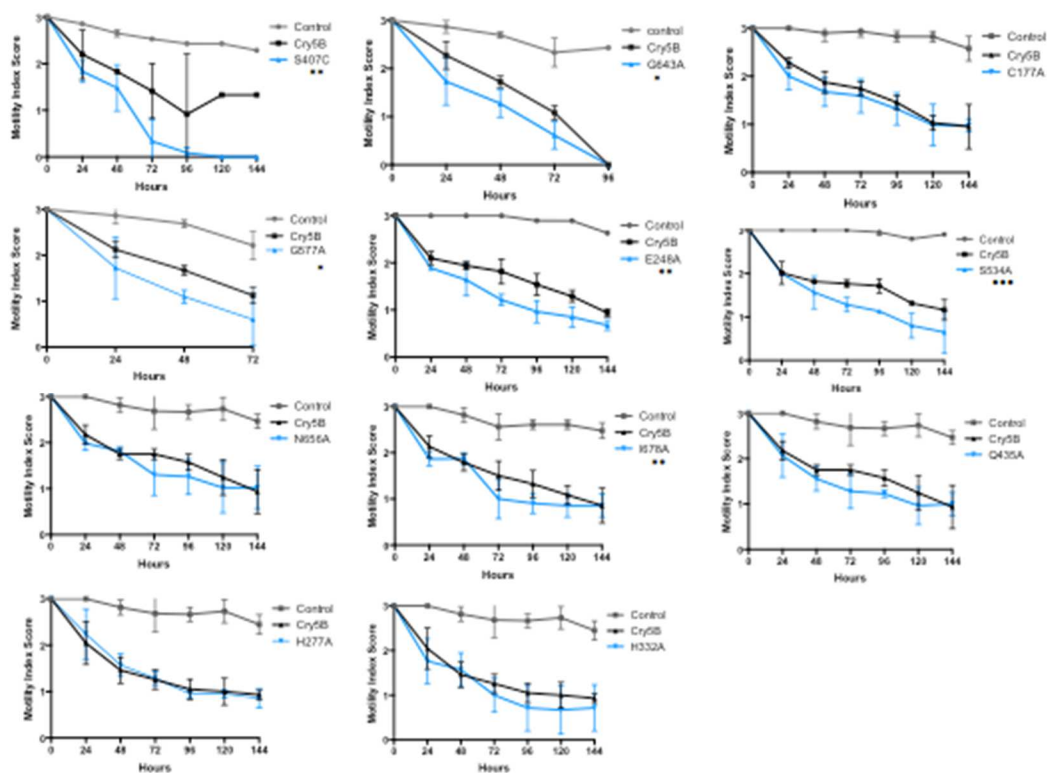
Figure 5.4: Eleven Cry5B variants tested against adult *A. ceylanicum* as spore crystal lysates at 1ug/mL

From the 11 variants tested against adult *A. ceylanicum* as spore crystal lysate, 6 of them were statistically significant through analysis with ANOVA with repeated measures. * $p<0.5$; ** $p<0.1$; *** $p<0.01$

From this set of data from the adult assays, in combination with the larval assays and the LC-50s, six variants stood out. The parameters used to determine this included: initially looking at the adult assays to narrow down from the 11 tested, specifically looking at variants that were fast hitting in the assay, more active than wild type, more active at each time point, and also showed to be more active in the LC-50s. These six

variants that were chosen for further testing were: S407C, G643A, C177A, E248A,

G577A, and S534A. All were tested at least once at the 3ug/ml dose in the adult *A.*

ceylanicum assay (Figure 5.5). Results show that two out of the six appear to be

significantly more active at 3 ug/ml, but it should be noted that while the G577A, G643A

(p=0.19), and S407C (p=0.005) results are from triplicate repeats, the other variants

(S534A, E248A, C177A) are single-trial experiments.
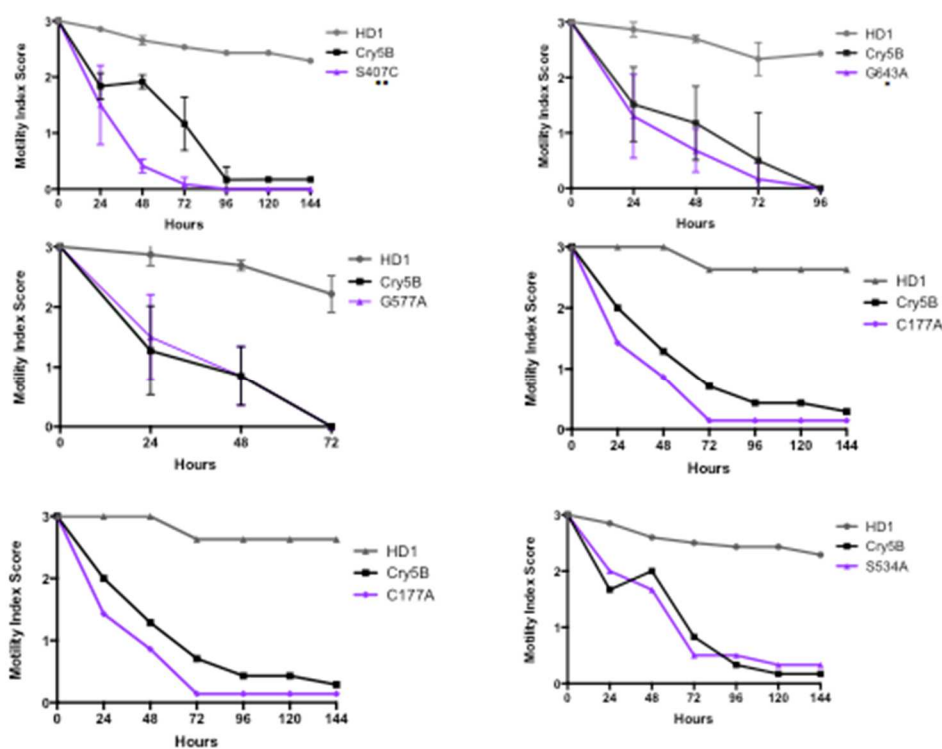
Figure 5.5: Six Cry5B variants tested against adult *A. ceylanicum* as spore crystal lysates at 3 ug/mL

Out of the three variants tested at 3 mg/kg in triplicate repeats (S407C, G643A, and G577A), both S407C and G643A demonstrated to be significantly more active as compared to the wild-type Cry5B when using ANOVA with repeated measures. In single trails, both C177A and E248A looked to potentially be hyperactive at the dose of 3 ug/mL, while S534A looked comparable to the wtCry5B. * p<0.5; ** p<0.1

5.5 *In vivo* assays

Ultimately, my goal was to identify a Cry5B single point mutant with increased activity *in vivo*. Since G643A was one of the first variants identified and confirmed, a small *in vivo* experiment was initially conducted to see if it was more active in a parasite-host system. I assisted with a small pilot *in vivo* experiment (in this experiment, I specifically prepped the samples for testing) performed by members of the Aroian lab with G643A at a dose of 3mg/kg with three hamsters/group (n=3) infected with *A. ceylanicum*. The wild-type Cry5B was tested at 1 mg/kg, 3 mg/kg, and 9 mg/kg (figure 5.6). Only the 9 mg/kg dose of Cry5B showed a significant reduction in worm burden compared to the HD1 spore only control (p=0.04). Although there appears to be a reduction in worm burden in the G643A dose, it is not statistically significant. However, this experiment must be taken with caution, since the Cry5B and the G643A were harvested from separate batches, and small differences in toxicity may be attributed to the innate variation that I have observed occurring in assays from different harvests.
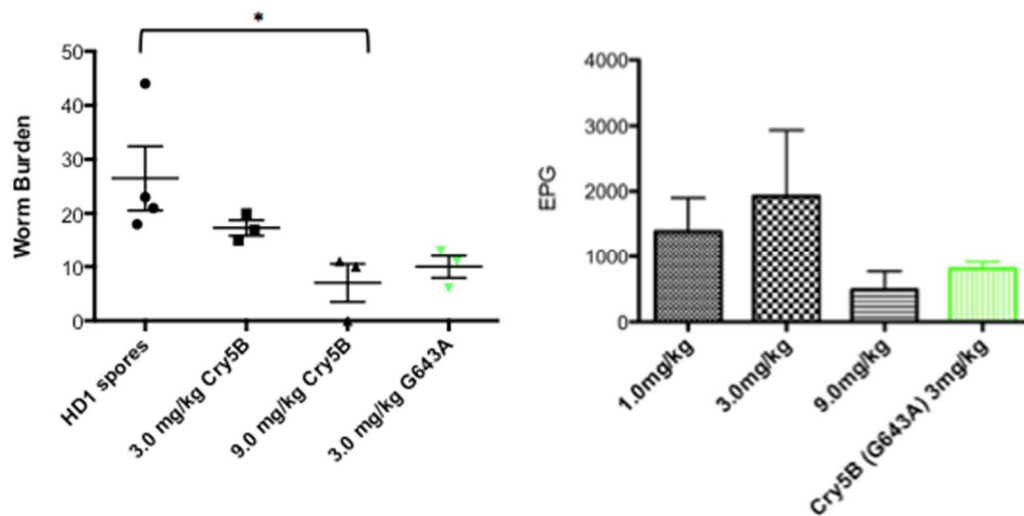
Figure 5.6: Preliminary *in vivo* testing of G643A against *A. ceylanicum* in hamsters

G643A was tested at a single dose of 3mg/kg in a preliminary *in vivo* test against hamsters infected with *A. ceylanicum* but did not significantly reduce the worm burden s compared to the wild-type Cry5B.

One of the reasons Cry5B is so promising as a future antihelmintic is that there are no observed ill-effects on vertebrates, due to the receptor for this protein being an invertebrate specific glycolipid receptor (Griffitts 2005). Additionally, crystal proteins have been studied in depth for years due to their application in agriculture. However, there is always the possibility that a single mutation might alter the receptor binding, and thus alter its safety profile. To test this, I utilized the bre-4 mutant *C. elegans*, which are deficient in a glycosyltransferase enzyme and cannot make the Cry5B receptor (Griffitts 2003). As a result, the worms are unable to be intoxicated by Cry5B. I tested G643A

against *bre-4 (ye13)* worms in an LC-50 well based assay at two fixed doses of 100

ug/mL and 11 ug/mL. I found that like the wild-type Cry5B, these worms showed no

signs of intoxication at any of the doses tested (figure 5.7). Additionally, the wild-type

Cry5B showed toxic effects to the wild-type *C. elegans*, and G643A was hyperactive as

compared to the wild-type.
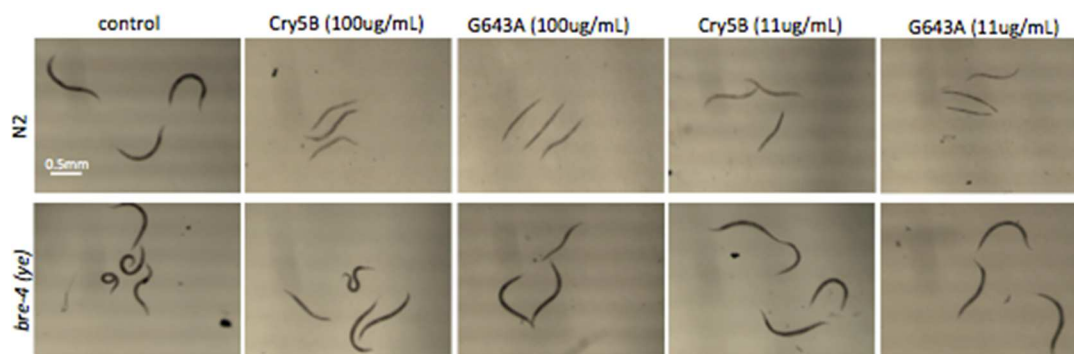
Figure 5.7: G643A tested against bre-4 *C. elegans*

G643A was tested against bre-4(ye13) and wild-type *C. elegans* in a well based assay
at two fixed doses of 100ug/mL and 11ug/ml to demonstrate that this single point
mutant had no effect on the Cry5B receptor, as seen through the resistance of the bre-
4 worms to this mutant protein.

The next *in vivo* assay was performed by members of the Aorian lab with my

assistance (I specifically prepped the samples, assisted with fecal collection and counting,

and assisted with euthanizing the hamsters, and the isolation of the intestines and

counting of the hookworm burden) with Cry5B, G643A, and S407C from the same

harvest batch, with 4 hamsters/group at two doses of 3 mg/kg and 9 mg/kg. 3 mg/kg is

considered to be a sub-therapeutic dose, while the 9 mg/kg would be predicted to reduce

the worm burden (Hu 2012) (figure 5.8). From the data, the 3 mg/kg dose of Cry5B

shows an increase in worm burden as compared to the HD1 control (not significantly),

but this is actually a common trend observed in the hamster-*A. ceylanicum* system at the

lower/sub-therapeutic doses. It has been hypothesized that this low dose will evoke the

worm host defense mechanism without being a high enough dose to hit the parasites hard

enough to clear them out of the small intestines. At the 3 mg/kg dose, the worm burden of

G643A and S407C has been decreased compared to the wild-type, though not

significantly. The same reduction pattern, but also not significant, of the wild type and

the two variants, though not as dramatic, can be seen at the 9 mg/kg dose as well.

Additionally, both the S407C and G643A doses of 3 mg/kg look to be behaving similar to

the wild-type Cry5B 9mg/kg dose by observation alone.
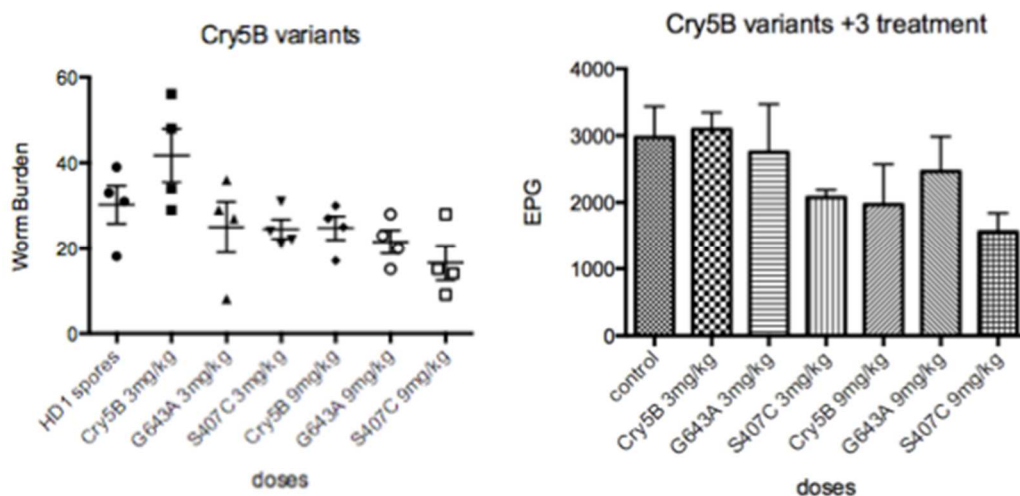
Figure 5.8: *In vivo* testing of G643A and S407C at two doses of 3 mg/kg and 9 mg/kg

Cry5B, G643A, and S407C were tested *in vivo* against *A. ceylanicum* at the doses of 3
mg/kg and 9 mg/kg. Neither variant at either dose displayed a significant decrease in (a)
worm burden or (b) eggs per grams of feces as compared to the wild type Cry5B.

A third *in vivo* study was conducted to test the six variants that are referenced above. Once these six variants were narrowed down using the previous assay data, they were categorized into three groups based on the *A. ceylanicum* egg to larval data: Group 1 (predicted to be the best) – S407C, G643A. and C177A. Group 2 – E248A. Group 3 – S534A and G577A. In addition to determining if any of these six variants had a significant impact on worm burden, this assay might also give us additional insight and further information as to which assay might be the best predictor of *in vivo* activity for Cry5B SCL. Although these six variants are hyperactive to some degree in at least one of the assays, they all behave very differently from each other in the different assays. For example, C177A is very hard-hitting against the larval *A. ceylanicum* at all doses, but is not hyperactive against the adult parasites, while S407C is statistically more active against the adult worms, and only moderately strong against the larval stages. Clearly, there are differences in the variants in the different systems, and we do not know at this point which assay correlates best with *in vivo* activity.

