Characterization of the Insecticidal Activity of *Chromobacterium subtsugae*

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ABSTRACT *Chromobacterium subtsugae* PRAA4-1 produces toxins orally active to Colorado potato beetle larvae and other pest insects. These toxins have proved difficult to characterize. In liquid medium, principal component analysis suggested that the best correlation of toxicity to Colorado potato beetle was to optical density (r = 0.78). Toxicity to Colorado potato beetle was not correlated to components of optical density such as viable cell number and cell mass, nor to protein concentration, pH, and violacein pigment production. Isolated violacein pigment was not toxic to beetle larvae. In a reduced-peptone liquid medium, non-pigmented colonies appeared on the titer plates after 11 serial transfers. Most colonies were unstable when transferred to peptone plates. One stable non-pigmented mutant, PRAA4-1B was not toxic to Colorado potato beetle larvae. Using phenotypic microarrays, it was observed that PRAA4-1B grew 15% faster than the parent strain, and differed in its use of many nitrogen and phosphate sources, suggesting a regulatory mutation. The antibiotic resistance of the mutant was identical to the parent strain, except this mutant was also more resistant to polymixin B, suggesting a membrane location for the toxin. The toxin could also be extracted from non-viable cells by detergents and was stable upon autoclaving, suggesting that it is not a protein.

KEY WORDS : Colorado potato beetle, insect biocontrol, phenotypic microarrays, violacein

INTRODUCTION

We have isolated a violet bacterium, *Chromobacterium subtsugae* Martin *et al*. (Martin *et al*., 2007a), from soil. This bacterium is toxic to Colorado potato beetle larvae (Martin *et al.*, 2004) as well as other pest insects (southern corn rootworm, diamondback moth, Martin *et al.*, 2007b). *C. subtsugae* produces a toxin, or toxins, that kill insect larvae independent of bacterial viability. The nature of the toxin or toxins is still unknown.

Loss-of-function mutants can be helpful in determining gene function. However, such spontaneous mutants are often point mutations, which are difficult to determine by molecular methods if the gene in question is not known (Black, 1996). Phenotypic microarrays are one tool that can help characterize events that lead to loss-of-function mutations, especially when multiple phenotypes are affected (MacLean *et al.*, 2004). These arrays use over 1900 independent substrates to characterize differences between strains. Because strains for phenotypic analysis are grown under identical conditions, differences in substrate utilization are caused by strain differences and not adaptation to media. These patterns of utilization, therefore, tend to be reliable and repeatable.

The most commonly observed mutants lost the ability to produce pigment. These white mutants also

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were not toxic to Colorado potato beetle and grew faster than the pigmented parent strain, thus indicating multiple phenotypic changes. The production of other insecticidal toxins, such as the crystal toxin of *Bacillus thuringiensis* Berliner and the tca proteins of *Photorhabdus luminescens* Thomas and Poinar, is often regulated along with other proteins, such as proteases and lipases, thought to have a role in insect toxicity (Schnepf *et al.*, 1998, Forst and Nealson, 1996). *Chromobacterium violaceum* Bergonzini is known to regulate a number of genes, including those involved in pigment synthesis, by cell density or quorum sensing (Chernin *et al.*, 1998).

The growth medium can make a difference in both the quality and quantity of toxin production (Mummigatti and Raghunathan, 1990) for other insect pathogens. During the testing of various media for increased toxin production, it was observed that on solid media, pigment production and toxicity were correlated (Martin *et al.*, 2004); but in liquid media, pigment production was not always indicative of toxicity. The violet pigment in *C. violaceum*, termed violacein (Duran *et al*., 1983), has a UV absorbance spectrum similar to that of the pigment produced by *C. subtsugae* (Martin *et al.*, 2004).

Characterizing the toxin or toxins of any potential insect pathogen is crucial if it is to be useful for insect control. Most bacteria associated with biological control of insects produce protein toxins, but initial observations indicate that the *C. subtsugae* toxins are heat stable and resistant to proteases (Martin *et al*., 2004). Knowing that other heat stable insecticidal toxins such as the *B. thuringiensis* β-exotoxin are not proteins (Sebesta *et al.*, 1981), we began to investigate other possibilities for the nature of the toxin.

