ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES ACTIVE AGAINST *BURKHOLDERIA GLUMAE*

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

ABSTRACT

Isolation and Characterization of Bacteriophages
Active Against *Burkholderia glumae*. (May 2015)

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Burkholderia glumae, the primary causal agent of Bacterial Panicle Blight (BPB), results in the abortion of infected rice kernels and grain rot that may result in 75% yield loss. The significance of this disease and finding a sustainable control is evident, as rice is the primary food staple in many countries and this disease threatens production worldwide. Presently there are no chemicals registered for protective or therapeutic treatment of rice with BPB in the US, and although some varieties of rice exhibit moderate resistance to BPB, most non-hybrid commercial varieties are still susceptible. The goal of this research was to isolate, characterize and determine the host range of virulent bacteriophages active against *B. glumae*, which would be the first steps in the possible development of a sustainable bacteriophage based treatment for BPB.

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CHAPTER I

INTRODUCTION

Burkholderia glumae, a Gram negative bacterium, is the primary causal agent of Bacterial Panicle Blight (BPB), which results in the abortion of infected kernels, causes grain rot that may result in 75% of yield losses in severely infected fields. This disease has been reported to be transmitted through seeds, flowers, leaves and crop residue (Ham et al. 2011). During the booting stage, the pathogen grows on stems and leaves, multiplying within the panicle and infecting the spikelets. The toxin produced by the pathogen reduces the growth of both leaves and roots of rice seedlings, inducing chlorotic symptoms on panicles in the grain rot phase and results in grain abortion. The bacterium produces toxoflavin (1,6-dimethylpyrimidio[5,4-e]-1,2,4-triazine-5,7 (1H,6H)-dione), which is the main toxin, that is essential for pathogenicity (Suzuki et al., 2004). The *tox* operon responsible for toxoflavin biosynthesis is polycistronic and consists of five genes (*toxA,B,C,D,E*). The synthesis of the toxin occurs at temperatures over 30°C and is maximal at 37°C, whereas no detectable amount of toxin is produced at 25°C-28°C (Suzuki et al., 2004). The BPB pathogen has been reported in major rice-growing countries in many parts of Asia (Goto and Ohata, 1956), Latin America (Nandakumar, 2007b), as well as being the most prevalent in developing nations, such as India, Korea, Vietnam, Indonesia, Philippines, Nicaragua, Guatemala, Columbia, Venezuela, Costa Rica, the Dominican Republic, and Panama, which lost 40% of rice crops due to BPB. This pathogen has also emerged in Texas, Louisiana and Arkansas (Nandakumar, 2005). Recent outbreaks of BPB in rice and other food crops such as sunflower, tomato, sesame, perilla and eggplant (Jeong *et al*., 2003) may be related with global warming and newly introduced susceptible varieties. The development of a

bacteriophage-based biocontrol would provide a sustainable, environmentally acceptable treatment of the disease. Here, we will report the isolation and partial characterization of bacteriophages active against *B. glumae*, which would be the first steps in the development of a bacteriophage based treatment for BPB.

CHAPTER II

MATERIALS & METHODS

Bacterial Cultures Used in This Study. *B. glumae* isolates that were isolated during previous studies are listed in Table 1.