The *in vivo* experiment with these six variants was conducted as a double-blind. Spore crystal lysates were grown up and harvested in parallel specifically for this *in vivo* experiment. One flask of each of the variants was inoculated and harvested, while two flasks of the wild-type were inoculated and harvested. The two wild-type flasks were treated as two separate samples for treatment, but the data was combined for the results. These six variants and the wild-type were tested against *A. ceylanicum* in hamsters at a 6mg/kg dose (figure 5.9), with n=4 for the variants, and n=8 for the control. No HD1 treatment was utilized here. The study was conducted as detailed below in section 5.7. Reductions in worm burdens were observed relative to wild type in G577A, S407C,

G643A, S534A, and E248A, but none were statistically significant (ANOVA; P=0.20 was the smallest P value seen). C177A did not reduce the worm burden either visually or significantly compared to the wild-type.
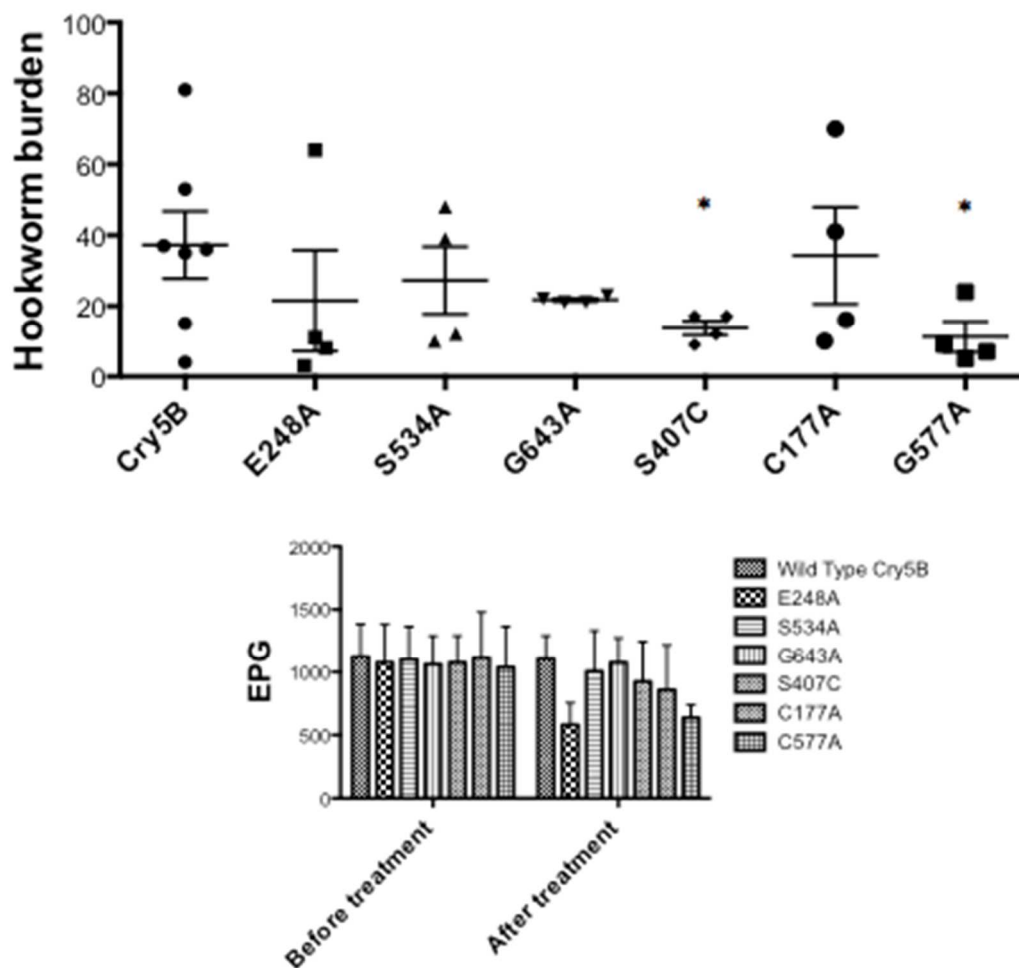
Figure 5.9: *In vivo* **testing of six Cry5B variants against hamsters infected with** *A. ceylanicum*

Two of the six variants investigated here (S407C and G577A) were found to statistically reduce the worm burden of *A. ceylanicum* in hamsters, while the other 4 (E248A, S534A, C177A, G643A) did not significantly affect the worm burden. * $p < 0.05$.

5.6 *In vivo* follow-up

Additional follow-up tests were conducted in the laboratory by current members, and the data was brought to my attention after the previous assays had been concluded. Triplicate repeats were conducted for the six variants of interest in both LC-50 killing assays with *C. elegans* and *E. coli* expressing Cry5B, and adult *A. ceylanicum* assays with SCL. Within the LC-50 assays, only S407C was more active. However, unlike the LC-50 data, all the variants are statistically are more active than the wild type in the adult *A. ceylanicum* assays (E248A p=0.0173; C177A p=0.0132; S534A p=0.0195; G643A p=0.0191; G577A p=0.0234; S407C p=0.0154). I believe that this may provide preliminary data that addresses the hypothesis that the point variants may have a slight reduction in stability, which allow them to be sensitive to small changes in the LC-50 assay conditions. Additionally, since these variants repeat in the adult *A. ceylanicum* system, the target parasite of interest, I would argue that is reason enough to pursue them forward.
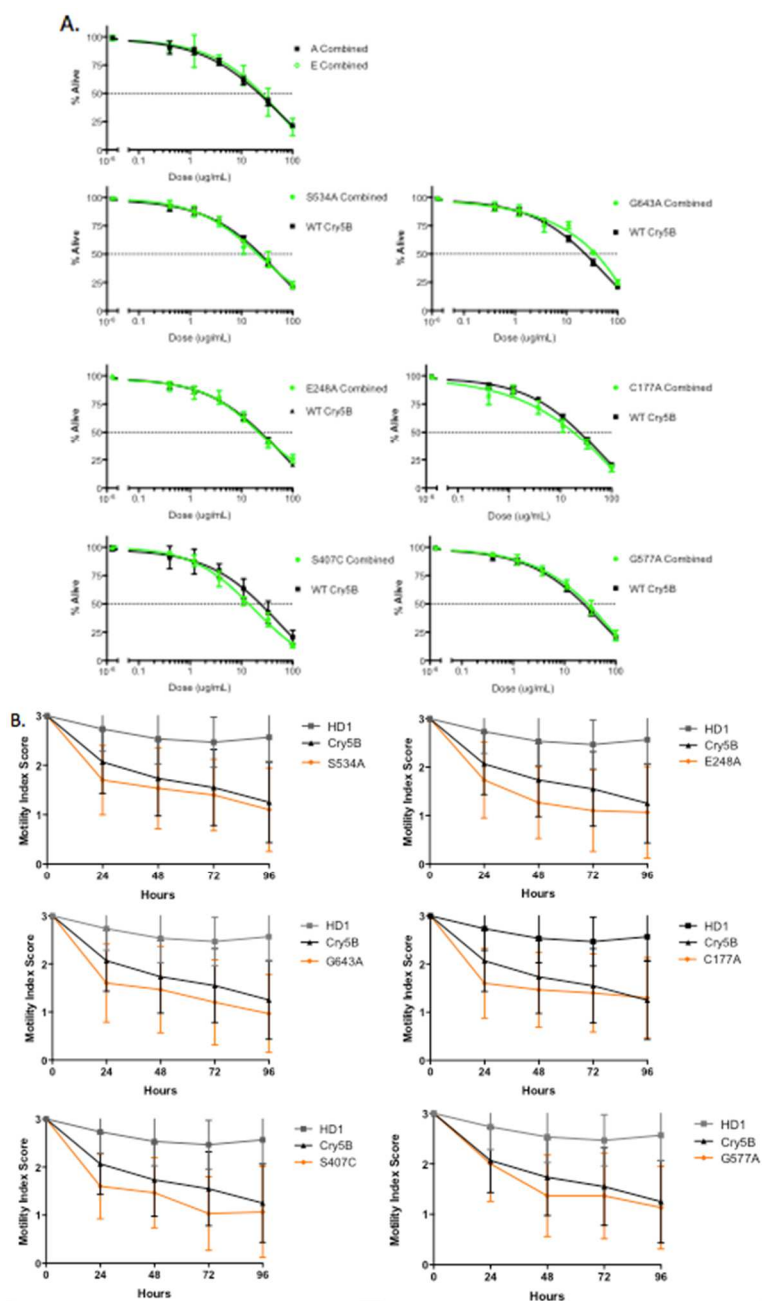
Figure 5.10: *In vivo f*ollow-up LC-50 and adult *A. ceylanicum* data

(a) In LC-50 assays conducted with the same SCL samples as the previous *in vivo* experiment, only S407C shows an increase in activity as compared to the wild-type. (b) In adult *A. ceylanicum* assays conducted with the same SCL samples as the previous experiment, all variants are significantly more active than the wild-type Cry5B.

5.7 Discussion

The set of assays testing the spore crystal lysates of the variants of interest against different stages of *A. ceylanicum* as compared to the wild-type Cry5B provided information on these variants in a parasite system. While there is still a lot to learn about these variants, how to best test for hyperactivity, and how they functionally behave, I believe that a handful of them are worth pursuing and may be more toxic to the hookworm as compared to the wild-type Cry5B. While an independent repeat of the *C. elegans* assays did not back up the original triplicate repeats, and the larval data overall was difficult to draw a conclusion from, I believe that the adult assays provide hints as to the future of the variants and how to test them. The adult assay does target the parasite at the closest development stage to mimic true treatment with a protein that can be quantified, eliminating any issue of expression. Although not statistically significant *in vivo*, it could simple be that the variants chosen to be tested in this case were not the best variants to be pursued for *in vivo* studies. Perhaps it is more difficult than originally thought to find a single variant with a striking hyperactive phenotype.

The egg to larval assays were set up almost identically to the ones in the previous chapter, except spore crystal lysates were used as the source of Cry5B instead of *E. coli* expressing Cry5B. Although still not a quantitative assay (which can be adapted in the future), images were taken and compared to the wtCry5B, and can be compared on a pound per pound basis due to the use of the SCL. To quantitate this assay in the future, the number larvae that develop into the L3i larvae are counted as compared to the number of eggs originally introduced in each well, or the number or larvae originally hatched. I

believe that in order to draw a definitive conclusion on this data, a quantitative approach should be undertaken.

Five variants were identified as being active in all three repeats tested for at least one dose – S407C, G643A, H332A, Q435A, and C177A. However, while all appeared to be reproducibility hyperactive under these conditions, they all had different doses that were discriminating. S407C for instance, was more active at the three highest doses, while G643A was only more active at the highest dose, though always repeated. C177A, was the only variant that was more toxic as compared to wild-type at all five doses tested. Although no cause for this was investigated, it is of interest to note that in the Cry5B structure, this residue is involved in one of two disulfide bonds that is formed in one of the loops of domain 1, and has been implicated in being important in pore formation. Other variants, G577A, N656A, and S534A, appear to be more active in two of the three repeats. Further repeats have only supported the inconclusive nature of the current data from this assay. Results such as this are illuminating to the limitations of the assay, and the lack of predictability and consistency that I have encountered in working with the parasites. To support this, variant I678A looks hyperactive in one assay, inconclusive in another, and definitely not in a third. No increased activity was observed with H277A. From these results, it is hard to draw any definite conclusions about many of the variants with their activity in the egg to larval *A. ceylanicum* system. While this system still has the potential to be optimized and explored further as an assay, the limitations of this assay have been illuminated through these results.

Overall, the egg to larval assays have both positive and negative traits. While this system provides an easy and accessible way to screen a large number of variants against a

parasite in the lab, the assay had some inconsistencies. Also, the one variant that appeared to be the most active at all doses tested reproducibility, C177A, showed no worm burden reduction as compared to wild type *in vivo*. This is perhaps an indication that this egg to larval *A. ceylanicum* assay may not be the best way to screen through Cry5B variants with the intention of narrowing down the number of variants in hopes to test the best potential candidates *in vivo*. However, if there was ever a reason to develop and find a variant with strong activity against the larval form of *A. ceylanicum*, there are several variants that might be potential candidates to continue testing.

The adult assays show six variants that are statistically more toxic than the wild-type at the single dose of 1ug/mL. While all of these are more active than wt Cry5B, they all have a different observed pattern of behavior. Some variants, like S407C and G643A, start to affect the worms at earlier time points, and can potentially be viewed as fast hitting. While others, such as E248A and S534A, while more active than wild-type, take longer for their effect to be observed. In looking at this data from a potential therapeutic point of view, the variants that hit at earlier time points might be of more use in the future. Any variant of Cry5B that would be used as a therapeutic will be exposed to the digestive system, with the potential to be degraded at a rapid rate by digestive juices (Hu 2012). Having a faster acting variant might be an important factor to consider, and one could hypothesize that it would have a stronger therapeutic effect than a variant that is overall more potent, but takes longer to act on the parasite. This idea also, once again, stresses the need and importance to test these variants in a system that not only is against the same invertebrate target, but also mimics the environment that they will be exposed to.

The first *in vivo* pilot experiment tested a single dose of G643A against three separate doses of Cry5B, where G643A SCL was harvested in a separate batch from the Cry5B SCL. No significant reduction in worm burden in G643A was observed compared to the wild-type Cry5B at the tested dose of 3mg/kg. However, because the wild-type and G643A were harvested from separate batches, no true conclusion can be drawn from this experiment. Due to this, I set to repeat this experiment, adding in the S407C variant and testing at 2 doses. Cry5B, G643A, and S407C were harvested from the same batch and tested at both a 3mg/kg and 9mg/kg dose. Once again, no signification reduction in worm burden at either dose for both G643A and S407C was observed as compared to the wild-type Cry5B. However, this is the second *in vivo* experiment in which G643A displays a reduction in worm burden compared to the wtCry5B that is not significant, indicating that perhaps G643A is just slightly more toxic, but not enough to be significant. If this were the case, however, it would also not have a high enough of an increase in activity to be worthwhile to pursue as a future drug therapy. While the 3mg/kg dose of the wild type of Cry5B behaved as expected, the 9mg/kg was not as active as observed in previous experiments.

The results from the final *in vivo* experiment (though one experiment is clearly not enough to come to a conclusion about which specific assays predict *in vivo* behavior) allows an opportunity to look back at all the data and start to see if any correlations could be used in the future to build upon. G577A worked well *in vivo*, but based upon the data from previous assays, was almost not included in this *in vivo* study. However, as noted, none of the in vivo experiments showed a statistically significant finding.

The one assay that gave reproducibility statistically significant results was the adult *A. ceylanicum* assay. There is not enough evidence or information to claim that it is predictive of *in vivo* behavior, but there is some preliminary evidence here that would be worth exploring in the future. One additional point of interest was that for G643A, G577A, and S407C, in addition to their individual data points being lower than the wild-type, their initial drop off had the largest gap of the 6 variants tested here from the adult assays. For C177A, which repeatedly hit hard at every dose in the larval *A. ceylanicum* assay, was not more active than the wild type Cry5B in this *in vivo* experiment. If the egg to larval assay was a good predictor, I would have hypothesized that C177A would be the best variant to test *in vivo*.

While there may be slight assay variances that could account for the changes in activity in the LC-50, S407C always continues to be hyperactive. There is preliminary data from chapter 3 that shows that the assay conditions and the buffer used to dissolve the protein does play a role on observed hyperactivity of the variants. As observed during the initial optimization of this assay, while G577A, G643A, and D553N fluctuate in whether they displayed as hyperactive or not, S407C continued to remain better than the wild-type Cry5B under all conditions. This preliminary data does indicate that these variants might not be as stable as the wild-type, or are sensitive to changing assay conditions that indicate perhaps changes with regards solubility, but none of these explanations actively account for why S407C continues to look strong in each and every assay, including this set of LC-50 data. One disconcerting aspect of this finding is that, although the mutants were found based on C. elegans screening, there is ultimately no

consistent data to support that they are statistically significantly better than wild-type, with the exception of S407C and C177A.

Looking specifically at S407C, the major difference between this variant and the other variants is that this is the only variant that I have worked with in which the original amino acid was changed to a cysteine, and not an alanine. However, I did make the S407A variant in my screen, and found it to be less active than the S407C variant, though still fairly more toxic than the wild-type in the plate assay. I would hypothesize that the CH3-SH chain that is now present on this amino acid may act to create additional disulfide bonds, either within the protein, or between Cry5B molecules. In looking at the structure, there are two cysteine resides that are structurally close by S407. Internal disulfide bonding may be occurring with this cysteine residue that allows this variant to be more stable in its conformation, since disulfide bonding is known for providing stability in protein structure, and helping with protein folding. Perhaps is it is this increase in stability that doesn't allow this variant to be sensitive to small changes assay conditions that potentially are affecting the other variants, and thus makes S407C a variant with high and consistent reproducibility. To investigate this in the future, I would propose doing a side by side comparison of S407C and S407A, as well as disrupting the disulfide bonds in S407C and comparing. I would investigate stability through western blots and assay for changes in activity against the worms in both the *C. elegans* and *A. ceylanicum* systems. Additionally, it has been proposed in the literature (Du 1994) that disulfide bonding can affect solubility in different pH solutions. I would test for solubility of S407C, S407A, and S407C with disulfide bond disruption for solubility in

different pH solutions, and then follow up with those solutions in an LC-50 assay agasnt *C. elegans*.

Additionally, it could be hypothesized that this cysteine residue forms a disulfide bond with another Cry5B molecule, thus forming a dimer, and bringing domains I of the two monomers closer together, or into a formation that better pore formation occurs. It could be this more robust pore formation that is stronger than small changes within assay conditions that the other residues are sensitive to. If one were to actively follow-up with this idea, experiments using a reducing agent, such as DTT or TCEP, could be used either within the LC-50 assay (as long as it did not affect worm health) to see if this effect goes away, or a gel could be run with both non-reducing and reducing conditions to look for dimer formation, and see if with a reducing agent, a dimer is disrupted.

Additionally, I did work a very small amount with the variant S407A in both the *C. elegans* plate assays and larval *A. ceylanicum E. coli* assays. One would predict that if it is the cysteine residue responsible for the observed results, that this effect would not be observed with S407A. Unfortunately, the two assays just mentioned are the only ones I have S407A data for, but there are indications that these two variants are behaving different from each other. For instance, S407C is more active than S407A in the initial *C. elegans E. coli* experiment, and in the egg to larval *A. ceylanicum* assay, I could not get S407A to express. This does hint to the importance of the amino acid that the original variant is being mutated to, and that this may be affecting the protein slightly differently. If one were to actively follow-up with this idea, further testing with S407A SCL in these two assays might help to provide more information on what is happening, and whether

the cysteine residue behaves quite differently from an alanine residue in this position in this protein.