MATERIALS AND METHODS

Bacteria and Media

Chromobacterium subtsugae strain PRAA4-1 was isolated from soil (Martin *et al.*, 2007a). PRAA4-1B was a non-pigmented spontaneous mutant derived from PRAA4-1 during growth on a reduced nutrient medium.

Bacteria were grown in L-broth (Atlas, 2004) or on L-agar at 25°C. Mutants were produced on RM (media which contained half the nutrients of L). Bacteria were harvested from plates into 15 ml of sterile distilled water. Cells were mixed into a suspension and used directly for insect bioassays.

For small scale liquid cultures, cells were grown in various media in 50 ml of broth in a 250 ml flask and shaken at 150 rpm for 48 h at 25°C. Cells were used directly or diluted in sterile water for insect bioassays. To obtain larger quantities of toxin for testing, cells were grown in 500 ml broth in 2 liter baffled flasks and shaken at 150 rpm at 25°C. Samples of the culture were removed at 24 h intervals and used in insect bioassays against *Leptinotarsa decemlineata* (Say) as described below to determine optimal conditions for toxicity. This experiment was done in triplicate and repeated twice to determine time to optimal toxin production. Cell mass, viable cell numbers, pH, optical density at 600 nm and protein concentrations were measured at each sample interval.

To test for heat stability, whole cultures were autoclaved for 10 min and tested for toxicity as described below.

Insects and Bioassays

Colorado potato beetle adults were fed potato foliage. The eggs were laid on potato foliage, hatched on IBL (Insect Biocontrol Laboratory) potato leaf diet (Gelman *et al.*, 2001), reared to 2nd instar in the dark (so that the larvae would feed on the diet), and then in a 16:8 L:D cycle with 46% RH at 24°C. For bioassays, the IBL diet was used as re-hydrated freeze-dried pellets (Martin, 2004).

Thirty-two diet pellets were used for each treatment in bioassays. Each diet pellet was placed in a well (1.5 cm) in white plastic trays (C-D International, Ocean City, NJ, USA) and re-hydrated with 0.3 ml of liquid using either water for controls or water containing dilutions of a bacterial suspension. This liquid was allowed to soak into the pellet before adding a single Colorado potato beetle 2nd instar larva to each pellet. Wells were sealed with film, and holes were made in the film with insect pins to permit air exchange. Insects were kept at 24°C at 46% RH on a

16:8 L:D cycle and mortality recorded at 24h intervals until 144 h had elapsed.

Mutant

Spontaneous non-pigmented mutants appeared after 11 passages on a reduced nutrient medium (RM). These non-pigmented colonies were streaked for isolation at least 5 times consecutively on L-agar to assess their potential to revert to a pigmented culture. To confirm that the non-pigmented mutant PRAA4-1B was derived from the parent strain, fatty acid analysis was performed with the Sherlock Microbial Identification System (MIDI, Inc., Newark, DE). Bacteria were grown and processed according to the standard MIDI method (MIDI Inc., 2002). Fatty acids were identified by gas chromatography using the TSBA40 method. The fatty acid profile of the mutant PRAA4-1B was compared to that of the parent PRAA4-1.

Extraction of violacein

Twenty-five ml of a deep violet *C. subtsugae* PRAA4-1 culture was extracted with an equal volume of ethyl acetate. The aqueous phase was discarded and the organic phase was evaporated under a stream of helium, and the extract was re-dissolved in 2 ml methanol and brought to the original volume with sterile distilled water. The ultraviolet/visible spectrum of the extract matched that of violacein. This solution was applied to diet pellets. Controls with the same amount of methanol were used in the assay against 2nd instar Colorado potato beetle larvae.

Phenotypic microarrays

Comparison of the parent PRAA4-1 and the mutant PRAA 4-1B were also done by phenotypic microarrays. Both were grown on the same medium for inoculation and then the growth of these two strains were compared on 1900 different substrates including: carbon, nitrogen, sulfur, phosphate, and peptide nitrogen sources; supplemental nutrients; osmolytes; pH; antibiotics; and heavy metals (Biolog, Hayward CA, USA).

Electron Microscopy

Five day old cultures of *C. subtsugae* grown on L-agar at 25°C were frozen and freeze fractured according to Steere *et al*. (1980) to observe biofilm formation.