Media, Culture Conditions and Strains. Tryptone nutrient agar with the addition of cycloheximide (TNA-Cyclo) (30mg/liter) was used for non-selective plating to obtain total bacterial CFU. Medium S-PG, as modified by Kawardani and designated medium CCNT (Kawardani, 2000), and *Burkholderia cepacia* selective agar, designated BCSA (Henry, 1996), were used for selective plating of seed extracts for the isolate of potential *B. glumae*. Tryptone nutrient agar (TNA) (20g/liter of agar) and tryptone nutrient broth (TNB) (TNA without agar) were then used to culture suspected *B. glumae* isolates. Liquid cultures were monitored for growth at absorbance of 600 nm and both solid and liquid cultures were incubated overnight at 37°C. Tryptone nutrient soft agar (TNA-SA) was also used for overlays at 0.4% agar. Rice seed samples were obtained from Texas and Louisiana rice growing areas and are listed in Table 2. Samples were processed by crushing of rice seeds (2g) with a sterile mortar and pestle in 20 ml of 0.125 M phosphate buffer, pH7.2. We then filtered the mixture using a sterile double layer of cheesecloth to remove debris. The samples were then serially diluted using in P-Buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM MgSO4) , and plated to TNA-Cyclo, CCNT and BCSA, respectively. After 72 hours, suspected *B. glumae* colonies were short streaked purified onto TNA-Cyclo, CCNT and BCSA and incubated overnight at 37°C to confirm phenotype. Individual short streaks were stab-inoculated on onion scales to confirm pectolytic activity. Controls consisted of known *B. glumae* (positive control) and a onion scale stab inoculated with

a sterile toothpick (negative control). Isolates that produced maceration of onion tissue were then streak purified to obtain isolated colonies for further analysis and identification via PCR using specific *toxC* primers. (See below)

PCR Analysis. Genomic DNA of putative isolates was obtained using Qiagen Genomic kit. B. glumae specific primers were used to determine the presence of toxin genes encoded by B. glumae isolates. The toxoflavin *toxC* primer set BG185-ToxC-F 5'-CTTCCTCGTCCGAC TACACC-3' and BG822-ToxC-R 5'-GGAATGGAAGGTCGAGAACA -3' were designed from the toxoflavin sequence (AB040403). Each PCR reaction (25 µl) using the B. glumae specific primers was performed using a Taq PCR Core kit (QIAGEN) and a GeneAmp 2700 (Applied Biosystems). Reaction parameters were as follows: Initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1 min; annealing at 56°C for 1 min; and extension at 72°C for 1 min with a final extension of 10 min. Resulting products were analyzed by agarose gel electrophoresis.

Bacteriophage Isolation, Titration and Single Plaque Purification. Macerated rice extracts were centrifuged (10,000 x g, 15 min at 5°C) in a J2-21 Beckman centrifuge and filter sterilized through a 0.22 um filter (Supor; Pall Life Sciences). Aliquots of individual filtrates were combined to make a pooled filtrate. The pool filtrate was added to log phase cultures of *B. glumae* isolated to enrich for bacteriophage. Briefly, cultures of *B. glumae* isolates were grown in TN broth (700 rpm at 37°C) to an optical density (OD) of 0.35 (A_{600}) at which time 2 ml of the pool filtrate and 2ml of 2X TNB were added to the culture. The enrichment was allowed to grow 18 h at 37°C (700 rpm). At 18 h the culture was centrifuged and the supernatant filter sterilized. The sterile enrichment was then assayed for bacteriophage, plating to the enrichment host by spotting the enrichment to agar overlays seeded with the enrichment host. To make

overlays, 100 µl of the host suspension (OD = 0.5 @600 nm) mixed with 5 ml of molten TNA-SA was poured onto a TNA plate and allowed to solidify. The plates were then spotted with 20 µl of the enrichment, allowed to dry, and then incubated overnight at 37°C. Plaque or zone formation was an indication of the presence on bacteriophage in the enrichment. Enrichments exhibiting plaque formation on spot plates were serially diluted in P-buffer, mixed with 100 µl of a bacterial host suspension and 100 µl of each serial dilution of enrichment with 5 ml of TNA-SA-4%, then poured onto a TNA plate, allowed to solidify and incubated overnight at 37°C in order to determine titer. Plaques observed the next day were evaluated for morphology and three representative plaques were excised and suspended in P-buffer and filter sterilized. Each excised plaque was plaque-purified 3X to obtain a pure bacteriophage isolate. High titer lysates (~1 x 10^9 PFU/ml) were obtained by harvesting overlays of plates that demonstrated confluent lysis, macerating the gel, centrifuging (10,000 x g, 15 min at 4°C) and filter sterilizing with a 0.22-µm filter. Both high titer lysates and purified plaque isolates were stored at 4°C.