Overall, as previously noted, it was seen that all the variants the adult *A. ceylanicum* assay were hyperactive as compared to the wild-type Cry5B, and the *in vivo* data shows hints of promising results, albeit none statistically significant. This data shows promise for identifying a single point variant of Cry5B with significant increase *in vivo,* as well as potential future directions as to what variants to potentially utilize in a multi-mutant combination Cry5B to search for an even higher exponential increases in activity.

5.8 Materials and Methods

Egg to larval *A. ceylanicum* Assays with Spore Crystal Lysates

Twenty *A. ceylanicum* eggs were added to a 96-well plate with 10ul of varying concentrations of variant Cry5B SCL, OP50 OD=3.0, and S-media in triplicate wells. Plates were incubated at 25°C for four days, and scored visually as compared to the wild-type Cry5B on the basis of size, motility, and coloration. Combining triplicate wells into one well in the 96-well plate, and cleaning the well completed imaging. This process was done by combining two wells (200 ul in total volume), and subsequently removing 80ul of media. The third well was added to the first well (220 ul total volume), and 80ul was removed again, leaving a final volume of 140ul of combined triplicate wells to be imaged.

Adult *A. ceylanicum* Assays

Adult golden Syrian hamsters were orally gavaged with 150 third-stage (L3i) *A. ceylanicum* infectious larvae and euthanized between days 18-22 pi. The small intestines were isolated, and adult *A. ceylanicum* were manually picked and allowed to recover in RPMI/HEPES at 37°C + 5% C0$_2$. The assay was set up in a 24-well plate with hookworm culture media (50% RPMI/HEPES 50% FBS) supplemented with antibiotics (100U penicillin/100 ug/mL streptomycin and 10ug/mL fungizone), spore crystal lysates of Cry5B diluted to 1 ug/mL in ddH$_2$0, and five worms/well separated by gender with two wells per variant. The assay was stored at 37°C with 5% CO$_2$. The assay was scored every day for a week based on a motility index score: 3 – robustly moving worms; 2 – worms moving on their own; 1 – worms moving when stimulated with an eyelash pick; 0 – no motility when stimulated.

Cry5B variants *in vivo* against *A. ceylanicum*

Golden Syrian hamsters are orally gavaged with 150 L3i stage *A. ceylanicum*. Seventeen days P.I, the hamsters are divided into groups based on fecal egg counts and are orally treated with spore crystal lysates of Cry5B or a Cry5B variant. Feces is then collected and the hamsters were euthanized on day 22 P.I. Small intestines are isolated from each hamster, and worm burden is manually determined. Eggs were counted to determine eggs per gram of feces.

Statistical Analysis

Data analysis for adult *A. ceylanicum in vitro assays* was plotted using GraphPad Prism 6 for Mac, GraphPad Software, La Jolla California USA. Statistical analysis was

carried out with one-way ANOVA with repeated measures. Averages refer to the average motility index score for all worms combined. Data analysis for the intestinal worm burden and fecal egg counts for the *in vivo* experiment were graphed using GraphPad Prism 6 for Mac, GraphPad Software, La Jolla California USA. Statistical analysis was carried out using one-tailed t-test assuming unequal variances. Averages refer to either the average worm burden in each treatment group, or the average egg count per group.

Chapter 5, currently unpublished, is, in part, co-authored. Sesar, Jillian; Hu, Yan; Aroian, Raffi. The dissertation author was the primary investigator and author of this material.

5.9 References

1. Michael Cappello, Richard D. Bungiro, Lisa M. Harrison, Larry J. Bischof, Joel S. Griffitts, Brad D. Barrows, Raffi V. Aroian, A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicum*. *PNAS*. **103(4)**15154-9. (2006).

2. Joel S. Griffitts, Stuart M. Haslam, Tinglu Yang, Stephan F. Garczynski, Barbara Mulloy, Howard Morris, Paul S. Cremer, Anne Dell, Michael J. Adang, Raffi V. Aroian, Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. *Science*. **307(5711)**922-5. (2005).

3. Joel S. Griffitts, Danielle L Huffman, Johanna L. Whitacre, Brad D. Barrows, Lisa D. Marroquin, Reto Muller, Jillian R. Brown, Thierry Hennet, Jeffrey D. Esko, Raffi V. Aroian, Resistance to a bacterial toxin is mediated by removal of a conserved glycosylation pathway required for toxin-host interactions. *J BioChem*. **278(46)**45594-602.(2003).

4. Yan Hui, Bin Zhan, Brian Keegan, Ying Y. Yio, Melanie Miller, Kathryn Jones, Raffi V. Aroian, Mechanistic and single-dose *in vivo* therapeutic studies of Cry5B anthelmintic action against hookworms. *PLOS NTD*. **6(11)**900d (2012).

5. Yan Hu, Sophia B. Georghiou, Alan J. Kelleher, Raffi V. Aroian, *Bacillus thuringiensis* Cry5B protein is highly efficacious as a single-dose therapy against an intestinal roundworm infection in mice. *PLOS NTD*. **4:3e614** (2012)

CHAPTER 6

DOUBLE VARIANTS

6.1 Summary

All 55 double variant combinations of the 11 variants previously mention in chapter 4 (narrowed down from the original 34) were made in the pQE80 vector, confirmed, and transformed into the XL10-gold *E. coli* strain. Fifty of these variants were tested in the *E.coli-C. elegans* plate assay at varying concentrations of bacteria to identify variants that appeared to be more active than their single variant counterpoints. Sixteen double variants were identified as meeting this criterion at multiple dilutions tested, as well as a single triple variant. These variants were then tested in *E. coli* OP50 against larval *A. ceylanicum*, without conclusive results. Five double variants were then subsequently subcloned, transformed, and harvested as spore crystal lysates out of *Bacillus thuringiensis* along with the relevant single variants in parallel. The SCL was tested against *C. elegans* in quantitative LC-50 assays – triplicate repeats for the double mutant G643A/S407C and a single preliminary trial for the other four mutants. Results

showed that none of these multi-variant combinations display an increase in toxicity as compared to the single variants. It was therefore concluded that either screening of the other doubles created or a different means of identification of potential doubles would most likely be the best way to find a double variant with a synergistic increase in toxicity over the wild type.

6.2 Introduction

While single point variants may be important in understanding Cry proteins better, specifically Cry5B, as well as potentially providing variant protein candidates to use in treating intestinal helminths, previous studies have shown that the potential for dramatic (100x-fold+) fold increase in Cry protein activity lies in multi combination variants. Such variant combinations are already in use in various transgenic crops across the US (1). There are several reasons that a double (or higher order) variant might be advantageous over a single mutation. First, the enhanced increase in toxicity would make it a better potential candidate therapeutically. An increase in toxicity would lend to a lower quantity of drug needed, which would be helpful in the regions of the world in which single dose drug treatments are necessary. This should also lead to lower costs. Additionally, multiple mutations might target difference steps in the protein's mechanism of action, making it harder for the host to develop resistance. One study showed a 2-3 fold increase with single variants, and an increase from there with multiple variant combinations (Rajamohan 1996). To address this further, in all practicality, one would be looking to develop a protein for which administration of a single dose would be enough to clear the worm burden. Studies have shown that the majority of the Cry5B is

degraded in the stomach, with only a small amount reaching the intestines, and thus the worms (Hu 2012). While there are many ways to address this issue – such as encapsulating the protein or finding alternative delivery methods – another way could be to increase the efficacy of the Cry5B, where the small amount that makes it through to the intestines is hard hitting and potent.

The main issue that I initially faced when starting to investigate making multiple mutant combinations in Cry5B was that there were 34 potentially hyperactive variants from the screen, and I wanted to find the best approach to maximize my chances of identifying a combination variant with a synergistic (greater than additive) increase in activity. The second issue was which assay to run these variants through. I decided to choose the 11 variants that potentially were most toxic as singles from previous chapters, make all the combinations of those, and test them in the *E. coli-C. elegans* plate assay, since it was the assay originally used to identify these variants. This leads to a total of 55 double variants to make and attempt to test in the assay.

6.3 S407C/G643A double variant

Based on the previous data on the single variants, specifically the second *in vivo* experiment, I first made the double variant of G643A/S407C. Both these single variants looked promising, and I hoped that the double might have a synergistic increase in toxicity. Preliminary results in the *E. coli* plate assay displayed no increase in toxicity as compared to the single mutants (figure 6.1). More specifically, while the assay doesn't display an increase in activity, I would argue that it is also inconclusive, as one can't interpret anything about this double variant except that in the toxin plate assay it is more

active than wild-type Cry5B. However, since this assay lacks quantification, and previous

data has shown that this assay doesn't necessarily predict the mutant's behavior in other

assays, I decided to subclone the variant into *Bacillus thuringiensis*, grow it up, and

harvest the SCL along with, in parallel, the single variants (figure 6.2a). This SCL was

tested against *C. elegans* in the LC-50 assay in triplicate repeats (figure 6.2b). This

double variant does not display an increase in toxicity as compared to the single variants

G643A and S407C (figure 6.2c). Both G643A and S407C still display an increase in

activity as compared to the wild-type Cry5B, and while not increasing in activity, the

double variant G643A/S407C remains more active than the wild type Cry5B.
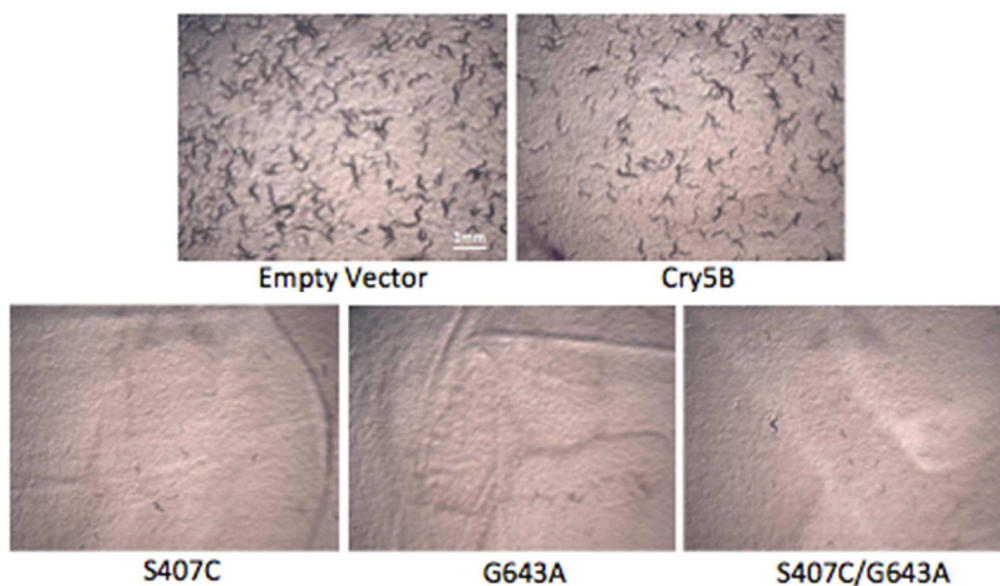
Figure 6.1: The double variant of S407C/G643A

The double variant of S407C/G643A was tested in the *E. coli – C. elegans* plate assay, but was not found to be more active than the single variants when visually compared.

Having found that the G643A/S407C double variant did not show an increase in toxicity against *C. elegans* in the LC-50 assays, I next tested it as SCL in a single trial, *A. ceylanicum* experiment. As with the LC-50 results, the double did not appear to show an increase in toxicity as compared to the single variants (not shown). This presented the challenge as to how to find a double variant with an exponential increase in toxicity as compared to the single variants.
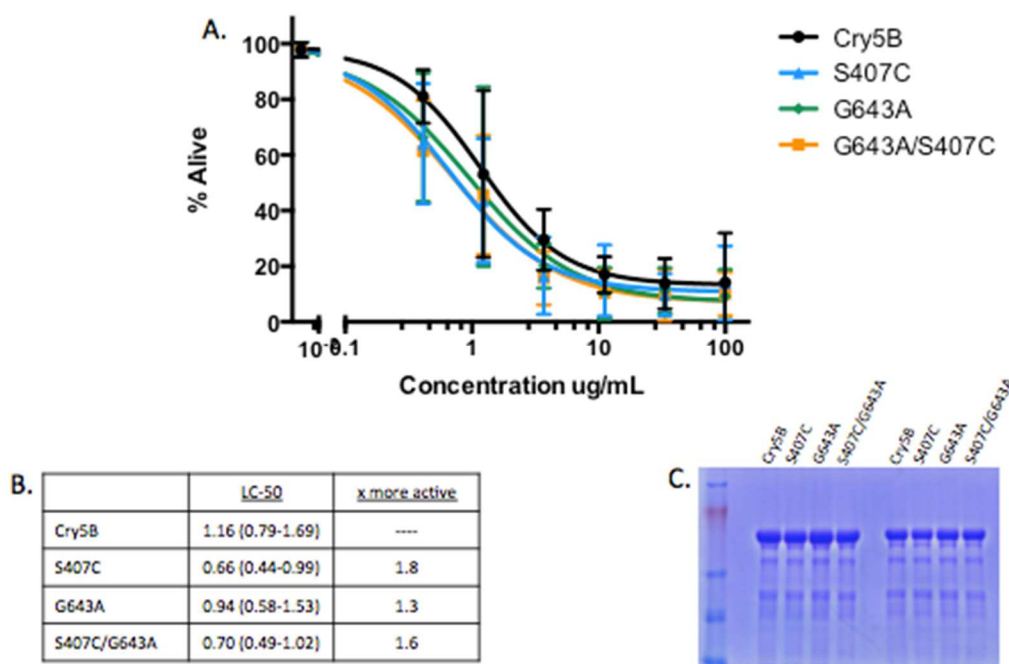
Figure 6.2: S407C/G643A is not more active than the single variants

(a) The double variant of S407C/G643A was transformed into Bt, and the spore crystal lysates were harvested in parallel with the single mutants and wtCry5B. (b) In combined triplicate repeats, S407C/G643A did not show an increase in toxicity compared to the single variants of S407C and G643A. (c) LC-50 values for S407C, G643A, and S407C/G643A as calculated by probit. (d) For each assay, a protein gel is run with the leftover SCL to ensure equal amount of protein is being used across the variants.

6.4 Double variants in *E. coli* plate assays

   With many single variants with potentially increased toxicity, how does one go about determining which ones to choose to make double mutants? Which have the highest chance of increasing the fold of toxicity as compared to the wild-type and the singles against the parasites? Because of the ease of making the doubles, and the simplicity of the *E. coli* plate assay to screen a large amount of variants at once, I decided to take the 11 variants that I had previously identified as promising, make every potential double mutant combination, and test them in this assay along with western blots to assay for expression. To optimize the assay, I dropped the percentage on Cry5B on the plate to 25%, in order to discern a difference between the single variants, and any potential increase in the double variants. I successfully made 50 double variants in the Cry5B-pQE80/XL10-gold system, and tested them in the *E. coli* plate assay against *C. elegans* along with testing for expression levels on the cultures. Figure 6.3a shows the variants that are either more active than their respective single variants or variants that might be more active (difficult to tell, but worth investigating further to discern) in this assay with expression levels. This assay revealed 15 potential variants with which to move forwar. Figure 6.3b shows the singles, and the doubles mapped out, and also provided one possible triple variant to include. However, while there are 15 double mutants that look more active than their respective single variants, these doubles are not necessarily more toxic than other single variants. Additionally, the 25% concentration used in this assay appeared to be too high to distinguish between some of the doubles and singles in a few of the cases.
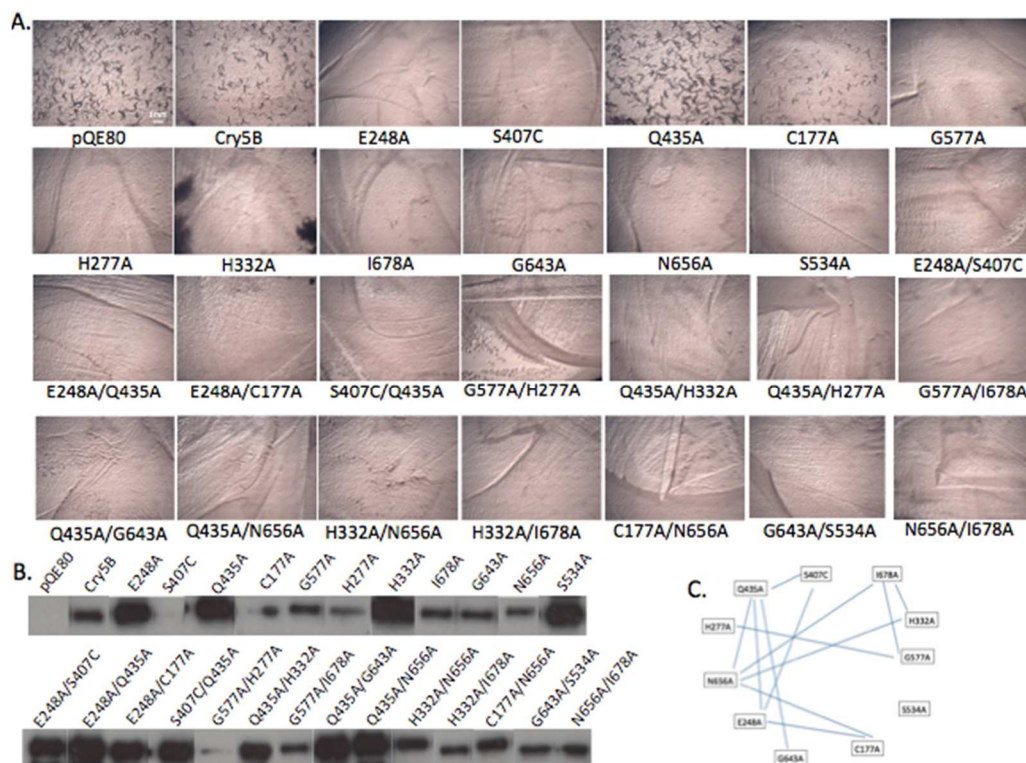
Figure 6.3: Fifteen double variants of interest from the first round of double mutant testing

(a) 15 double variants looked to be more active than their respective single variants in *C.elegans-E.coli* plate assays when Cry5B is diluted out with empty vector to 25%. (b) The cultures used to make these toxin plates were run on an SDS-PAGE gel and probed with a Cry5B antibody to investigate expression levels. (c) A web was constructed to view the double mutant combinations of interest from this assay.