Separation and Extraction of Toxin

As a first step in the purification of the toxin or toxins, cells grown on L for 48 h were separated from the supernatant by centrifugation (10,000 x g for 15 min) and the supernatant was filtered through a low protein binding 0.45µm PVDF filter (Millipore, Bedford MA USA). The pellet was then extracted with the same volume of water or 1% v/v Triton X 100 (other detergents caused significant mortality to Colorado potato beetle larvae) and shaken for 30 min on a vortex mixer at room temperature. The separation into particulate and soluble fractions was repeated and fractions were tested against Colorado potato beetle larvae.

The toxin in the supernatant was also separated by a series of molecular weight filters (Whatman Inc., Florham Park NJ USA) starting with the exclusion of material with molecular weights of greater than 300kD. Both the filtrate and retentate were tested against Colorado potato beetle larvae.

Statistical analysis

LC_{50}s (concentration needed to kill 50% of the larvae) were determined using the PROBIT procedure from SAS (SAS Institute, 2004). Previous assays indicated that viable cell number was not directly related to toxicity, so we measured a number of parameters including titer, optical density at 600 nm, protein concentration, wet weight, and pH to determine correlation to toxicity using Principal Component Analysis (SAS Institute, 2004).

RESULTS

Toxicity in Liquid Cultures

To obtain additional material for characterization of the toxin or toxins that are made by PRAA4-1, we performed an experiment growing 500 ml cultures in 2 liter shake flasks. Multiple parameters were measured to determine those that could be correlated with mortality. Using principal component analysis, it was found that the best correlation with mortality

was optical density at 600 nm over all times (r = 0.7774). The LC_{50}, as measured by dilution, decreased with increasing optical density (regression analysis n = 14, r = 0.81, Fig. 1A). The age of the culture at harvest was also correlated with mortality (r = 0.6278). However, viable cell titer, which is a component of optical density, was more poorly correlated with mortality and the correlation was negative (r = -0.3890), that is, as the culture aged the viable cell number declined while the toxicity to Colorado potato beetle larvae increased (regression analysis n = 13, r = 0.45, Fig. 1 B). Other components of optical density, wet weight (Fig. 1C) and whole culture protein concentration (Fig. 1 D), were also poorly correlated with toxicity. *C. subtsugae* produces a distinctive biofilm which also contributes to the optical density (Fig. 2).

When optical density (600 nm) was used as a measure of dose, then the LC_{50} of liquid cultures at day 2 (1.27 ± 0.41) declined to 0.74 (± 0.012) at day 6, but was not significantly different. For this reason all bioassays were performed using cultures ranging in age from 2–6 days.

Previously we have observed that microbial adaptation to liquid culture can result in a decreased ability to kill target insects, therefore we tested twenty successive 500 ml serial passes for toxicity in liquid media, L and RM (Table 1). For RM broth, the mortality was always less than L-broth, regardless of cell titer. For example, in passage 8 where the titer for both media was greater than 8.8×10^9 colony forming units/ml, the mortality for Colorado potato beetle larvae for undiluted RM broth was about half of the mortality on L broth. Although the violet pigment was usually produced in both media, the amount of pigment, as determined by the overall intensity of the color, was not correlated with toxicity. The white culture of pass 17 (L) and the violet culture of pass 19 (L) had 93.6% and 100% mortality, respectively, suggesting that the pathways for toxicity and pigment production are not the same. The toxicity to Colorado potato beetle did not decrease with serial passage in L broth.

From these successive liquid subcultures 10 ml samples were taken and autoclaved. The toxicity of samples of whole cultures to Colorado potato beetle larvae that were harvested and autoclaved immediately did not decrease compared to samples of the same cultures that were not autoclaved.

Mutants and Toxicity

During pass 11 in RM broth white colonies spontaneously appeared after titering. Initially, these white colonies made up only 10–20% of the colonies recovered. However, by the 20th pass 85% of the colonies on a given plate were white. Several of these white colonies were sub-cultured onto L-agar. Most (8 out of 10) non-pigmented mutants reverted to violet with a single subculture. One mutant, PRAA4-1B that remained white on subsequent subculture was shown by fatty acid analysis to be identical to the violet parent strain. When 2nd instar Colorado potato beetle larvae were fed freeze-dried diet containing this mutant, mortality did not increase above the control mortality (< 6%).

