Screening *Bacillus* spp. for Crystalline Delta-Endotoxins

Haig John Kassabian

Writer's Comment: Last summer I interned at the Lawrence Berkeley National Lab on a fellowship from the Department of Energy. My mentor, Dr. Tamas Torok, guided me throughout the ten-week program and taught me much about bioinsecticides. In UWP 104E (Science Writing), Brad Henderson gave me the perfect opportunity to share the results of my research in a scientific paper. In addition to sharing my results, I wanted to inform the reader about the role of bioinsecticides in agriculture and possible future studies related to it. Writing the paper was very challenging; however, Mr. Henderson's guidance and advice during office hours proved to be great help. I thank Mr. Henderson for giving our class a solid foundation for communicating science. Lastly, I dedicate this paper to my Great Uncle, Dr. John E. Amoore, whose footsteps I follow today.

—Haig John Kassabian

Instructor's Comment: Writing a winning primary research article (also known as an IMRAD paper: Introduction, Methods, Results, and Discussion) is both a daunting and peculiar challenge, and Haig met with and conquered this challenge nobly, developing an original scientific hypothesis, laying out his methods clearly, discovering insights in his test data, and ultimately connecting those observations to his initial premise and prediction. The result is a paper that presents significant new knowledge on natural bioinsecticides as an excellent and "green" alternative to artificial toxins. Mr. Kassabian's writing—section-by-section, and sentence-by-sentence—is impeccable. As methodical and precise in his writing as in his research, Haig avoided both errors in logic and garbled sentences. And I know first-hand how earnestly Haig labored to produce such a polished and finessed paper.

—Brad Henderson, University Writing Program

Abstract

Members of the bacterial genus *Bacillus* are microorganisms that, when deprived of nutrients, have the capability to develop endospores and concurrently produce delta-endotoxins. The crystalline delta-endotoxins are toxic to insects but not to humans because insects have an enzyme that breaks down the crystalline structure and releases the toxic protein. The high insect specificity of the toxins makes the producing bacilli good candidates for use as natural bioinsecticides. In this study, extremophilic *Bacillus* spp. were screened for the production of crystalline delta-endotoxins. The strains were grown in 96-well plates containing TB medium for one day and stressed in C2 medium for six days. We used high-throughput screening to detect crystalline toxins. Crystal-positive strains were grown in larger volume for further screening of the novel compounds. So far, 30.5% of the 583 strains screened showed positive crystalline toxin production. The two most prevalent toxin-producing species were *B. subtilis* and *B. megaterium*. The area where the highest numbers of positive crystal-producing bacilli were collected was Solovetskie Island, Russia, with 30.3% of the 178 strains screened. Continuing work will concentrate on screening more toxin-producing strains, sequencing the novel proteins, and detecting their new insect specificities that will benefit crop protection.

Introduction

The use of chemical-based insecticides for agriculture has had a tremendous impact on the ecosystem. Due to synthetic insecticide use, 37% of lakes and rivers in the United States are too polluted for basic activities such as swimming and fishing (Engelking, P., 2005). Chemical insecticides benefit crop growth by killing insects that are harmful to plants; however, negative effects, such as bioaccumulation and water contamination, far outweigh their usefulness. One of the best known synthetic insecticides to cause extensive environmental damage is DDT. In 1972 DDT was banned by the EPA, and agriculture would have to look elsewhere to protect their crops from insects while also preserving the environment.

Microorganisms and their natural products promise to be a safe alternative to chemical insecticides. One particular rod-shaped bacterium, called *Bacillus thuringiensis*, can form spores when placed in stressful environments. Along with the spores, a crystalline protein is sometimes produced that is toxic only to specific insects but not to humans. The toxin is called delta-endotoxin and is

lethal to many insects such as butterflies, beetles, and grasshoppers (Schneph, E. et al., 1998). Insects possess an enzyme that breaks down the crystalline structure, releasing the toxin inside their gut (Schneph, E. et al., 1998). Higher level organisms, such as mammals, do not have the same enzyme to break down the crystalline structure, thus no toxin is released. The toxin is ultimately safe to both humans and the environment.

Microorganisms that survive harsh extremes are commonly referred to as extremophiles. These organisms thrive at extreme levels of pH, temperature, and pressure, due in part to unique biocatalysts they express. In this study, strains of extremophilic *Bacillus* spp. collected earlier was screened for novel compounds that may be important to agriculture. The increased possibility of finding novel natural products expressed by microorganisms found in harsh environments is the reason we are exploring extremophilic bacilli. We predict that if we stress other species of bacilli, they too will produce the crystalline delta-endotoxin. If a compound is found specific to an insect, it can soon become another alternative to harmful chemical-based insecticides.