The 55 doubles were then re-tested in this system at three separate *E. coli* expressing Cry5B percentages – 7.5%, 15%, 30%. The altered concentrations would hopefully better distinguish between the doubles of interest and their respective single mutants. I also included the triple variant S407C/E248A/Q435A. Figure 6.4 shows the

variants that appear to have an increase in toxicity against *C. elegans* as compared to the singles at each of these three percentages tested, as well as the respective double mutant maps. While for the most part the same doubles appeared as hyperactive at all three concentrations, the 7.5% concentration was the most revealing with regard to a difference between the singles and the doubles. To summarize from the plate assays, 17 variants (16 doubles and 1 triple), emerged as potentially being more active than their respective singles.
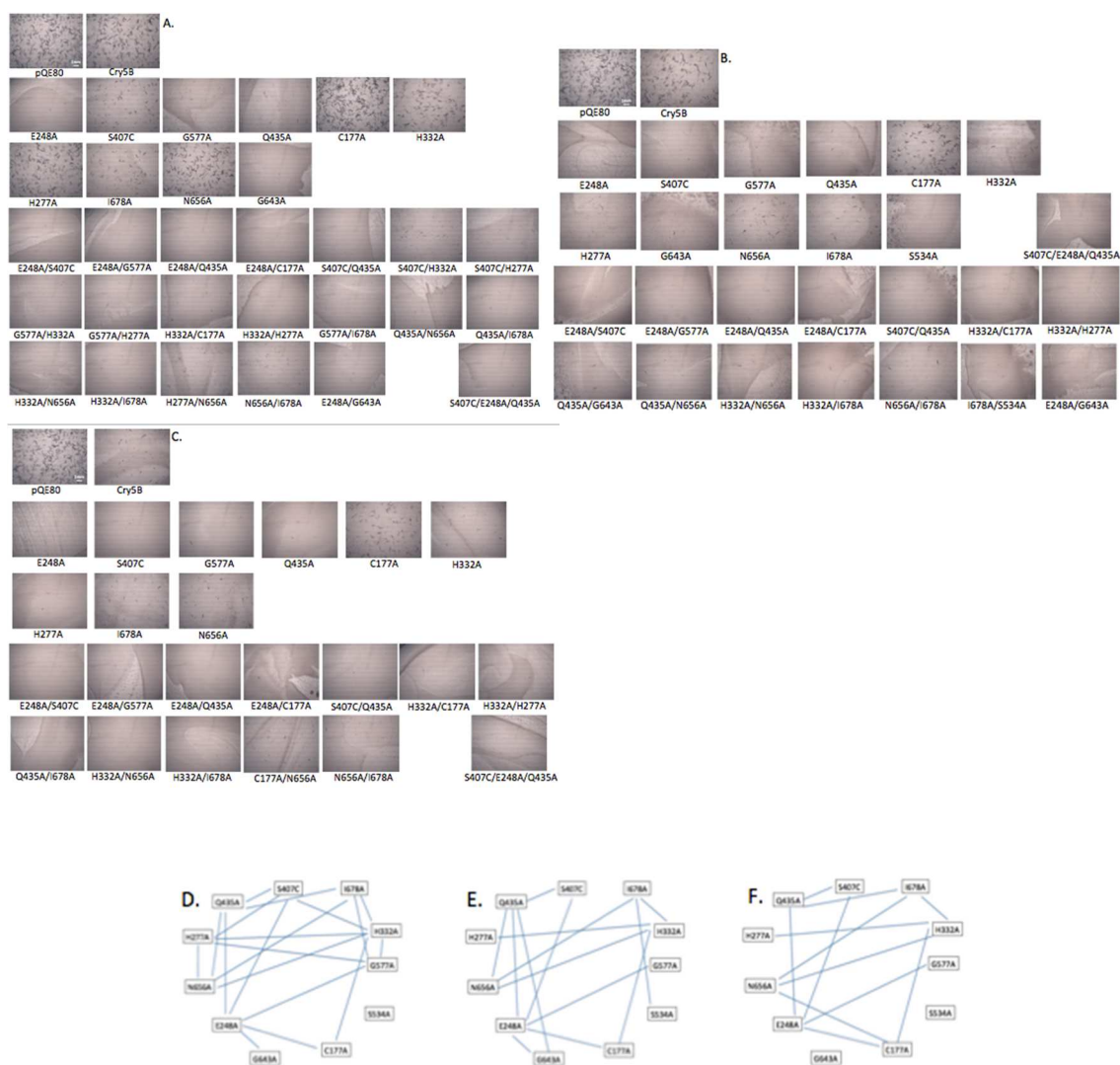
Figure 6.4: Potential hyperactive variants from the 55 doubles tested

55 double variants of interested were tested in the *C.elegans-E. coli* plate assay at three separate dilutions – 7.5%(a), 15%(b), and 30%(c). Variants that appear to be more active than their single variants are displayed here for each dilution, along with their associated web diagrams (d,e,f, respectively).

6.5 *A. ceylanicum* **egg to larval assays**

The next step I pursued with these 17 variants was to test them in a parasite system, since previous data has shown that while *C. elegans* does predict the parasites to some extent, there is clearly, and predictably, some difference. Since all of these variants were in *E. coli* (XL-10 gold cells), I decided to test them in the *A. ceylanicum* larval assay due the ease of running the variants through the assay and obtaining data on how they behave against the hookworms.

I transformed all 17 variants that stood out in the plate assays into OP50, and set-up the assay as described in chapter 4, with the Cry5B variants in *E. coli* diluted out with vector control to 5% and 10% (figure 6.5). From these 17 different variants tested, only 4 looked more active than their single counterparts (Figure 6.x). Ten double variants looked just like the single variants, indicating that either they have similar levels of activity as the singles, or the dilution % used was not ideal to distinguish between them. Three variants, while displaying increased activity as compared to the wild-type Cry5B, showed decreased activity as compared to the single mutants. This assay was not repeated, however, since improved data could be gathered by focusing on subcloning and transforming the variants into *B. thuringiensis*. Through harvesting the SCL, quantitative assays could be performed to see if these 17 doubles were statistically more active than the singles.
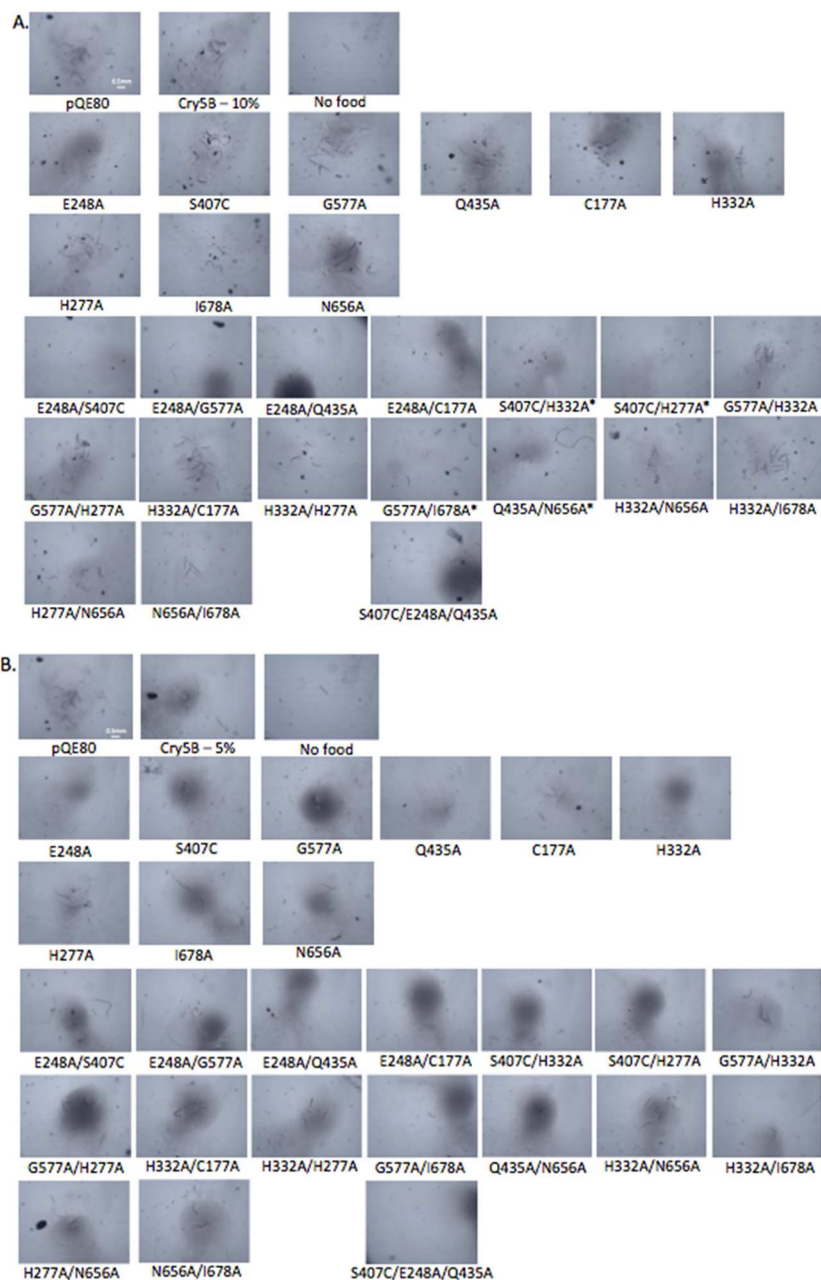
Figure 6.5: Seventeen hyperactive variants tested in the *A. ceylanicum* egg to larval assay

16 double variants and one triple variant of interest from the *C. elegans – E. coli* plate assay were tested against *A. ceylanicum* larvae at two separate dilutions – 10%(a) and 5%(b). Variants that appear to be more active than their single variants in this assay are denoted with a *.
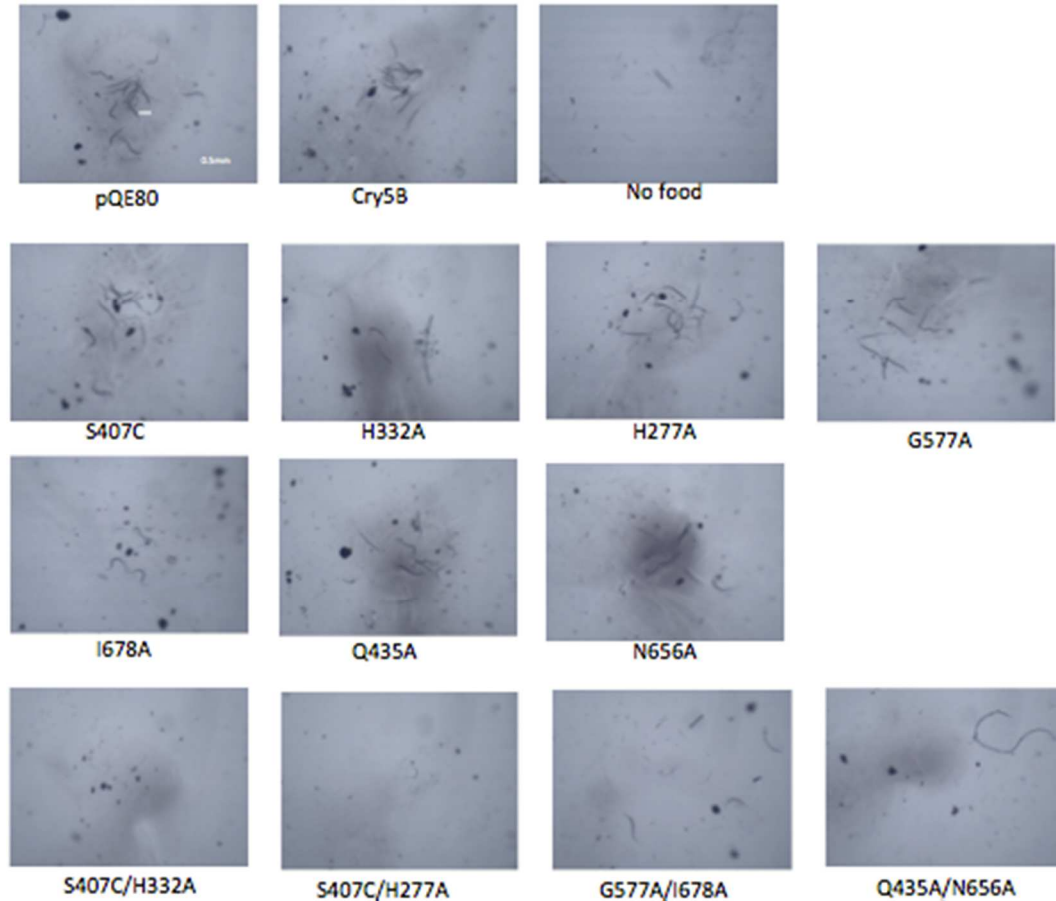
Figure 6.6: 4 Combination variants are more active than their single variants in the *A. ceylanicum* egg to larval assay

14 double Variants are more active than their single variants at th 10% Cry percentage on the plate against *A. ceylanicum* larvae.

6.6 *C. elegans* LC-50 assays

The *E. coli* plate assay illuminated 17 variants that were potentially (make same edits to figures here as above) more toxic than the singles mutants, however, from my previous data, it was known that this did not always translate over into the quantitative

LC-50 assays, or into the parasite systems. Therefore, I decided to also include all the combinations of the six that were previously tested *in vivo* to increase my chances of identifying a double mutant of interest. So this halved the number of double variants (from 50 to 25) that I was going to subclone and transform into *B. thuringiensis* for quantitative assays. My approach was to tackle as many as I could, in no specific order.

I first subcloned three (E248A/Q435A, S407C/Q435A, S407C/Q435A/E248A), harvested the SCL, and tested these variants in an LC-50 assay. Neither of the two doubles, or the one triple appeared to be more active than the single variants (Figure 6.6). The plan originally was to run them all through this assay in single trial experiments, looking for a variant that would dramatically shift the curve to the left. However, based on my data on the double variants so far, I decided that perhaps finding a double variant required a different approach. The one piece of positive data that this assay does provide, however, is confirmation of the set of singles being more active than the wild-type Cry5B.
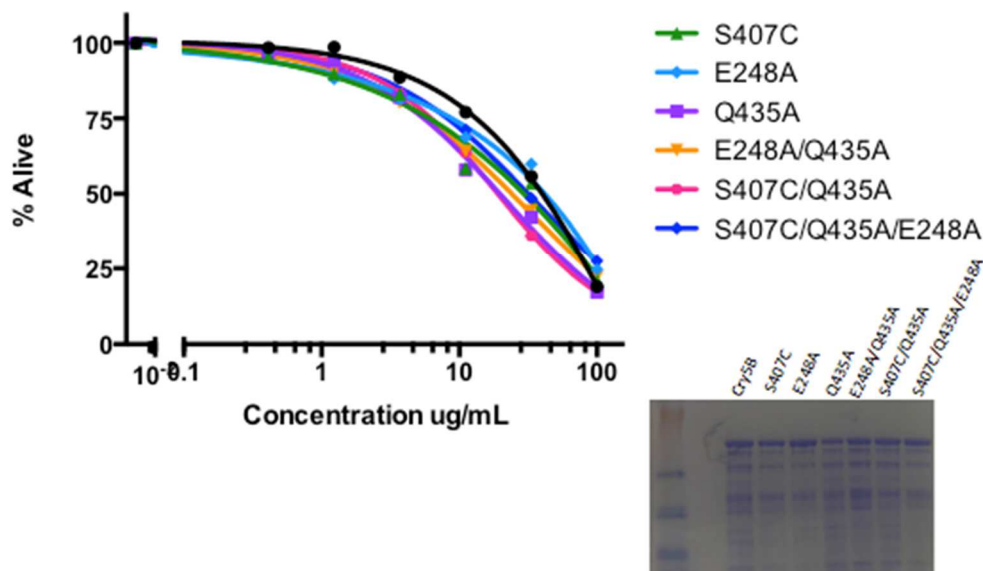
Figure 6.7: Three separate multi-Cry5B mutants do not look more active than the respective single mutant in a single trial

Spore crystal lysates of two Cry5B double mutants, E248A/Q435A and S407C/Q435A, and one Cy5B triple mutant, S407C/Q435A/E248A, were tested against *C. elegans* in a LC-50 assay, but neither of these three variants are more toxic than the Cry5B single mutants in this single trial.