Host-range Study and Transmission Electron Microscopy (TEM). Bacteriophage isolated and purified was then spot diluted to overlays seeded with a variety of hosts of *B. glumae*, both virulent and avirulent, as well as hosts isolated during this study to determine host-range. The bacteriophage high-titer lysate (~1 x 10^9 PFU/ml) made earlier was used for TEM by diluting stock with P-buffer. Bacteriophages were applied onto thin 400-mesh carbon-coated Formvar grids, stained with 2% (wt/vol) uranyl acetate, and air-dried. Specimens were observed on a JEOL 1200EX transmission electron microscope operating at an acceleration voltage of 75 kV to determine morphological characteristics. I acknowledge the help of Dr. Mayukh Das for his assistance with TEM.

(TABLE 1) Laboratory *B. glumae* Strains Used In This Study

Strain	Relevant Characteristic	Source
379gr-1-b	Avirulent	Laboratory Stock
99SH-7	Avirulent	Laboratory Stock
366gr-2	Avirulent	Laboratory Stock
98gr-1	Avirulent	Laboratory Stock
191SH-1	Virulent	Laboratory Stock
171gl-7-a	Virulent	Laboratory Stock
261gr-9	Virulent	Laboratory Stock
201SH-1	Virulent	Laboratory Stock
189-gr-4	Unknown	Laboratory Stock
AG128N2	Unknown	Laboratory Stock

(TABLE 2) Rice Seed Varieties Assayed

Variety	Source	*B. glumae* positive
Cocodrie	Beaumont, TX	+
Presidio 1	Beaumont, TX	-
CL-152	Beaumont, TX	-
XL-752	Beaumont, TX	-
Antonio	Beaumont, TX	+
Sierra	Beaumont, TX	-
Dixiebelle Sill	Beaumont, TX	-
Catahoula	Crowley, LA	+
SAR-CL-161	Lake Arthur, LA	-
VAR-CL-152	Lake Arthur, LA	+
M202	Crowley, LA	+
CL-261	Crowley, LA	+
VARJAZZMAN2	Crowley, LA	+
VAR-CL-111	Crowley, LA	+

(+) = Confirmed *B. glumae* isolate by PCR

CHAPTER III

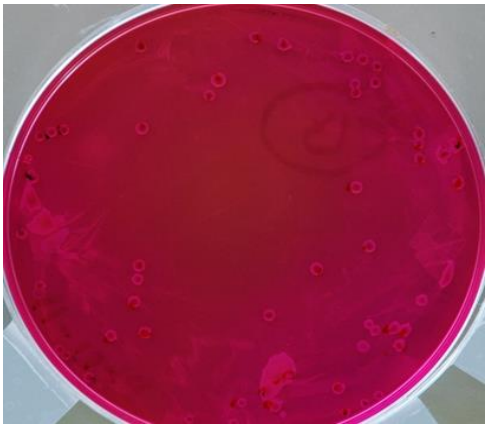
RESULTS

***B. glumae* Isolation and Characterization.** Using TNA-Cyclo, as a non-selective medium, for total CFU counts per gram of seed were obtained ranging from 10 to ~10^{-5} per gram of seed. We observed putative *B. glumae* isolates on semi-selective medium, BCSA, which produced characteristic pigmented colonies on BCSA's agar, as shown in Figure 1. Growth was noted from a dilution series ranging from 10 to ~10^{-5} on this medium. If growth of bacterial was observed on both media, the strains were short streak purified for further testing, as shown in (Figure 2). Pectolytic activity was then confirmed by stab inoculation of the purified putative *B. glumae* isolates producing maceration on onion tissue that is characteristic of *B. glumae* (Figure 3). Finally, using PCR analysis for the *toxC,* which is a part of the polycistronic pathway that encodes for toxoflavin and required for pathogenicity (Figure 4), and gel electrophoresis, we verified putative isolates were indeed *B. glumae* (Figure 5). All characteristics of confirmed *B. glumae* isolates are listed in Table 3. *B. glumae* isolates were successfully isolated from 2 of the 7 Texas samples and 6 of the 7 Louisiana samples and characteristics were listed in Table 3 below.