Because the non-pigmented mutant was not toxic to beetle larvae, we tested an enriched violacein fraction for toxicity. An organic extract containing violacein was not toxic to Colorado potato beetle larvae. At 96 h only two of 32 larvae died (6.3%) when fed the enriched violacein fraction, whereas control mortality was 3.1%. The original culture had 100% mortality for larvae over the same time period.

Phenotypic Microarrays

The spontaneous non-pigmented mutant PRAA4-1B was compared to the parent strain using a phenotypic microarray. This comparison of approximately 1900 tests compared the growth of this loss-of-function mutant to the parent strain. Differences in substrate utilization and antibiotic sensitivity were expected to give clues as to the defect in the mutant strain. The mutant strain grew about 15% faster in the control microtiter plate wells than the parent strain. Compensating for differences in growth rate, the parent and mutant were identical in 91.5 % (1739 of 1900) of their tests including most carbon sources, nutrient supplements, osmolytes and pH. However, even compensating for the faster growth rate, the mutant grew to a higher optical density than the parent on a variety of nitrogen sources including L-histidine, L-leu-

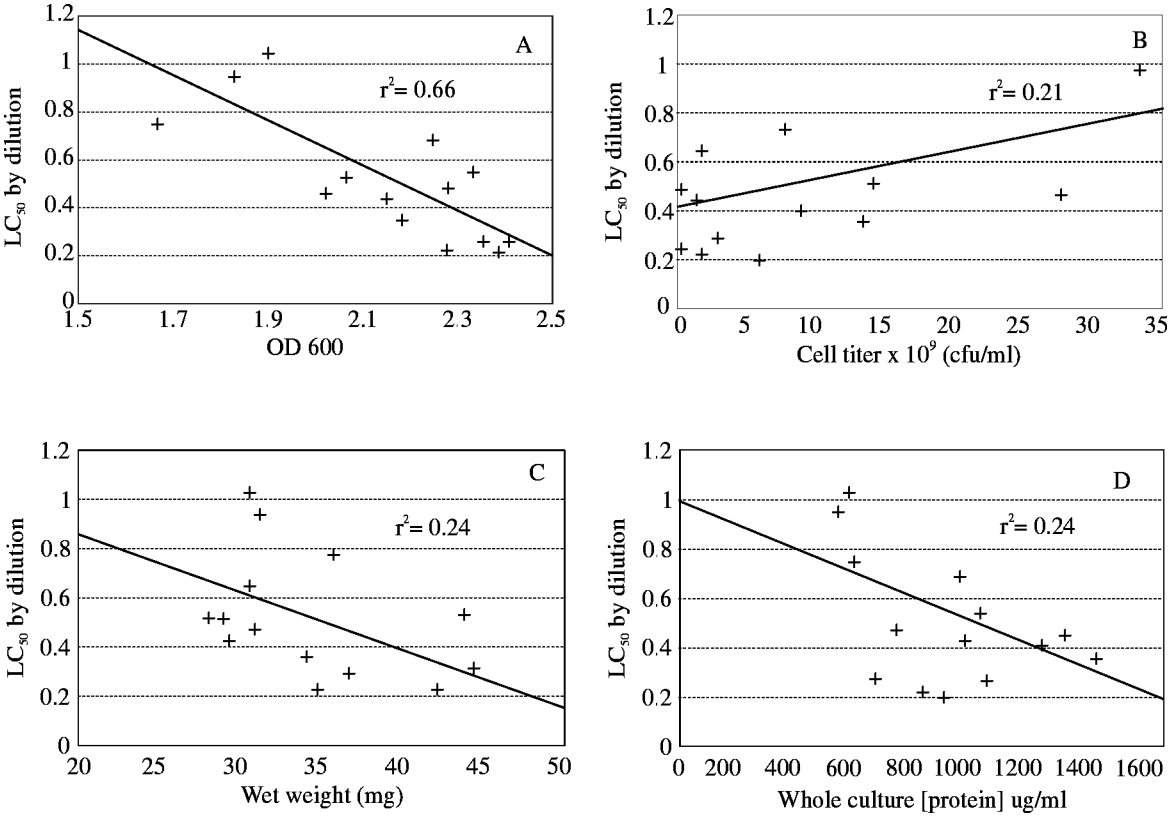

Fig. 1. (A) Correlation between optical density and LC_{50} based on dilution of whole culture. (B) Correlation between cell number and LC_{50} based on dilution of whole culture. (C) Correlation between wet weight and LC_{50} based on dilution of whole culture. (D) Correlation between protein concentration and LC_{50} based on dilution of whole culture.