6.7 Discussion

Overall, through these methods, I was unable to find a double variant that had synergistic activity against either *C. elegans* or *A. ceylanicum* as compared to the single variants. In fact, all of the combination mutants that were tested in the quantitative LC-50 assay appeared to have levels of toxicity similar to the singles. Several reasonable

explanations can be hypothesized. If the variants are acting in the same steps or parts of the mechanism of action, then targeting this step repeatedly may not increase activity further. One conclusion that can be drawn is that to determine how to understand how to make double combinations better, it might be best to further understand what effect these single variants are having on the activity and mechanism of Cry5B. Additionally, although no further increase in toxicity was observed, there was no loss of toxicity in these combinations tested.

The lingering question that remains is what is the best way to find double (or triple) variants that show a synergistic, potentially therapeutic, increase in toxicity as compared to the single mutants and the wild-type Cry5B. I approached the doubles here the same way I approached my original screen – using the *C. elegans-E. coli* system to identify variants of interest, and then move forward with the ones of interest in further testing. I believed that this would be the best way forward here, as most of my results are based off the initial results of the screen here and the methods used to identify the single variants. While there are definitely limitations of this assay that I have previously acknowledged and addressed, I would reason that it successfully helped to identify single point variants that show an increase in activity as compared to the wild-type. However, this system may not necessarily be the best way to identify double variants of interest. These results may simply be illuminating the limitations of using the plate assay to screen for double variants of interest. Preliminary results so far have demonstrated that four variants that have appeared to be more active than the singles in the plate assay do not demonstrate this in the quantitative LC-50 assay when tested as spore crystal lysates against *C. elegans*. On the other hand, these results could simple be attributed to bad luck

that the four that were tested in the LC-50 assay were not hyperactive, and maybe several of the others to be tested are.

One way to continue forward would be to continue to screen through the rest of the 25 that stood out from the plate assay/combination of the six variants. Another thought to find doubles to assay would be to use the structure to chose targeted single variants that perhaps clustered together, or looked to target different steps in the mechanism of action. Perhaps pursuing an understanding of what is happening to the Cry5B structure/function relationship with these point variants should be pursued as a means to specifically know which point variants to pick and choose to make a multi-variant combination. There is precedent in the literature for multi-variant combinations showing 10-fold+ activity against their target host in crystal proteins (Rajamohan 1996), and based on this, one would reason that such combinations should exist in Cry5B as well. Another way to approach this could be to take all 55 doubles, and develop a new, throughput, quantitative assay to screen them all either against *C. elegans* or *A. ceylanicum* as spore crystal lysates. Additionally, there is always the potential that a good double variant combination could exist through combining the single variants that did not necessarily stand out individually in the initial screen. There are many directions that can be taken to pursue the double variants in Cry5B, and any future work in the area will have to determine what the best strategy would be to pursue.

6.8 Materials and Methods

Site-Directed Mutagenesis – Double Variants

Mutations were made using a the plasmid of the single variants in the pQE80 vector that had already been confirmed through sequencing , using the QuikChange II XL-site directed mutagenesis kit. Primers were used that were originally designed for the screen. If this method yielded no results, the opposite plasmid/primer combination was used, and solved the problem. The rest of the protocol follows as states in section 2.8.

C. elegans Plate Toxicity Assays

Qualitative toxicity assays were performed as previously referenced in section 2.8. Changes were made to the dilutions of the plates, using 25%, 7.5%, 15% and 30% to conduct the assay.

Western Blots on *E. coli* expressing Cry5B

Overnight *E. coli* cultures used to make the toxin plates in XL-10 gold cells were normalized to OD=2.0 and run on a 8% gel, and then blotted with an anti-Cry5B antibody at 1:1000. Protocol details can be found in section 1.8.

Egg to larval *A. ceylanicum* Assays with *E. coli* expressing Cry5B

Assays were set up as described in section 3.7, except the bacteria was diluted out to 10% and 5% with the pQE80 vector.

6.9 References

1. A Review of the Environmental Safety of the Cry3bB1 Protein. Center for Environmental Risk Assessment, ILSI Research Foundation. 2014.

2. Francis Rajamohan, Oscar Alzate, Jeffrey A. Cotrill, April Curtiss, Donald H. Dean, Protein engineering of *Bacillus thuringiensis* delta-endotoxin: Mutations at domain II of Cry1Ab enhance receptor affinity and toxicity toward gypsy moth larvae. *Proc Natl Acad Sci* **93:14338-43**. (1996).

3. Yan Hu, Sophia B. Georghiou, Alan J. Kelleher, Raffi V. Aroian, Cry5B protein is highly efficacious as a single-dose therapy against an intestinal roundworm infection in mice. *PLOS NTD.* **4:3e614** (2012)

CHAPTER 7

FUNCTIONALITY ASSAYS OF CRY5B

7.1 Summary

Thirty-four potentially hyperactive variants were isolated from the alanine scan and narrowed down with follow-up assays against different worms at different stages. However, nothing was known why these variants might increase the activity of Cry5B. I attempted to investigate several of these variants into why they displayed an increase in toxicity, focusing on three specific steps from their mechanism of action – receptor binding, solubility, and protease processing. Although all three assays were altered and attempted to be optimized for working with spore crystal lysates, ultimately, no conclusive results were drawn from these experiments.

7.2 Introduction

I identified 34 putative hyperactive variants from the original alanine scan, subsequently narrowed down to 11 through LC-50 assays, and then to six through assays

with *A. ceylanicum.* However, there was no data that pointed to why they display an increase in toxicity. The common thread in the literature is to look at different steps in the mechanism of action that might relate to the variant in question, and assay for changes in that step as compared to the wild type. In insect studies, multiple steps have been implicated in the increase in toxicity for different mutants.

Receptor binding has been implicated in Cry protein specificity and level of toxicity. However, much of the data has come from insect studies, which differ from functional assays in worms with regard to receptor binding. In an insect, the midgut can be isolated, and brush boarder membrane vesicles (BBMVs) can be prepped. These BBMVs contain the toxin receptors, and can be incubated with the toxin to assay for changes in receptor affinity. For example, two variants of Cry1Ab were shown to have 8-fold increased toxicity against gypsy moths, and were shown to have an enhanced binding affinity to BBMVs of 4-fold (Rajahoman 1996). There is an established protocol in the lab to test for receptor binding of Cry5B to isolated glycolipids from *C. elegans*, as well as nematode parasites of interest (2-4).

Additionally, changes in solubility have also been implicated in changes in toxicity. In one study it was shown that crystals from a non-insecticidal Bt were soluble only at pH=12. When tested against an insect host with an intestine of pH=9, this Cry protein was not toxic. However, when pre-solubilized at a high pH, this crystal protein displayed toxicity to the host insect (Du 1994). This may be relevant to the activity of Cry5B variant N586A that displayed an increase in toxicity to worms. The harvested crystals, when tested at a range of pH's and compared to wild-type Cry5B, were more soluble at a lower pH. It was hypothesized that the increase in toxicity was due to the

ability of this variant to solubilize at the lower pH's, as is closer to the pH of the *C. elegans* intestine (Want 2012).

A third step in the mechanism of action that has been of interest is protease processing. Cleavage at the C and N terminus is required for activation of the pro-toxin to the toxin form, and it has been hypothesized that single amino acid changes can change the processing profile of the protein, leading to an increase in activity. Once again, the Cry5B variant N586A was compared to the wtCry5B with regards to its sensitivity to trypsin. It was concluded that both variant and wild type showed the same sensitivity to the protease, however, differences were seen in their degradation product, perhaps indicating a difference in protein conformation.

Over the course of my thesis, I attempted to investigate three of these steps in the Cry5B mechanism of action – receptor binding, solubility, and protease binding – from a number of different angles. However, despite various methods, all assays will need to be refined or changed slightly in order to provide potential useful information into why these single point variants confer an increase in activity to the protein.

7.3 Receptor binding

As mentioned above, a protocol for testing receptor binding in Cry5B against its glycolipid receptor has previously been established. Therefore, I ran through the protocol to ensure I could demonstrate positive and reproducible Cry5B binding to glycolipid receptors isolated from *C. elegans*. Figure 7.1 displays full-length (un-activated) Cry5B binding to different glycolipid species from mixed staged *C. elegans*.
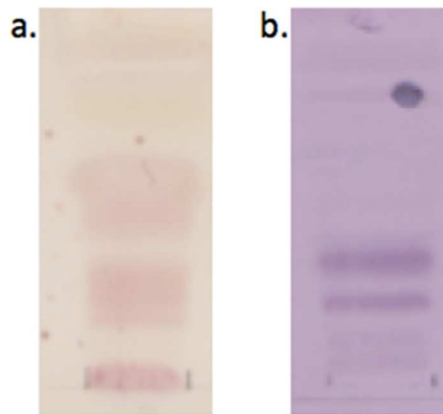
Figure 7.1: Binding of Cry5B to *C. elegans* glycolipid species
(a) Glycolipid species isolated from *C. elegans* visualized with oricinol, which stains carbohydrates non-specifically. (b) TLC assay revealing Cry5B binding interaction with the isolated glycolipid species.

The next step I attempted to pursue was to test a few variants of interest in this assay, specifically variants from domains II and III, as they would be the most logical to initially pursue. More specifically, domain II is thought to be involved in receptor recognition and binding, and domain III is thought to play a role in receptor binding (DeMaagd 2001, Burton 1999).

However, as discussed in section 3.4, I was then troubleshooting issues relating to isolating purified protein of the variants, and so I utilize spore crystal lysates instead. This overlay assay had not been previously successfully tested with SCL, and therefore I wanted to make sure that a positive signal could be detected using spore crystal lysates. The assay was repeated with full-length un-activated Cry5B spore crystal lysates, however, no signal was detected with the SCL, while one was observed for full length Cry5B run in parallel. This result was most likely an indication of two possible things:

either the overlay assay needed to be further optimized for the use of spore crystal lysates

successfully, or level one protein was the only option to use going forward in this assay.

To pursue the SCL assay, the initial approach I would take would be to look for a positive

biotinylation reaction with the SCL. I would also investigate whether the SCL might be

reacting differently in the buffers, putting the protein in a form or a conformation that

may not be accessible for a specific step in the protocol.

7.4 Protease processing and stability

Protease processing is important for toxicity as well, to convert the pro-toxin into

the active toxin fragment, which can then affect conformation, oligomerization, etc. The

first assay I did with regard to protease processing was to set up a digestion profile of

Cry5B SCL using elastase. Both level 1 Cry5B and SCL Cry5B were exposed to elastase

at over the course of 18hr in both 20mM HEPES at pH=8.0 and 20mM citrate at pH=3.0

at two different ratios of protein:protease (figure 7.2). As observed, the level 1 cry5B

showed sensitivity to the protease at the previously established conditions of pH=8.0,

producing the anticipated 61kDa fragment. However, no change was observed with the

level 1 protein at pH=3.0, or with the SCL. Since I wanted to do my assays with the spore

crystal lysates, these conditions were not suitable for use with the variants of interest.
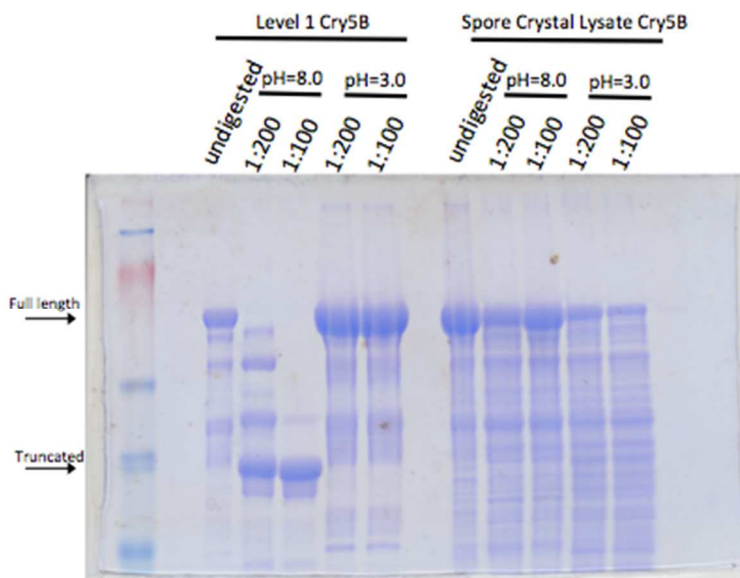
Figure 7.2: Cry5B SCL protease processing
Cry5B SCL exposed to elastase at varying ratios at different pHs. As
compared ot the positive control of level 1 Cry5B at pH=8.0, which showed
Cry5B activation, there was no processing observed with the Cry5B SCL.

Based on the results in section 4.7, I took the SCL of wtCry5B and six variants of

interest (S534A, E248A, Q435A, H332A, H277A, S407C), and left them on my bench

over the course of 72 hours to observe a natural degradation and stability pattern. I chose

the variants that appeared to have an increase in expression from section 4.7, as well as a

few that didn't in hopes of potentially observing a difference between them. Since the

assay referenced in section 4.7 was over the course of 48 hr, and I had hypothesized that

some of these variants might be more stable during this time (thus resulting in the

observed higher level of protein present in the wells), I tested this hypothesis. I left the

SCL on the bench top for 72 h and removed aliquots every 24 hr, in both water and

HEPES (figure 7.3). For the wt Cry5B, only a small amount appeared to have degraded

from the 0 time-point in water, and then no further degradation was observed. For the

variants tested, a large amount was lost by the 24 hr mark, and then no protein was

observed after 48 hr. While this is showing a difference between these variants and the

wild type, there is no apparent difference between the variants that indicate that some of

them my be more stable than the others, and may be a source of their increase in toxicity.

In HEPES, all samples looked similar, with about half of the protein being degraded by

two hours, and completely gone after 48 hr.
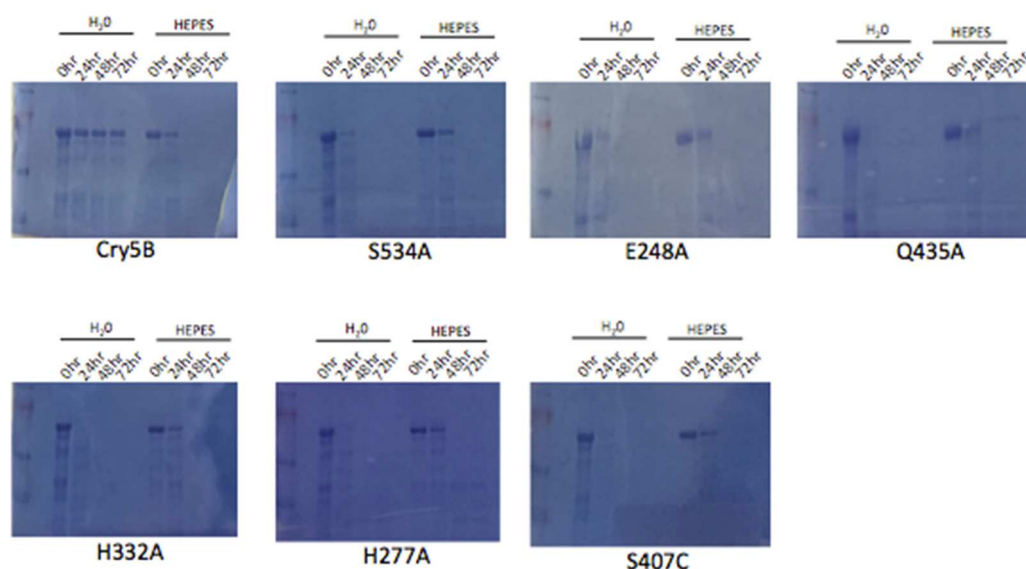
Figure 7.3: Cry5B SCL and variants at room temperature over time
Spore Crystal lysates of Cry5B and select variants were left at room temperature over 72 hours in two separate buffers to observe natural stability and degradation patterns.

7.5 Discussion

Overall, the limitation in these experiments was my inability to establish assay conditions for the Cry5B spore crystal lysates. While most of these assays investigated have been optimized and established for use with level 1 protein, my preliminary data suggest that they cannot simply be applied to Cry5B spore crystal lysates, whether that be due to the state of the protein, or perhaps a form of inhibition from the presence of the spores. Therefore, in order in the future to move forward, either the idea of utilizing level 1 Cry5B protein must once again be investigated, or these assays must be further optimized and altered for use with the spore crystal lysates.

As previously mentioned in sections 3.8 and 4.7, some of the observed results could potentially be attributed to alterations in solubility. I attempted to observe solubility of Cry5B and select variants over a range of pHs, as well as a using a fixed pH over time to see if the solubility profile was changed. Specifically I tested the wild type Cry5B SCL from pH 3-10 in increments of one for one hour at room temperature, and used the fixed pH=3.9 (the pH of the *C. elegans* intestine (3.92±0.22) to incubate the samples from 1 min to 2 hr. For both assays, the samples were spun down and the pellets and the supernatants were run on an SDS-PAGE gel and stained with coomasie. It was observed, hoever, that the protein remained in the pellet. This is contrary to what one would expect, as the standard protocol to isolate level 1 protein has the SCL solubilized in a pH=3.0 buffer, Therefore, the results of the solubilty testings were inconclusive to set up a profile for the wild type. This issue must first be resolved, and then alterations to the protocol, such as time or temperature, could be introduced to further optimize this set-up for Cry5B SCL.