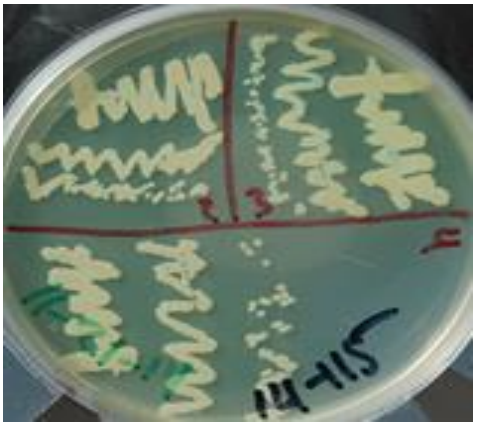
(TABLE 3) *B. glumae* Isolates and Characteristics, This Study

Source Variety	Experiment #	Location	CCNT Growth	BSCA growth	Acid/Base Production on BCSA	Onion Maceration	PCR Product-*toxC*-F/R
Cocodrie	BG14-105	Beaumont, TX	+	+,P	Acidic	+, P	+
Antonio	BG14-108	Beaumont, TX	+	+,P	Acidic	+, P	+
VAR-CL-152	BG14-117	Lake Arthur, LA	+	+,P	Acidic	+, P	+
M202	BG14-119	Crowley, LA	+	+,P	Acidic	+, P	+
CL-261	BG14-120	Crowley, LA	+	+,P	Acidic	+. P	+
VARJAZZMAN2	BG14-122	Crowley, LA	+	+,P	Acidic	+. P	+
VAR-CL-111	BG14-123	Crowley, LA	+	+,P	Acidic	+, P	+
Catahulla	BG14-115	Crowley, LA	+	+,P	Acidic	+, P	+

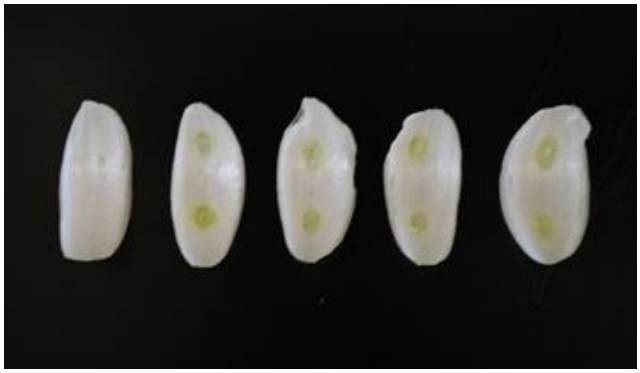
(+) = Growth, presence and/or maceration, P = Pigmentation



(**Figure 1) -** Characteristic pigmented colonies on BCSA medium

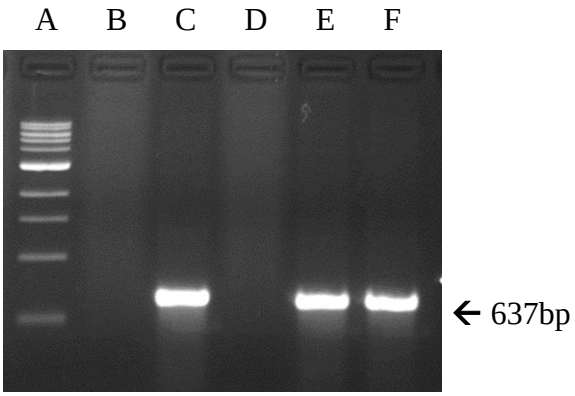


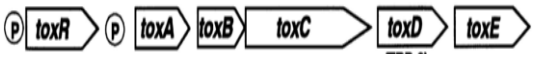
(Figure 2) – Short streak purified *B. glumae* isolates



(Figure 3) - Maceration of onion tissue by putative *B. glumae* isolates A. Phosphate buffer control; B. Tex-105-9A; C. LA-115-3A; D. LA-119-3A;