cine, L-lysine, L-proline, L-threonine, D- and L-valine, L-citrulline and L-ornithine. PRAA4-1B also showed improved growth on the 3'5' cyclic monophosphates of adenosine, guanosine, cytidine, uridine, and thymidine as phosphate sources. While the non-pigmented mutant grew better on a variety of peptide-nitrogen sources, the only peptide-nitrogen source that increased growth of the parent strain was di-tryptophan. The two strains were identical in their response to a variety of antibiotics and inhibitors with the exception that the mutant was more resistant to polymixin B and less resistant to the protease inhibitor phenyl-methyl sulfonyl-fluoride (PMSF). The mutant also used Tween 20, Tween 40, and Tween 80 as carbon sources more efficiently than the parent strain.

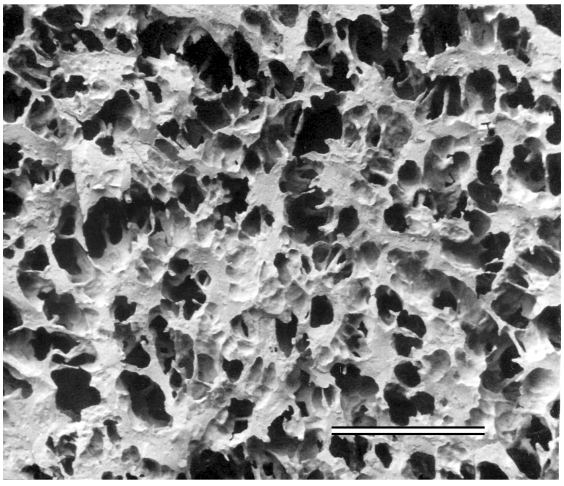

Fig. 2. Freeze fracture of biofilm produced by *Chromobacterium subtsugae* after 5 days. Bar is 150 µm.

Toxin Extraction

When 5 d cultures grown on solid media were harvested and immediately centrifuged, the supernatant on average was only half as toxic as the whole culture. When allowed to sit for 20+ days both the supernatant and the pellet, when fed individually to Colorado potato beetle larvae, caused 100% mortality within 120 h.

When the pellet from a 5 d culture was harvested and extracted with Triton X 100, the supernatant from the pellet became violet, unlike the supernatant from a water-extracted control which only had a faint violet color. The toxicity of this Triton-extracted supernatant was equivalent to the whole culture or the re-suspended pellet. The supernatant (light violet) of the second Triton-extraction also gave 100% mortality. The supernatant (clear) of the third Triton-extraction gave only 37% mortality

Upon ultrafiltration of a 5 d culture, the mortality of Colorado potato beetle larvae to the greater than 300 kD fraction was 93.8% when diluted 1:5 from the original volume. The mortality of Colorado potato beetle larvae to the greater than 100 kD fraction but less than 300 kD was 80% when reconstituted to its original volume when assays were performed immediately after harvest. In undiluted fractions less than 30 kD the mortality was 18%.

DISCUSSION

Because toxicity of *C. subtsugae* was not correlated with cell number (Martin *et al*., 2004), we conducted a replicated experiment that measured multiple parameters. The only parameter measured that was correlated with $LC_{50,}$ as measured by dilution, was optical density at 600 nm. Optical density consists of cells, whether viable or not, and biofilm.

The toxicity of the parent strain PRAA4-1 remained consistently high on continuous passage in liquid media (20 passes over 80 days) confirming that toxicity is stable over time . However, when the strain was subcultured in a reduced nutrient liquid medium, overall toxicity decreased compared to full strength medium (Table 1). Our experience with *C. subtsugae* has shown that changes in growth conditions can lead to a temporary reduction in toxin production. Adaptation to media and environmental conditions appear to stabilize toxin production as measured by mortality.

Attempts to adapt PRAA4-1 to lower nutrient conditions favored the growth of non-pigmented mutants. Because these mutants grew faster than the parent strain under these conditions, they soon became 85% of the culture. This faster growth may be attributed to the mutant directing more energy to growth rather than toward the production of the non-essential pigment or the toxin of the parent strain. Most of these mutants were unstable and quickly reverted to produce violet pigment. One mutant remained non-pigmented and was also non-toxic. These simultaneous changes from the parent strain suggested a connection or a common pathway for toxin and pigment production.