7.6 Materials and Methods

Lipid Isolation and Receptor Binding

Lipids were purified from mixed life stage *C. elegans* following the protocol established by Svennerholm and utilized by Griffitts (Griffitts 2006, Svennerholm and Fredman 1980). The isolated lipids were dried down with nitrogen, and resuspended in methanol. The receptor binding was carried out as described in griffitts. A HPTLC plate was prepped, and loaded with the isolated lipids on two parallel plates. One plate was stained with orcinol, to stain the glycolipids nonspecifically. The second plate was probed with a biotinylated Cry5B (22x excess molar Biotin) to reveal the species that were binding to Cry5B.

Solubility

To test Cry5B solubility over a range of pHs, the SCL was spun down in water, resusupended in a 50 mM buffer of the desired pH, and incubated for 1 hr at room temperature. The solution was then spun down, and the supernatant was separated from the pellet, and both components were run on a 8% gel and stained with coomassie.

To test Cry5B solubility at a single pH, the SCL was spun down in water, and resuspended in 50 mM citrate buffer pH=3.9. The sample was incubated for 1 min, 5min, 10 min, 30 min, 60 min, and 120 min, and at each time point, 61 ul of the sample was removed (200 ug of protein), spun down, and the supernatant and the pellet were immediately boiled with SDS-sample buffer. The samples were run on an 8% gel, and stained with coomassie.

Protease Processing

To test Cry5B wild-type sensitivity to elastase, both level 1 and SCL were spun down, and resuspended in both 20mM citrate pH=3.0 and 20 mM HEPES at pH=8.0. The samples were then incubated with elastase for 18 hr as both a 1:100 and 1:200 ratio at room temperature. 2X SDS-sample buffer was added, and the samples were run on a 8% SDS-PAGE gel, and then stained with coomassie.

To test Cry5B SCL stability to natural proteases, equal amounts of both wtCry5B and variants were spun down, and resuspended in either water or 20 mM HEPES pH=8.0. The samples were left at room temperature for 72 hr. Aliquots were taken every 24 hr, and immediately boiled with SDS-sample buffer. The samples were run on an 8% gel, and stained with coomassie.

7.7 References

1. Francis Rajamohan, Oscar Alzate, Jeffrey A. Cotrill, April Curtiss, Donald H. Dean, Protein engineering of *Bacillus thuringiensis* delta-endotoxin: Mutations at domain II of Cry1Ab enhance receptor affinity and toxicity toward gypsy moth larvae. *Proc Natl Acad Sci* **93:**14338-43. (1996).

2. Joel S. Griffits, Stuart M. Haslam, Tinglu Yang, Stephan F. Garczynski, Barbara Mulloy, Howard Morris, Paul S. Cremer, Anne Dell, Michael J. Adang, Raffi V. Aroian, Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. *Science*. **307(5711)**922-5. (2005).

3. Fan Hui, Ulrike Scheib, Yan Hu, Ralf J. Sommer, Raffi V. Aroian, Partho Ghosh, The structure and glycolipid-binding properties of the nematicidal protein Cry5B. *Biochem*. (2012).

4. Joseph F. Urban,, Yan Hu, Melanie M. Miller, Ulrike Scheib, Ying Y. Yiu, Raffi V. Aroian, *Bacillus thuringiensis*-derived Cry5B has potent anthelmintic activity against *Ascaris suum*. *PLOS NTD* **7:6** 2263 (2013).

5. Du, Cheng, Martin, Phyllis A. W., and Kenneth W. Nickerson. Comparison of disulfide contents and solubility at alkaline pH of insecticidal and noninsecticidal *Bacillus thuringiensis* protein crystals. *Appl Environ Microbiol*. **60(10):** 3847-3853. (1994).

6. Fenshan Wang, Yingying Liu, Fengjuan Zhang, Lujun Chai, Lifang Ruan, Donghai Peng, Ming Sun,Improvement of Crystal Solubility and Increasing Toxicity against *Caenorhabditis elegans* by Asparagine Substitution in Block 3 of *Bacillus thuringiensis* Crystal Protein Cry5Ba. *AEM*. **78(20);**7197 (2012).

7. Svennerholm, L, and Fredman, P. A procedure for the quantitative isolation of brain gangliosides. *Biochim Biophys Acta*. **617(1)** 97-109. (1980).

8. Ruud A. de Maagd, Alejandra Bravo, Neil Crickmore, How Bacillus thuringiensis has evolved specific toxins to colonize the insect world. *Trends in Genetics* **17**, 1993-1999 (2001).

9. Sarah L Burton, David J Ellar, Jade Li, Dean J Drbyshire, N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a Bacillus thuringiensis insecticidal toxin. *Journal of Molecular Biology* **287**, 1001-1022 (1999).

CHAPTER 8

CONCLUSIONS, IMPLICATIONS, AND

FUTURE DIRECTIONS

8.1: Summary

To summarize, I alanine scanned the entire toxin domain of Cry5B, and found 34 potential hyperactive variants from the 698 amino acids attempted that I further pursued. I tested 18 of these in triplicate repeats as spore crystal lysates harvested from *Bacillus thuringiensis* against *C. elegans* in quantitative LC-50 killing assays, and found that nine were statistically more toxic as compared to the wild-type Cry5B, although this was reproduced in independent assays for only two. I next tested all 34 in *E. coli* against the larval stage of *Ancylostoma ceylanicum*, and from these results in conjunction with the LC-50 data, narrowed down the initial 34 to 11 variants of interest to further test against the hookworms. One important caveat was the lack of quantitation with this assay, which impedes making any firm conclusions.

I tested these eleven variants as spore crystal lysates in triplicate repeats against *A. ceylanicum* larvae and adults, and used the results of this data to narrow down the 11

mutants to six. While not quantitative, the larval assay results showed seven out of the eleven to be more active than the wild-type. However, there were issues with consistency of these assays. The adult assays showed six out of the eleven significantly more toxic than the wild-type, although none to a high degree. I then tested six variants *in vivo* against hamsters infected with *A. ceylanicum*, and found no mutants statistically significantly reduced the worm burden. The attempts to find a double variant with an exponential increase in toxicity were not successful.

Although at the end there are no firm conclusions about hyperactivity of the mutants as none are consistently hyperactive in all assays, with the possible exception of S407C. There are, however, several variants of interest that may have been identified that could be further pursued for both their potential value as a therapeutic, as well as to understand what is happening with the Cry5B protein on a structure-function based level.

8.2: Alanine scanning and *C. elegans* as tools to identify hyperactive variants

Overall, the alanine scan identified 34 variants that may be more active than the wild-type Cry5B based on initial screening. In regards to this screen, I looked only for mutants that showed an increase in activity as compared to the wild-type, since the larger goal of my study was to potentially find a variant with a therapeutic level effect against a parasitic nematode. However, with regards to understanding Cry5B as a protein better, the other toxicity categories of variants hold just as much potential information. There are frozen stocks of all of these variants that are available for future testing. This could be important to pursue, because I belive that the best way to improve this protein's efficacy (against the parasites at a therapeutic level) is to further understand how the

single amino acid change is altering the protein, and then utilize that information to understand better how to manipulate it. The group of variants that lost all toxicity is a category that holds interest to me. Although these variants may not directly be used to treat the worms, they almost certainly hold key information with regards to important steps in the protein's mechanism of action, such as receptor binding and potentially the site of binding. If a single amino acid change to alanine can completely disrupt the activity of the protein, the original amino acid must be playing a necessary role in the protein. Additionally, it is important to note that these loss of toxicity mutants were only screened against *C. elegans*, and therefore it still remains unknown if any of these would have a different effect against *A. ceylanicum* or any other parasite system.

A potential limitation to this screen as a means to find variants that increase the activity of Cry5B is the use of alanine as the sole substitution. Alanine is commonly used for large scale mutagenesis screens because it perturbs function of the residue that it is replacing while not adding any additional side chains to the protein. In regards to doing a large scale screen, such as with Cry5B, alanine is the simplest, best choice structurally, to chose to change each amino acid to. However, preliminary data has shown a difference in the levels of activity between S407C and S407A, indicating that while a change in that specific amino acid residue results in an increase in activity, the specific amino acid to which it is mutated has an effect as well. Perhaps a better way to approach finding a useful Cry5B variant is to use the alanine scan as a starting point, and then follow that up with testing alternative amino acid residues other than alanine to determine which variants to move forward into more quantitative testing. This method, however, does not directly address the parasites right away, but provides the potential to find the best amino

acid substitution of an identified residue of interest. To support this idea, I have preliminary data that indicates that more hydrophobic substitutions may increase the activity of a variant of interest (figure 8.1). Specifically, in the plate assay, it appears that for both G643A and S407C, that changing the amino acid to a longer, aliphatic hydrophobic side change increases the activity as compared to the alanine variant, while changing it to a polar amino acid, such as aspartic acid, decreases its activity as compared to the alanine variant (but still hyperactive as compared to the wild-type). In a previous study, the authors conclude that the activity profile of a specific substitution is not fixed, because the activity of the variant will always relate back to the amino acid used in the substitution (Mourez 2003). While I do believe that alanine remains the best amino acid to perform a large scale screen with for Cry5B, I also believe that after variants of interest are identified, other residues should be investigated to find a mutant with increased activity with regards to alanine that may be beneficial to be identified earlier rather than later.
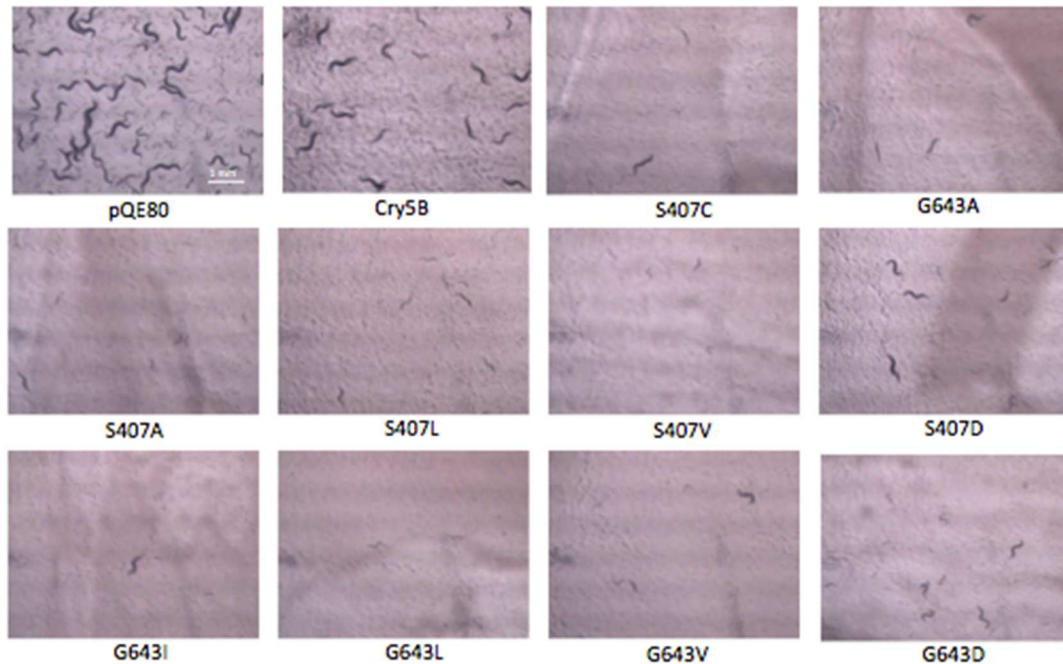
Figure 8.1: Preliminary data – amino acids with longer aliphatic hydrophobic side chains will increase activity

For variants S407C and G643A, additional mutants were made to the alaphatic hydrophobic side chains in a preliminary assay against *C. elegans* as expressed through *E. coli* on plates. Preliminary data supports that these variants show an increase in toxicity as opposed to the alanine variants, and that toxicity is reduced as compared to the alanine variant when mutated to an aspartic acid.

An additional question and potential implication worth exploring is that if there are so many single amino acid changes that seemingly increase the toxicity of this protein, why don't these variants naturally exist? There may be a reason that this protein is not optimized already in nature. One hypothesis is that such a variant may be too toxic to the host. If these Cry proteins exist in nature to be active against their respective

invertebrate hosts, then it is important to maintain a balance between the host and the protein in order for the protein to continue to exist and evolve naturally. A second hypothesis would be that these mutations have an adverse affect to the protein in its natural conditions that are not apparent in a laboratory setting.

The Cry5B structure is known and published (Hui 2012), and I can take these variants and map them on the structure. With the right resources, one could potentially use this structure to view these mutants from a structure point of view, and begin to hypothesize into their possible functions or interactions. I would predict and advise that any future direction taken with regards to these mutants should include utilizing the structure as a resource. Observations can be made as to whether these variants are located on the surface, are clustered together, located in any loop regions of interest, or participate in disulfide bonding. Additionally, modeling techniques could be applied to predict receptor binding locations, or to see how these amino acid changes can alter the conformation of the protein, its folding, or its formation of oligomers. Visualization of the 34 hyperactive variants on the structure can be seen in figure 8.2a, while the six that were carried through the *in vivo* studios can be seen on the structure in figure 8.2b.
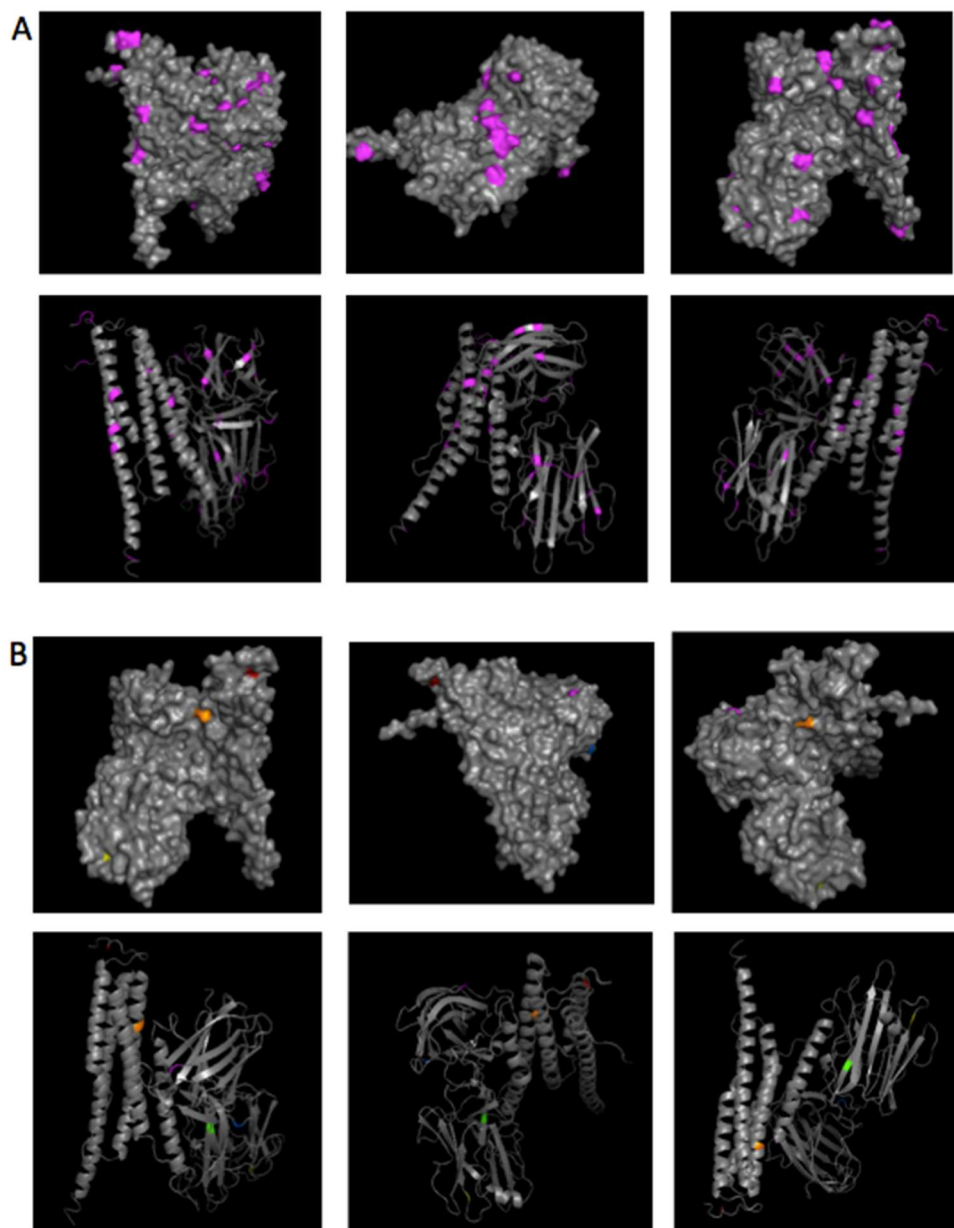
Figure 8.2: Cry5B hyperactive variants on the structure

The 34 hyperactive variants can be seen on the Cry5B structure in both a surface and a ribbon diagram (a). The six variants that were tested in the *in vivo* studios can be seen in both a surface and ribbon diagram (b) and are as follows: C177A – red, E248A – orange, S407C – yellow, S534A – green, G577A – blue, G643A – purple.