E. Positive Control (*B. glumae* 191 sh-1)





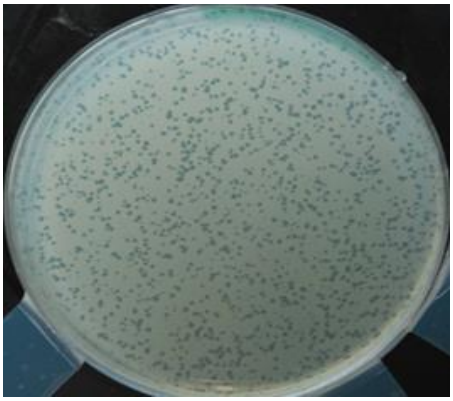
(Figure 4)- Genetic map of *B. glumae* toxRABCDE genes

(Figure 5) - PCR analysis of *B. glumae* isolates using toxC specific primers. Lanes A. 1kb Ladder; B. Tex-5; C. Tex-105-3A; D. LA-4 ; E. LA-115-3A; F. LA-2; Expected PCR product for *toxC* specific primers is 637bp

Bacteriophage Isolation, Determination of Titer and Characterization. Seventeen different enrichments were spotted to overlays seeded with seven confirmed *B. glumae* isolates obtained from seed samples (Table 3). Enrichments of the rice seed extract from hosts Catahulla, M202,

and CL-261 produced clear zones and plaques when spot diluted to their respective hosts on TNA-SA (0.4%) (Figure 6). TNA-SA (0.4%) was used over TNA-SA so plaque formation could be observed. These enrichments were then serially diluted and plated to their respective hosts to determine titer (Figure 7). Catahulla and M202 yielded small clear plaques, whereas CL-261 yielded medium-sized clear plaques. Three representative plaques were excised from each host and plaque purified x 3 and were designated as ⍉ 813 for Catahulla, ⍉ 843 for M202 and ⍉ 173 for CL-261. The titer of these enrichments was determined to be 1.57 x 10^9 PFU/ml, 4.4 x 10^8 PFU/ml and 6.5 x 10^8 PFU/ml, respectively. After plaque purification x 3, we made a high titer lysate (~1 x 10^9 PFU/ml) determination of morphological characteristics by transmission electron microscopy.

(Figure 6) – Spot Dilution plating of bacteriophage enrichment on host 119-3A



(Figure 7) – Individual plaques of ⍉ 843 on host 119-3A.

Bacteriophage Host-Range and Transmission Electron Microscopy (TEM). Purified bacteriophage isolates of ⍉ 813, ⍉ 843, and ⍉ 173 were spot diluted onto seventeen confirmed *B. glumae* hosts that were isolated in this study, as well as previously isolated in other studies, as

indicated in Table 4, to determine the host range of each bacteriophage. ⍉ 813 and ⍉ 843 each produced approximately the same number of plaques on all representative hosts that demonstrated lysis, with the exception of host 191SH-1, for which ⍉ 843 did not produce plaques, despite ⍉ 841 producing plaques on that particular host. ⍉ 173 produced plaques only on its original host, demonstrating a very narrow host range.