Materials and Methods

Retrieval and revival of Bacillus *spp.*

Strains of *Bacillus* spp. were isolated from samples collected from extreme environments, predominantly from regions in Russia. Pure cultures of the strains were inoculated on nutrient agar (Difco), placed into microcentrifuge tubes, and sent to the Berkeley Lab, where they were stored in a refrigerator at 4°C. Each tube was labeled with an accession number that identified its strain and collection and isolation information. *Bacillus* strains were revived by streaking them on nutrient agar (Difco) with a sterile loop and incubating them at 30°C for 24 hours. Strains that showed contamination or did not grow were re-streaked. For strains requiring re-streaking, 500 µl of nutrient broth was added to the microcentrifuge tube (1.5 ml eppendorf) and incubated for 24 hours at 30°C. Then, the strains were re-streaked and incubated just as before.

Inducing sporulation and crystalline delta-endotoxin formation

First, we added 200 µl of TB medium (12 g tryptone, 24 g yeast extract, and 4 ml of glycerol added to 900 ml of deionized water, autoclaved for 20 minutes, and buffered with 100 ml of sterile 0.17 M KH2PO4 and 0.72 M K2HPO4) into each well of a 96-well plate with an Oxford Multi-12 channel pipettor. One colony of each individual strain was picked by a sterile toothpick and mixed into separate wells. The plate was incubated at 30°C for 24 hours. Then, 10 µl of the TB culture were transferred to a new 96-well plate containing 200 µl of C2 medium (4.66 g K2HPO4, 3.11 g KH2PO4, 2 g yeast extract, 5 g casamino acid, 2 g peptone, 25 ml of 40% glucose, 100 ml of 10 X MgCl2 · 6H2O, and 100 ml of 10 X stock of salts for C2 medium added to 750 ml of deionized water, mixed, and filter sterilized). Strains in the C2 medium were incubated at 30°C for 6 days. On the sixth day, the strains were screened for crystalline delta-endotoxin formation using a Zeiss Axioskop light microscope in phase contrast mode. The strains were viewed at 1000 X total magnification with oil immersion. Digital images were taken of strains containing crystals by a mounted digital camera (Optronics MacroFire) connected to a computer. The computer uses a visualization program called PictureFrame to display and store the pictures taken.

Cultivation of crystal-forming Bacillus *spp. for novel compound screening*

Once strains of *Bacillus* spp. forming crystals were identified, the microorganism was cultivated in larger volume. A colony of each strain was put into a 14-ml Falcon tube containing 1 ml of TB medium. The culture was placed into a 30°C incubating shaker for 24 hours. Two hundred and fifty microliters of the culture was used to inoculate a 125-ml baffled Erlenmeyer flask containing 25 ml of C2 medium. The flask was incubated in the same shaker and temperature for 6 days. On the sixth day, the strains were screened for crystal production using the same microscope. Crystal-forming strains were centrifuged for 20 minutes in a Beckman Coulter centrifuge model Avanti J-25 using a JA-17 rotor at 20,000 RCF (x g) and 4°C. The supernatant was poured out and 25 ml of sterile deionized water was added to the tube. The pellet was re-suspended

in the water via a Vortex-Genie 2 and centrifuged once more with the same settings mentioned above. The supernatant was poured out and the pellet stored at -20°C until further screening of the novel compounds.

Results

Figure 1 displays a relative distribution of crystal-producing *Bacillus* strains found in various regions of Russia. Of the strains screened, the largest number (44%) belonged to *B. subtilis.* Seven of the eleven sampled regions had *B. subtilis* as the major producer of crystalline delta-endotoxins. *B. thuringiensis* was the lowest producer of crystals with 0.56% of the 178 strains. In Figure 2, the percent distribution shows that the largest number of crystal-producing *Bacillus* spp. (30.3%) were collected on Solovetskie Island. Hakasia and Shoria were the regions with the lowest numbers of crystal-producing bacilli with 2% and 1%, respectively. Finally, Figure 3 is a picture of bacilli with an endospore and a crystalline endotoxin.