A future direction of this assay would be to tighten up this system to either make it more reliable, in terms of the direct correlation between the plate assays and the LC-50 assays, or the plate assays and how the identified variants act on the parasites. The beauty of the *C. elegans*-Cry5B *E. coli* system also cannot be ignored here. It is the interaction in this system that allowed me to screen through all 698 amino acids of the Cry5B toxin domain in a timely and efficient manner. However, there are a lot of variables that may play into the reasons why these 34 variants didn't always appear to be hyperactive in the LC-50 assays or against the hookworms, such as the form of the protein being tested, the worm being used, how the protein reacts in the assay conditions, or the stage of worm being tested. A screening system that utilized pure protein, or a way to quantitate the protein, would be advantageous, while additionally, a system that would screen directly against the parasites would clearly be the best future option for finding variants of potential therapeutic value. However, the beauty of staying within the *C. elegans* system, besides the ease of working with the worms, is that experimentally a lot of tools exist to work with *C. elegans*. Through the use of this system, I believe that this is the best way to approach a screen of this size, especially to learn more about Cry5B from a structure point of view.

8.3: Cry5B variants against *C. elegans* in LC-50 assays

My intention in testing the Cry5B variants in the LC-50 assay against *C. elegans* as spore crystal lysates was to take the variants that stood out from the screen, and test them in a quantitative assay in order to be able to determine if the variants were truly more active. The assay would then identify the variants that were more toxic than the

wild type, allow for comparison, tell how much more active than the wild-type they are, and then help to narrow down the variants to a smaller subset to test against the parasites. Once the assay conditions were established and settled, I was able to do this for 18 of the variants. From the 18 that were tested, I found that half were statistically more active than the wild-type Cry5B on approximately a 2-3 fold basis. While 2-3-fold is not a high enough increase to be considered useful on a therapeutic level, it is what would be expected from a single amino acid change. However, nine of them were not hyperactive in this assay compared to wild-type Cry5B. Moreover, independent replication could not confirm hyperactivity of many of the mutants. Indeed, the only mutant that consistently stood out in all assays was the previously identified S407C.

When establishing the conditions of the assay, the decision to use spore crystal lysates stemmed from two separate pieces of information: 1) the difficulty of harvesting out pure level 1 protein of the variants from *Bacillus thuringiensis*, and 2) the lack of hyperactivity observed by the G577A and G643A as pure protein. . Following the level one harvest protocol detailed above, barriers were initially encountered when trying to purify out the level one protein from the variants. Specifically, once the protein was solubilized in the citrate buffer, it failed to precipitate out when the 1M tripotassium citrate was added. Several studies were conducted to investigate into this (1-a time course to determine the peak time of protein expression to alter harvest time, 2-adjusting incubation times and temperatures, and 3-adjusting the volume and incubation time with the tribasic buffer), but none of the approaches appeared to make a consistent difference. Other protocols exist that laboratories use to harvest out crystal proteins, such as using a sodium-bromide gradient centrifugation technique, that could be adapted in the future to

pursue the use of level one protein (Komano 2005). To recap and further address this issue, when tested as level 1 protein in the LC-50 assay, S407C and D553N, the two variants already known in the lab and confirmed to be more active in this assay, both confirmed and repeated in my hands (chapter 3). However, when I tested the variants G577A and G643A as level 1 protein, using S407C as a control, these two variants were not more active. Due to this result, I switched to using spore crystal lysates in the LC-50 assay. This time, G643A, G577A, and S407C were more active, while D553N shifted to being less active than the wild-type. Clearly, there is indication that something is happening in this assay that affects the behavior of these variants. The question then arises: are these variants really hyperactive if they only appear to be so under specific conditions?

To address this, I have a few hypotheses to begin to account for this observation that could be tested in the future. This LC-50 assay uses a system buffered to a pH 6.0 with citrate buffer. When level 1 protein is used, it is pre-dissolved in 20 mM citrate buffer pH 3.0, while when SCL is used, the protein is simply diluted out in water, and is not dissolved. Literature on Cry proteins has demonstrated altered levels of toxicity of the same Cry variant just by varying whether it is pre-dissolved or not before treatment on a specific target host (Du 1994). If these variants are sensitive to changing conditions of the assay, maybe because the mutation changes the solubility or stability of the protein, then its activity level may also be affected in different assay conditions, or sensitive to the solution it is pre-dissolved in. For S407C, I have hypothesized (as mentioned in chapter 5) the idea of additional disulfide bond formation, leading to a more stable variant that is not affected by changing conditions.

The direct follow-up to this observation leads to this question: is the LC-50 assay the best assay to use to validate the results of the screen and narrow down the variants? If the variants are sensitive to changes in assay conditions, however small, then the assay may not be the correct or reliable method to use here. Looking at the future direction of the LC-50 assay, I believe that further understanding of the Cry5B protein will help to unlock more information on the observed results. While performing these assays, my focus was on confirming these variants in a quantitative manner, and narrowing down the initial list of 34 to find a smaller subset to test against *A. ceylanicum*. Clearly, finding a variant with therapeutic effect has direct and important possibilities and potential. In terms of moving forward with these mutants, I believe that the role they are playing and how that relates back to the structure is far more important that I originally anticipated, and should not be so easily overlooked. While it is possible to simply screen a large number of variants through sheer force and effort, I believe that understanding the subtleties of this protein will lead to further understanding of which variants to intelligently pursue.

The LC-50 data doesn't seem to correlate well with the *in vivo* data. All variants tested *in vivo* were more active in one iteration of the LC-50 assay, but in looking solely at the LC-50 data, it would be impossible to predict which ones would have an effect in the *A. ceylanicum* hamster system. Since only 6 variants that looked good in this assay were tested *in vivo*, there is no way to know if any of the variants that were not more active than the wild-type in the LC-50 assay would have had any effect *in vivo*. However, looking at the data that I do have, the six tested appeared to be better or as equally toxic as the wild-type *in vivo*, showing that the LC-50 did not identify variants that had any

reduced toxic activity. However, it needs to be pointed out that none of the proteins were actually better statistically *in vivo*.

8.4: Cry5B variants against a parasite – *A. ceylanicum*

Cry5B variants of interest were tested against the hookworm *A. ceylanicum* in four separate assays: egg to larval assays with Cry5B expressed in *E. coli*, egg to larval assays with Cry5B as SCL, adult assays with Cry5B SCL, and in *in vivo* assays with Cry5B SCL. All four assays were performed with a specific purpose in mind, and had their advantages and disadvantages.

The *E. coli* egg to larval assay is the simplest assay to run against all the variants against a parasite, but there is a question of how predictive it is of *in vivo* behavior. For example, G577A shows almost no difference from the wild-type Cry5B in this assay, while it displayed a higher (albeit not significant) reduction in worm burden *in vivo*. On the other hand, C177A displays a very toxic effect in the egg to larval assay, but showed no difference *in vivo* as compared to the wild-type. While I might not be able to conclude that this assay is helpful for determining *in vivo* behavior, and it is clearly complicated by expression inconsistencies and the role expression may play in hyperactivity, I believe that it serves as a quick and efficient way to run all Cry5B variants of interest through a parasitic system, and narrow things down. Hopefully a variant that would be potent *in vivo* would also be hard hitting against larval and adult stages of these worms, and thus would not be missed by this assay. Even if a variant were to be missed through this assay, the screen provided so many variants for possible testing, that I believe it is useful to have this assay as a simple tool to narrow down the list of variants, especially in

conjunction with another assay. For example, I used this assay in conjunction with the

LC-50 assay to take the initial 34 and narrow it down to 11 variants. I believe that these

assays together were helpful in narrowing down a larger list to a smaller subset for

further testing in other assays that are not so easily utilized with a large number of

variants.

While expression levels were originally probed as a way to eliminate variants that

displayed increased expression, they might actually shed some light on what is happening

with the protein. Observed increased in expression may point to variants that are perhaps

more stable, or resistant to degradation. Additionally, while not quantitative, I believe this

assay served as a simple, consistent, and reproducible way to run a large amount of

Cry5B variants through a parasite system early on in the screen. Although working with

the pure protein is preferred, it is also necessary to balance the importance of testing these

variants against the hookworms as early on as possible.

This same *A. ceylanicum* egg to larval assay was repeated using spore crystal

lysates as a means to test these variants against the hookworms and compare them in a

quantitative, dose dependent manner. However, unlike when using *E. coli,* the assay

using spore crystal lysates was difficult to repeat and lacked consistency, making it

unreliable I believe. While certain variants, like C177A, were always very toxic as

compared to the wild-type at all doses tested, other variants, like G643A, looked toxic at

only a select dose or two in one assay, and then not at all in a repeat assay. I continued to

set-up additional assays to try to find a consistent result, but the additional repeats did not

appear to help provide clarity. It is interesting to note, however, that this assay, like the

LC-50 assay, uses S-media as the media to perform the assay. And in both the LC-50

assay and this *A. ceylanicum* larval assay with spore crystal lysates, the data has suggested that the variants may be sensitive to slight changes in assay conditions. This observation is not seen in the *A. ceylanicum* larval assay with *E. coli* expressing Cry5B, which also uses S-media, or in the adult *A. ceylanicum* assays, which used a different media, but spore crystal lysates. While not performed as a quantitative assay in this study, it could be improved upon by counting the number of developed L3i worms to add a quantification component as seen in the literature to quantify (citation needed). If this technique were applied to this assay, it may provide better distinction into the inconclusive nature of the assay.

There were a few interesting pieces of data with the *A. ceylanicum* egg to larval assay. C177A, which hit hard at every dose tested in the assay, was not more active than Cry5B *in vivo*. G577A, which only really looked good in this assay at a single dose, reduced the worm burden *in vivo*, albeit not significantly. From this data alone, the egg to larval assay may not be the best predictor of *in vivo* behavior.

Eleven of the variants were tested against adult *A. ceylanicum* as spore crystal lysates at a 1ug/mL concentration. Overall, I would conclude that this assay is reproducible, consistent, and the most accurate of the assays that I have utilized. In addition, there is a potential benefit to using adult worms – this is the stage of the hookworms that will be exposed to the drug *in vivo*. I also found the results of this assay to be best correlated to the *in vivo* results. However, a limitation of this assay is the availability of the adult worms, and thus I would not suggest using this assay as an initial screening method. I believe, looking at my preliminary data though, that this assay is a good predictor for *in vivo* behavior, and should definitely be utilized before *in vivo*

testing is conducted. In support of this, specifically, C177A looked to be the least potent in this assay (no significant increase), and also was not more active than wild-type Cry5B at all *in vivo*.

For variants G643A, G577A, and S407C, in addition to their individual data points being lower than the wild-type, their initial drop off had the largest gap of the six variants tested here. Additionally, both G577A and S407C were significantly more active than the wild-type *in vivo*. As mentioned, this could indicate that these variants are faster acting, which would be a positive quality to have in a potential therapeutic. I would conclude based on my data that the adult *A. ceylanicum* assay is the most predictive of what is happening *in vivo*.

Three *in vivo* experiments were conducted over the course of this study, with two of them being properly controlled and analyzed. The first one of the properly controlled *in vivo* experiments tested S407C and G643A at two separate doses. While not statistically significant, both variants decreased the worm burden in the hamsters. The second *in vivo* experiment investigated six different variants at a single dose, compared only to the wild-type Cry5B. From this experiment, two variants, S407C and G577A, significantly reduced the worm burden. Once again, G643A lowered the worm burden, but not significantly. Whether this is a reproducible trend or not, it remains unclear, but two variants did lower the worm burden in the hamsters even further than the wild type Cry5B. While we don't know why these variants are behaving in this manner, this is the first set of data that I have that shows that two of these single point variants have some effect *in vivo*.

It has been previously demonstrated in the lab that over 90% of the Cry5B used in treatment never makes it to the small intestines of the hamsters, and that it is degraded by the acidic stomach juices (Hu 2012). Although I have data that several variants reduce the worm burden *in vivo*, I have no data that explains exactly why they are behaving this way. One hypothesis would be that these variants have better protection from the stomach juices, leading to more protein surviving to target the worms. From this hypothesis, this opens up the possibility that some of the other variants from the original screen, that fell into the other categories of toxicity, could potentially have potent effects *in vivo* due purely to a change in protection from degradation.

8.5 Multiple Variant combinations

I investigated into an attempt to find a double mutant with an exponential increase in activity as compared to the wild-type, but overall at this time, there was no success. As discussed in chapter 6, variants were made based off the singles of interest from the original screen, and then 55 doubles were tested against *C. elegans* in the plate assay. From there, 18 variants of interest stood out, and four were tested in an LC-50 assay against *C. elegans*, where it was found that they all had similar toxicity levels compared to the single mutants. This leaves me with a lingering question – how do we find a double mutant with increased activity as compared to the single mutants in Cry5B? This thought was further explored in section 6.7, and in the future directions section below.

8.6 Future directions

There are many immediate future directions that could be taken to learn more about these variants – whether to pursue a mutant with therapeutic potential or understanding Cry5B further. To address the first statement, future pursuit of the double mutants would be the best way to find a mutant with a larger fold increase in toxicity. I believe that the best way to approach this would be from two different sides simultaneously: test more of the 18 doubles that stood out from the plate assay and to also use the structure to make targeted choices of combinations to create and test. Additionally, running these doubles through a telling parasitic system much earlier would be helpful to start to gauge how they are actually acting on the hookworms, as opposed to simply just *C. elegans*.

A clear direction of this project is to investigate into the roles that these amino acids actually play in the structure/function of this protein. I believe that understanding the protein in more depth will ultimately help us to be able to intelligently select amino acids to mutate to find higher acting variants. There are many assays that currently have parameters set-up in the lab that could be used to start to investigate these variants, such as receptor binding, protease processing, solubility, and pore formation. My thoughts, based on the data, would be that solubility and pore formation may be playing key roles in the behavior of some of these variants. The variants could be tested in different pH buffers, at one pH over time, or observing the crystals as they solubilize. With regards to pore formation, changes in voltage can be measured using clamping or voltage sensitive dye, as well observations of propidium iodide. Also in this category would be investigation into the variants that lost all activity – what role are these playing in the protein? Are any of these the site of binding of Cry5B to the receptor? The Cry5B

structure could be utilized to help find variants in domain II that abolish activity, but still form crystals, and could be potential binding sites. Are any of these toxic against any of the parasites (what was missed by screening only against *C. elegans*?)? Why did so many lose toxicity and what can this tell us about the protein? I believe a variety of questions can be asked and hypothesizes can be pursued from this category of variants from the screen. Additionally, to pursue variants that may have a strong *in vivo* effect, it may be worthwhile to run them through an assay in which their sensitivity to simulated gastric fluids (SGF) is tested and compared to the wild-type in pursuit of a variant that is more resistant to the acidic stomach environment of the host. The other steps in the mechanism of action may be valuable to investigate as well with the hyperactive variants, such as pursuing the glycolipid overlay assay to test for changes in binding. Additionally, one could potentially look into applying the insect BBMV technique through dissecting midguts from larger parasitic worms, and using them to look at binding, as well as potentially other processes, such as protease processing and oligomerization.

Additionally, while some of these variants were shown to have potential against *A. ceylanicum*, there is no data for these variants against other parasites of interest. While I have been using *A. ceylanicum* as a parasite system to test, we do know that Cry5B, like other known anthelmintic, affects the different parasitic worms very differently (Hu 2013). Specifically, *Ascaris suum* is very susceptible to Cry5B, but it is unknown if these variants are also hyperactive against this giant roundworm. While *T. muris* are not as susceptible to Cry5B, it would actually be quite worthwhile to find out if any of these 34 variants have a strong effect on this worm, especially since we currently have the least

effective drugs to treat and target this worm. In addition, there are a whole host of veterinary parasites that potentially could benefit from a hyperactive variant.

Another aspect to pursue would be to look at conserved residues between Cry5B, and the other Cry proteins that target nematodes, as well as the whole list of Cry proteins active against insects. There may be some resides that are conserved between Cry proteins, whose since amino acid change in Cry5B lead to an increase in toxicity, that could then be applied and tested in these other Cry proteins to potentially increase their toxicity against their respective host. Figure 8.3 shows an alignment between Cry5B, Cry21A, and Cry1Ac by domain, with the 34 hyperactive residues highlighted red. Cry21A is another crystal protein that has been shown to be active against nematodes (Wei 2003), and from this alignment, ten of the hyperactive variants identified from the screen are conserved here. Cry1Ac is a crystal protein that is active against lepidopteran, and from this alignment, two of the 34 hyperactive variants are conserved here. To pursue this, these variants could be mutated to an alanine in these Cry proteins, and assayed for increased activity against their respective hosts. Additionally, domain swapping could be investigated as a way to increase toxicity, or alter specificity of the protein.