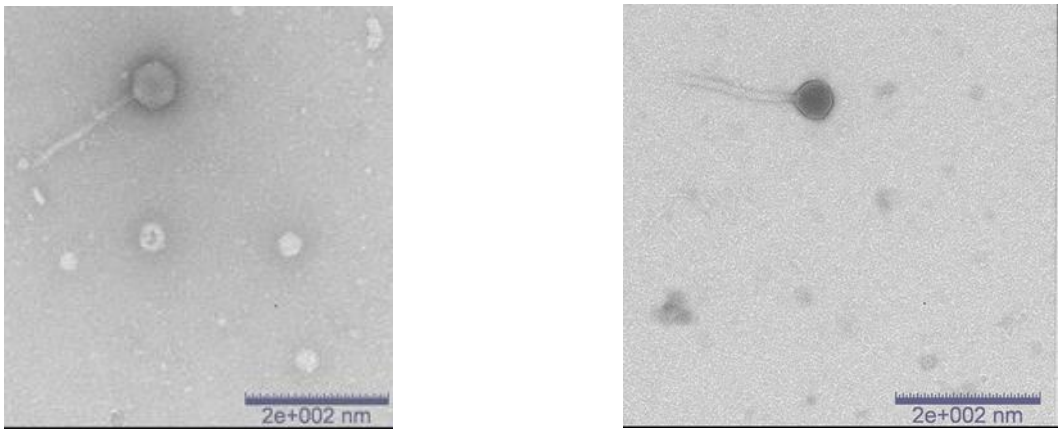
TEM studies confirmed that ⍉ 813 and ⍉ 843 both exhibited siphophage morphology, a long, non-contractile tails and had isometric heads that measured ~50 nm (Figure 8). We were unable to obtain a TEM micrograph of ⍉ 173, so we did not determine the morphology.

(Table 4) Host Range of Purified Phage

Host	Host Characteristics	⍉ 813	⍉843	⍉173
105-9A		+*	+	-
108-1A		+	+	-
115-3A		+	+	-
117-1A		NC	NC	NC
119-3A		+	+*	-
120-B1A		+	+	+*
122-5A		NC	NC	NC
191SH-1	Virulent	+	-	-
171gl-7-a	Virulent	+	+	-
261gr-9	Virulent	+	+	-
201SH-1	Virulent	+	+	-
189-gr-4		+	+	-
AG128N2		+	+	-
379gr-1-b	Avirulent	+	+	-
99SH-7	Avirulent	+	+	-
366gr-2	Avirulent	+	+	-
98gr-1	Avirulent	+	+	-

+= indicates individual plaque formation, *= bacteriophage production host
NC= not completed

ᴓ813 ᴓ843



(Figure 8) TEM of bacteriophages ᴓ 813 and ᴓ 843, showing siphophage morphology with long non-contractile tails and isometric heads that measure ~50 nm.

CHAPTER IV

CONCLUSIONS

In this study, we confirmed the seed borne nature *B. glumae* isolates in rice seed samples. Using a semi-selective medium, we identified putative *B. glumae* that were confirmed by phenotypic testing and PCR using primers specific for the *toxC*. We determined that 2 of 7 Texas and 6 of 7 Louisiana seed samples were infested with *B. glumae*. Although less Texas samples were infested, Louisiana is a neighboring state, and I believe the environmental conditions of Texas can easily allow BPB to spread to other rice growing areas. Enrichment of 17 seed extracts yielded three bacteriophages. Two bacteriophages (ϕ 813 and ϕ 843) exhibited long non-contractile tails and isometric heads, typical of siphophage morphology. No TEM studies were conducted with ϕ 173, since a high titer lysate could not be obtained. Preliminary host range studies indicated that bacteriophages ϕ 813 and ϕ 843 have a broad host ranges, whereas ϕ 173 has a narrow host range, only causing lysis on the original strain that was used for propagation. The narrow host range may indicate that the receptor site for ϕ 173 may be unique. The broad host range of bacteriophages ϕ 813 and ϕ 843 may indicate that a receptor site common to *B. glumae*. Further studies are needed to confirm receptor diversity.

The use of a single bacteriophage to protect plants is not a scientifically valid approach, since resistance to a single bacteriophage can be predicted. A large bank of bacteriophage with a diversity of receptors are need to develop a bacteriophage treatment. The bacteriophage receptor sites of bacteria, such as *B. glumae,* can include major proteins or sugars components of the bacterial surface. The future goal is to identify as many receptor sites as possible. This strategy

will develop an expanded pool of bacteriophages with different receptors that can be mixed for the formulation of diverse cocktails.

Bacteriophage therapy offers the possibility of an environmentally friendly, sustainable and effective treatment method. The research conducted in this study is a step forward in the possible development of a bacteriophage based biocontrol system for BPB.

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