The differential growth data from the phenotypic microarrays support the possibility that the differences between PRAA4-1 and PRAA4-1B are the result of a regulatory mutation, perhaps in ni-

Table 1. Mortality of Colorado potato beetle larvae after feeding on PRAA4-1 grown in L and RM broth

Pass Number	Medium					
	L			RM		
	Titer[1]	Color[2]	% Mortality	Titer	Color	% Mortality
1	7.3	Violet	31.3	3.7	Violet	12.5
4	3.8	Lt. violet	40.6	3.2	White	25.8
8	9.6	Tan	90.6	8.9	Violet	50.0
14	8.5	Violet	21.9	0.005	Violet	18.8
17	12.3	White	93.6	7.0	Tan	37.1
19	6.2	Violet	100	8.0	Violet	68.8

[1] Colony forming units per ml × 10^9 growth for 96 h
[2] Color indicates color of culture in flask

trogen regulation. There were too many differences between the two strains to be the result of a mutation in a structural gene unlike the multiple phenotypes of a *Legonella* mutant elucidated by phenotypic micorarrays which were traced to a single peptide transporter (Sauer *et al*., 2005). The mutant PRAA4-1B grew faster and to a higher optical density than the parent strain PRAA4-1 on a variety of nitrogen substrates. The only peptide-nitrogen source on which the parent strain grew better than the mutant was di-tryptophan. The violet pigment violacein, produced by the parent strain PRAA4-1, is a modified di-tryptophan molecule (Duran *et al.*, 1994) and thus di-tryptophan could be utilized directly for pigment production allowing the parent to put energy into growth rather than of pigment production. The differences in the mutant's utilization of Tween compounds and susceptibility to polymixin B, which both affect membranes, suggest that the membrane of this spontaneous mutant has been altered compared to the parent, but not in fatty acid composition.

Although the nature of the toxin(s) produced by *C. subtsuga*e is still not clear, several characteristics have been noted. Toxin production, while paralleling pigment formation on solid media, could not be directly correlated to pigment production in liquid media. Protein synthesis by this pathogen also appears to be necessary for toxin formation as the presence of antibiotics which inhibit protein synthesis also decrease toxicity to Colorado potato beetle (data not shown). While protein synthesis may be necessary for toxin formation, the toxin itself demonstrates properties not normally attributed to proteins, such as heat stability (55°C) and resistance to exoproteases (Martin *et al*., 2004). The present research has extended the heat stability to 121°C. While live cells are not necessary for toxicity, the toxin(s) seem to be associated with the cell membrane, as suggested by the loss of toxicity upon filtration and the migration of toxicity from the particulate to the soluble fraction by extraction with detergent. The toxicity to other insects, such as whiteflies increases as bacterial suspensions are allowed to age (D. Gelman, unpubl. data). During this "aging" process, it is believed that the toxin is leached out of the membrane or the biofilm or both.

As the violacein pigment also has the above properties, we tested the pigment alone for toxicity to Colorado potato beetle. Violacein, the molecule that gives *C. subtsugae* its violet pigmentation, is known to be toxic to bacteria (Duran *et al.*, 1983) and trypanosomes (Duran *et al.*, 1994), therefore violacein could also be the source of toxicity to beetle larvae. However, an enriched violacein fraction only killed beetle larvae at a lower rate than the whole culture and *C. violaceum*, another violacein producing bacterium was not toxic to Colorado potato beetle larvae (Martin *et al*., 2004). While pigment production paralleled toxin production on solid media, it did not in liquid culture. This was probably due to differences in aeration as oxygen is essential for violacein formation (Hoshino *et al*., 1987). However, the timing of violacein and toxin production suggested that both processes may be regulated by a common mechanism. In a closely related organism, *C. violaceum*, production of pigment, as well as antibiotics, exoproteases, and chitinolytic activity are regulated by an endogenous AHL (acyl homorserine lactone, Chernin *et al.*, 1998) indicating regulation by quorum sensing. In future research, we will determine if AHLs are produced by our strain and if exogenous AHL will restore toxicity to non-pigmented mutants. Exogenous AHL may also be a way to increase toxin production.

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