Domain 1

```
Cry5B    -MATINELYPVPYNVLAHPIKEVDDPYSWSNLLKGIQEGWEEWGKTGQKKLFEDHLTIAW 59
Cry21A   -MTNPTILYPSYHNVLAHPIRLDSFFDPFVETFKDLKGAWEEFGKTGYMDPLKQHLQIAW 59
Cry1Ac   MDNNPNINECIPYNCLSNPE------------VEVLGGERIETGYTPID----------- 37

Cry5B    NLYKTGKLDYFALTKASISLIGFIPGAEAAVPFINMFVDFVWPKLFGANTEGKDQ-QLFN 118
Cry21A   DTSQNGTVDYLALTKASISLIGLIPGADAVVPFINMFVDFIFPKLFGRGSQQNAQAQFFE 119
Cry1Ac   ----------ISLSLTQFLLSEFVPGAG----FVLGLVDIIWG-IFGPSQ--------WD 74

Cry5B    AIMDAVNKMVDNKFLSYNLSTLNKTIEGLQGNLGLFQNAIQVAICQGSTPERVNFDQNCT 178
Cry21A   LIIEKVKELVDEDFRNFTLNNLLNYLDGMQTALSHFQNDVQIAICQGEQPG-LMLDQTPT 178
Cry1Ac   AFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEADPTNPA--------- 125

Cry5B    PCNPNQPCKDDLDRVASRFDTANSQFTQHLPEFKNPWSDENS-TQEFKRTSVELTLPMYT 237
Cry21A   ACTPT---TDHLISVRESFKDARTTIETALPHFKNPMLSTNDNTPDFNSDTVLLTLPMYT 235
Cry1Ac   ----------LREEMRIQFNDMNSALTTAIPLFA---------VQNYQVP----LLSVYV 162

Cry5B    TVATLHLLLYEGYIEFMTKWN--FHNEQYLNNLKVELQQLIHSYSETVRTSFLQFLPTLN 295
Cry21A   TGATLNLILHQGYIQFPAERWKSVNYDESFINQTKVDLQRRIQDYSTTVSTTFEKFKPTLN 295
Cry1Ac   QAANLHLSVLRDVSVFGQRWG---FDAATINSRYNDLTRLIGNYTDYAVRWYNTGLERVW 219

Cry5B    NRSKSSVNAYNRYVRNMTVNCLDIAATWPTFDTHNYHQGG-------------------- 335
Cry21A   PSNKESVNKYNRYVRSMTLQSLDIAATWPTLDNVNYPSNV-------------------- 335
Cry1Ac   GPDSRDWVRYNQFRRELTLTVLDIVALFPNYDSRRYPIRTVSQLTREIYTNPVLENFDGS 279
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Domain 2

```
Cry5B    ---KLDLTRIILSDTAGPIEEYTTGDKTSGPEHSNITPNNILDTPSPTYQHSFVSVDSIV 57
Cry21A   -DIQLDQTRLVFSDVAGPWEGNDN-------ITSNIIDVLTPINTGIGFQES-SDLRKFT 51
Cry1Ac   QGIERSIRSPHLMDILNSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNA 60

Cry5B    YSRKELQQLDIATYSTNNS--NNCHPYGLRLSYTDGSRYDYGDNQPDFTTSNNNYCHNSY 115
Cry21A   YPRIELQSMQFHGQYVNSKSVEHCYSDGLKLNYKNKTITAGVSNIDESNQNNKHNYGPVI 111
Cry1Ac   APQQRIVAQLGQGVYRTLSSTLYRRPFNIGINNQQLSVLDG-TEFAYGTSSNLPSAVYRK 119

Cry5B    TAPITLVNARHLYNAKGSLQNVESLVVSTVNGGSGSCICDAWINYLRPPQTSKNESRPDQ 175
Cry21A   NSPITDINVNSQNSQYLDLN------SVMVNGGQKVTGCSPLS----SNGNSNNAALPNQ 161
Cry1Ac   SGTVDSLDEIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFN 179

Cry5B    KINVLYPITETVN----KGTGGNLGVISAYVPMELVPE 209
Cry21A   KINVIYSVQSNDKPEKHADTYRKWGYMSSHIPYDLVPE 199
Cry1Ac   NIIASDSITQIPA------VKGNFLPNGSVISGPG--- 208
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Domain 3

```
Cry5B    NVIGDVNADTKLPLTQLKGFPFEKYGSEYNNRGISLVREWINGNNAVKLSNSQSVGIQIT 60
Cry21A   NVIGDIDPDTKQPSLLLKGFPAEKG---YGDS-IAYVSEPLNGANAVKLTSYQVLQMEVT 56
Cry1Ac   ------FTGGDLVRLNSSGNNIQNRG--YIEVPIHFPSTSTRYRVRVRYASVTPIHLNVN 52

Cry5B    NQTKQKYEIRCR-YASKGD--NNVYFN-VDLSENPFRNS-ISFGSTES-SVVGVQGENGK 114
Cry21A   NQTTQKYRIRIR-YATGGDTAASIWFHIIGPSGNDLTNEGHNFSSVSSRNKMFVQGNNGK 115
Cry1Ac   WGNSSIFSNTVPATATSLDNLQSSDFG-YFESANAFTSSLGNIVGVRN-----FSGTAGV 106

Cry5B    YILKSIT-TVEIPAGSFYVHITNQGSSDLFLDRIEFVPKIQ- 154
Cry21A   YVLNILTDSIELPSGQQTILIQNTNSQDLFLDRIEFISLPS- 156
Cry1Ac   IIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTN 148
```

Figure 8.3: Cry5B alignment with Cry21A and Cry1Ac

Cry5B alignment with Cry21A and Cry1Ac by domain, with the 34 hyperactive variants identified from the original alanine scan highlghted in red.

A final avenue of focus would be to look at the intracellular responses of these mutants as compared to the wild-type Cry5B, such as the p38 MAPK pathway, or the unfolded protein response (UPR), however it is unclear if only a 2-3 fold increase in activity would lead to a detectable difference in these assays if there is a change to observe.

To summarize, there are many different roads and avenue one could pursue with the data generated by this project to continue to find a Cry5B variant that is more active and potentially therapeutic, or to simply understand how Cry5B works and functions. I believe that these two concepts are linked together, and should be pursued in parallel to support each other. This would help to not only gain more information on a very interesting crystal protein, but to also generate a Cry5B variant that could be used to address our ultimate goal of finding a cure for intestinal parasitic worms.

8.7 References

1. Michael Mourez, Ming Yan, D. Borden Lacy, Lisa Dillon, Lori Bentsen, Amy Marpoe, Clemence Maurin, Eileen Hotze, Darran Wigelsworth, Ruth-Anne Pimental, Jimmy D. Ballard, R. John Collier, Rodney K. Tweten, Mapping dominant-negative mutations of anthrax protective antigen by scanning mutagenesis. *PNAS*. **100, 24** (2003).

2. Fan Hui, Ulrike Scheib, Yan Hu, Ralf J. Sommer, Raffi V. Aroian, Partho Ghosh, The structure and glycolipid-binding properties of the nematicidal protein Cry5B. *Biochem*. (2012).

3. Yan Hu, Sophia B. Georghiou, Alan J. Kelleher, Raffi V. Aroian, *Bacillus thuringiensis* Cry5B protein is highly efficacious as a single-dose therapy against an intestinal roundworm infection in mice. *PLOS NTD*. **4:3e614** (2012)

4. Yan Hu, Brian L. Ellis, Ying Y. Yiu, Melanie M. Miller, Joseph F. Urban, Linda Z. Shi, Raffi V. Aroian, An extensive comparison of the effects of anthelmintic classes on diverse nematodes. *PLoS One*. **8-7**. (2013).

5. So Takebe, Shinji Morinaga, Akira Mizuhashi, Tohru Komano, Improved Technique for Refining the Crystal of *Bacillus thuringiensis* by NaBr Gradient Centrifugation. *6th Pacific im Conference on the Biotechnology of Bacillus thuringiensis and its Environment impact*. (2005)

6. Du, Cheng, Martin, Phyllis A. W., and Kenneth W. Nickerson. Comparison of disulfide contents and solubility at alkaline pH of insecticidal and noninsecticidal *Bacillus thuringiensis* protein crystals. *Appl Environ Microbiol*. **60(10):** 3847-3853. (1994).

7. Wei, Jun-Zhi, Hale, Kristina, Carta, Lynn, Platzer, Edward, Wong, Cynthie, Fang, Su-Chiung, Aroian, Raffi V. *Bacillus thuringiensis* crystal proteins that target nematodes. *PNAS*. **100(5)**: 2760-2765. (2003).

Table S1: Full results of the alanine scan

Full results of the alanine scan of the toxin domain of Cry5B. Listed below are the individual amino acids, and the observed result when mutated to an alanine (or cysteine). 0 – loss of activity. + - retained some activity. ++ - like wild-type Cry5B. +++ - hyper-active. ++++ - very hyperactive (red box).

Amino Acid	Result	Amino Acid	Result	Amino Acid	Result	Amino Acid	Result	Amino Acid	Result
W59A	n/a	T109A	0	Q159A	+	P209A	0	F259A	n/a
N60A	+	E110A	0	V160A	+	E210A	0	H260A	n.a
L61A	n/a	G111A	0	A161C	+	F211A	0	N261A	n/a
Y62A	n/a	K112A	0	I162A	+	K212A	0	E262A	n/a
K63A	+++	D113A	0	C163A	n/a	N213A	0	Q263A	n/a
T64A	+++	Q114A	+	Q164A	0	P214A	0	Y264A	n/a
G65A	0	Q115A	0	G165A	n/a	W215A	0	L265A	n/a
K66A	+	L116A	++++	S166A	n/a	S216A	+++	N266A	n/a
L67A	0	F117A	0	T167A	++++	D217A	0	N267A	n/a
D68A	0	N118A	0	P168A	0	E218A	0	L268A	+
Y69A	n/a	A119C	0	E169A	++	N219A	0	K269A	0
F70A	n/a	I120A	0	R170A	n/a	S220A	++	V270A	+
A71C	n/a	M121A	0	V171A	++	T221A	0	E271A	n/a
L72A	+	D122A	0	N172A	n/a	Q222A	+++	L272A	n/a
T73A	+++	A123A	0	F173A	n/a	E223A	0	Q273A	n/a
K74A	++	V124A	n/a	D174A	++++	F224A	0	Q274A	n/a
A75C	0	N125A	0	Q175A	++++	K225A	n/a	L275A	n/a
S76A	n/a	K126A	0	N176A	++++	R226A	n/a	L276A	n/a
I77A	0	M127A	n/a	C177A	++++	T227A	+++	H277A	++++
S78A	0	V128A	0	T178A	++	S228A	0	S278A	0
L79A	n/a	D129A	0	P179A	+	V229A	0	Y279A	n/a
I80A	++	N130A	n/a	C180A	0	E230A	0	S280A	+
G81A	0	K131A	n/a	N181A	+	L231A	0	E281A	0
F82A	n/a	F132A	n/a	P182A	++	T232A	n/a	T282A	0
I83A	0	L133A	0	N183A	+++	L233A	0	V283A	n/a

Table S1: Full results of the alanine scan, Continued

Amino Acid	Result	Amino Acid	Result	Amino Acid	Result	Amino Acid	Result	Amino Acid	Result
R284A	n/a	V309A	+	G334A	n/a	K359A	0	S384A	++++
T285A	0	R310A	0	G335A	0	T360A	++	F385A	0
S286A	0	N311A	0	K336A	n/a	S361A	+++	V386A	0
F287A	0	M312A	0	L337A	0	G362A	++	S387A	0
L288A	0	T313A	n/a	D338A	0	P363A	+	V388A	0
Q289A	n/a	V314A	0	L339A	0	E364A	0	D389A	0
F290A	0	N315A	0	T340A	0	H365A	0	S390A	+
L291A	0	C316A	++++	R341A	0	S366A	0	I391A	0
P292A	0	L317A	0	I342A	0	N367A	0	V392A	n/a
T293A	0	D318A	++	I343A	0	I368A	0	Y393A	n/a
L294A	0	I319A	0	L344A	0	T369A	n/a	S394A	0
N295A	0	A320C	0	S345A	0	P370A	++++	R395A	0
N296A	0	A321C	0	D346A	0	N371A	+++	K396A	0
R297A	0	T322A	0	T347A	0	N372A	+	E397A	n/a
S298A	n/a	W323A	0	A348C	0	I373A	0	L398A	n/a
K299A	0	P324A	0	G349A	0	L374A	0	Q399A	n/a
S300A	n/a	T325A	0	P350A	0	D375A	++	Q400A	n/a
S301A	0	F326A	0	I351A	0	T376A	+++	L401A	n/a
V302A	0	D327A	n/a	E352A	0	P377A	0	D402A	0
N303A	++	T328A	+++	E353A	++++	S378A	++	I403A	n/a
A304C	0	H329A	0	Y354A	0	P379A	0	A404C	n/a
Y305A	++	N330A	0	T355A	0	T380A	+++	T405A	n/a
N306A	0	Y331A	0	T356A	0	Y381A	++++	Y406A	++
R307A	n/a	H332A	++++	G357A	0	Q382A	++++	S407A	++++
Y308A	0	Q333A	0	D358A	0	H383A	++	T408A	n/a

Table S1: Full results of the alanine scan, Continued

Amino Acid	Result	Amino Acid	Result	Amino Acid	Result	Amino Acid	Result	Amino Acid	Result
N409A	+++	N434A	+++	A459A	n/a	S484A	++	D509A	0
N410A	n/a	Q435A	++++	R460A	0	G485A	+++	Q510A	n/a
S411A	+	P436A	0	H461A	0	S486A	n/a	K511A	n/a
N412A	0	D437A	n/a	L462A	0	C487A	0	I512A	0
N413A	0	F438A	0	Y463A	0	I488A	+	N513A	0
C414A	0	T439A	0	N464A	0	C489A	n/a	V514A	0
H415A	0	T440A	++	A465C	0	D490A	0	L515A	0
P416A	0	S441A	n/a	K466A	n/a	A491C	+++	Y516A	n/a
Y417A	0	N442A	n/a	G467A	0	W492A	0	P517A	+++
G418A	0	N443A	n/a	S468A	+++	I493A	n/a	I518A	n/a
L419A	0	N444A	0	L469A	0	N494A	n/a	T519A	n/a
R420A	0	Y445A	++++	Q470A	0	Y495A	0	E520A	0
L421A	0	C446A	+++	N471A	0	L496A	n/a	T521A	0
S422A	0	H447A	+	V472A	0	R497A	0	V522A	0
Y423A	n/a	N448A	0	E473A	++	P498A	+	N523A	0
T424A	0	S449A	0	S474A	++	P499A	++++	K524A	0
D425A	0	Y450A	0	L475A	n/a	Q500A	++	G525A	0
G426A	0	T451A	++	V476A	0	T501A	0	T526A	0
S427A	0	A452A	0	V477A	0	S502A	0	G527A	++
R428A	n/a	P453A	0	S478A	0	K503A	+++	G538A	+
Y429A	n/a	I454A	0	T479A	0	N504A	0	N529A	0
D430A	+++	T455A	+	V480A	0	E505A	++	L530A	0
Y431A	n/a	L456A	0	N481A	+	S506A	++++	G531A	0
G432A	0	V457A	0	G482A	++	R507A	0	V532A	0
D433A	0	N458A	0	G483A	n/a	P508A	n/a	I533A	0

Table S1: Full results of the alanine scan, Continued

Amino Acid	Result	Amino Acid	Result	Amino Acid	Result	Amino Acid	Result	Amino Acid	Result
S534A	++++	T559A	++	W584A	0	Q609A	0	N634A	n/a
A534C	0	Q560A	0	I585A	0	K610A	0	P635A	n/a
Y536A	0	L561A	+	N586A	0	Y611A	0	F636A	n/a
V537A	0	K562A	0	G587A	+	E612A	++	R637A	+++
P538A	+	G563A	0	N588A	++	I613A	0	N638A	++++
M539A	0	F564A	0	N589A	0	R614A	0	S639A	+++
E540A	0	P565A	0	A590C	++	C615A	+++	I640A	++++
L541A	0	F566A	0	V591A	0	R616A	0	S641A	+++
V542A	0	E567A	+	K592A	++	Y617A	0	F642A	n/a
P543A	0	K568A	0	L593A	0	A618C	+++	G643A	++++
E544A	0	Y569A	0	S594A	++	S619A	++	S644A	+++
N545A	0	G570A	+	N595A	+	K620A	++	T645A	+++
V546A	+	S571A	+	S596A	+	G621A	+	E646A	+++
I547A	0	E572A	++	Q597A	+	D622A	++	S647A	+++
G548A	0	Y573A	0	S598A	++	N623A	+	S648A	+++
D549A	0	N574A	+	V599A	+	N624A	0	V649A	+++
V550A	0	N575A	++	G600A	0	V625A	+	V650A	+++
N551A	n/a	R576A	+++	I601A	n/a	Y626A	0	G651A	++
A552C	+	G577A	++++	Q602A	0	F627A	n/a	V652A	0
D553A	++++	I578A	++	I603A	0	N628A	n/a	Q653A	0
T554A	0	S579A	+++	T604A	0	V629A	+++	G654A	0
K555A	+	L580A	0	N605A	0	D630A	+	E655A	0
L556A	0	V581A	+++	Q606A	+	L631A	0	N656A	++++
P557A	+	R582A	0	T607A	0	S632A	n/a	G657A	0
L558A	+	E583A	0	K608A	+	E633A	+++	K658A	+

Table S1: Full results of the alanine scan, Continued

Amino Acid	Result	Amino Acid	Result
Y659A	0	S684A	++
I660A	++++	D685A	+
L661A	0	L686A	+
K662A	+	F687A	n/a
S663A	n/a	L688A	+
I664A	++	D689A	0
T665A	0	R690A	0
T666A	++	I691A	0
V667A	+++	E692A	0
E668A	+++	F693A	+
I669A	n/a	V694A	++++
P670A	n/a	P695A	0
A671C	+	K696A	+++
G672A	0	I697A	0
S673A	0	A698A	0
F674A	++		
Y675A	0		
V676A	++		
H677A	0		
I678A	++++		
T679A	++		
N680A	++		
Q681A	++		
G682A	++		
S683A	++		