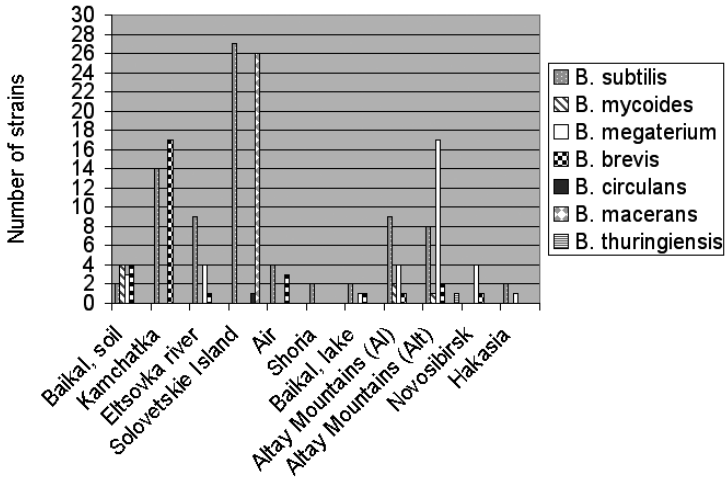

Figure 1. Comparing the types and number of crystal-producing strains found to date in different regions of Russia.

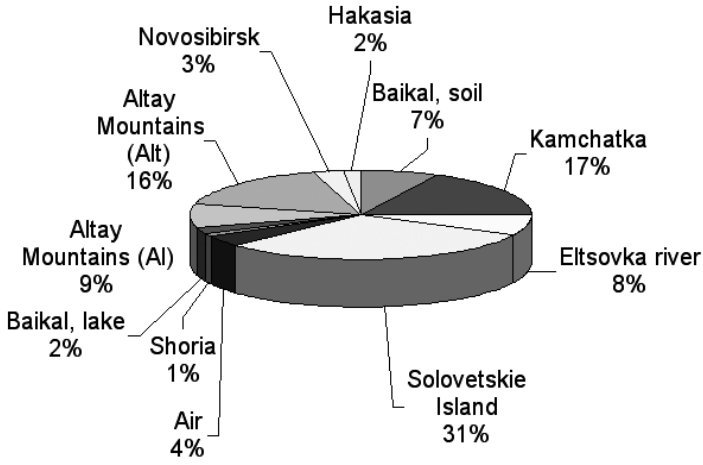

Figure 2. Number of toxin-forming strains found in different regions of Russia of the screened 178 strains to date.

Discussion and Conclusion

Eco-physiological differences among the sample collection regions may be due to the fact that more crystal-producing strains were found on Solovetskie Island than some of the other places. The island is not secluded by any means but is less inhabited compared to other regions.

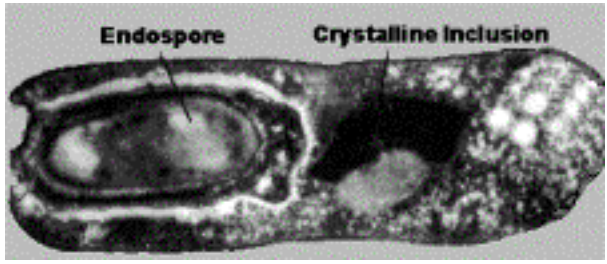

Figure 3. A stressed bacilli with an endospore and crystalline endotoxin.

B. thuringiensis is a well known producer of crystals, but in the experiment only one strain was available for screening. The number of toxin-producing *B. subtilis* strains tested was substantial. This suggests that it could be applied as a bioinsecticide just like *B. thuringiensis*. Furthermore, *B. subtilis*–derived natural product coding sequences

could also be used in the future for insect-resistant transgenic plants, considering that this species is the most extensively studied among all bacilli (Pimentel, D., 1991). Unfortunately, insects have the capability to evolve and adapt to the toxin. The Aronian lab, at the University of California, San Diego, is studying the mode of action the toxin takes in the insect's gut (Aronian, R.V., 2003). By studying how the protein works, the group hopes to find ways of inhibiting the insect's ability of adapting to the toxin.

Our study has shown that many *Bacillus* spp. are capable of producing crystalline δ-endotoxins just as we hypothesized; however, many more tests must be performed to see if the crystals we have harvested can be viable bioinsecticides in the future. Some of the tests performed will be assays on insects to determine if the crystals have any toxic effects on them. If toxicity is observed then we must sequence the amino acids of the protein inside the crystal. A three-dimensional model can also be determined via X-ray crystallography, which will give us insight as to how these novel proteins may function.

Many of the produced compounds may be novel, thus this high-throughput screening is important. Microbial-based natural products may reduce crop losses caused by insects. Currently, the amount of lost crops due to insect damage globally is projected to be 35% (Pimentel, D., 1991). This figure needs to be reduced to fulfill the growing worldwide demand for food. Natural-based insecticides can be one of the best ways to increase yields in agriculture and simultaneously reduce water and runoff pollution to the environment. In conclusion, rich and poor nations alike can use natural products as effective tools for helping people attain a healthier life.

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