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Isolation of Bacteriophages and Their Potential as a new Biological Weapon for Controlling Bacterial Disease of Rice in Bangladesh

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Abstract

Rice bacterial diseases are one of the major hindrances to achieving crop production across the world, particularly in Asian countries. Bacterial brown stripe (BBS) caused by *Acidovorax oryzae* (Ao) is one the major diseases of rice responsible for high economic losses. To combat the diseases, this study aimed to isolate and characterize lytic phages infecting Ao, which would serve as potential biological agents for the management of BBS. In this regard, three lytic bacteriophages were isolated from diseased rice leaves in Bangladesh. In this study, the 10 bacterial strains of Ao were identified based on 16S gene sequence analyses, which were screened with their respective phages isolated in this study. Phage AP-1 infected 6 strains of Ao bacteria out of 10 with the most increased titer of plaque in AP-3. All phages showed different titer ranging from $1.19x10^{10}$-$3.28x10^{10}$ for Ao phages. In the host exopolysaccharide production, the Ao phage AP-3 had the highest inhibition of biofilm formation of strains TNGAo-01 of Ao bacteria but the phage AP-2 inhibited significantly by 48.08% as compared to control. Interestingly, all the phages used behaved differently with regard to each of the experiments conducted. These results suggest that all the phages need to be combined in a cocktail to serve as efficient biological control agents in the control of bacterial brown stripe disease caused by *A. oryzae* in rice.

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Introduction

Acidovorax oryzae (formerly known as *Acidovorax avenae* subsp. *avenae*) causes diseases in many plants species, including rice, corn, oats, sugarcane, millet, and foxtail with massive economic losses, particularly in rice (Xie *et al.*, 1998; Song *et al.*, 2004; Santa Brigida *et al.*, 2016). In Bangladesh, bacterial brown stripe disease (BBS) caused by *Acidovorax oryzae* (Ao) is now considered a significant disease of rice due to its heavy incidence in different parts of our country. In most cases, the diseased seedlings become stunted and even die before the two-leaf stage. Plant bacterial diseases have been hard to control since there have been very few reliable, economical bactericides. Moreover, extensive use of bactericides has caused contamination of the atmosphere and resulted in resistant bacterial strains. The biological control of plant disease, which is increasingly needed for sustainable agricultural practices, is now being given greater attention.

Phage therapy has been shown to be effective against many kinds of bacterial diseases due to the specificity character of the virus. Phages kill target host bacteria during application due to the host

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specificity, making them good candidates for plant protection with great potential to replace chemical control measures. It is noteworthy that some studies have shown that bacteriophages can effectively control bacterial diseases in plants as an effective biocontrol agent (Balogh *et al.*, 2003; Lang *et al.*, 2007; Rahimi-Midani *et al.*, 2020). However, no information was found about its use on *A. oryzae* in Bangladesh. The present study has therefore been undertaken to isolate and identify the rice pathogens causing bacterial brown stripe (BBS) in Bangladesh and to characterize the phages by evaluating their effect on biofilm formation and the host bacteria's extracellular polysaccharide (EPS) production.

Materials and Methods

Isolation and identification of pathogenic bacteria in rice

The diseased samples showing BBS symptoms were randomly selected and collected separately from the fields in different rice-growing areas, including Gazipur and Tangail in Bangladesh. After surface sterilization followed by washing twice in double-distilled water, the diseased samples were vertically cut into pieces into a 1.5ml microcentrifuge tube containing 1%(w/v) peptone water and then shaken at 180 RPM/min for 3-5h in a shaker. The suspensions were then diluted 10-fold to 10^{-4}, and 100 µl diluents were then streaked onto Luria-Bertani (LB) agar media and incubated in a 30^{o}C incubator for 48h. The bacteria were purified two times by subculturing. The genomic DNA from bacteria was extracted using the Monarch DNA purification kit following the protocol. The suspected strains of Ao were identified by PCR amplicon using a universal primer (16SF 5′-AGAGTT TGATCCTGGCTCAG-3′ and 16SR 5′-GGTTACCTTGTTACGACT T-3′) (Turner *et al.,* 1999). The amplification of the DNA was performed in a reaction volume of 50µl with One Taq Quick-Load 2xTSINGKE Master Mix (Biolabs) while the PCR conditions were 94°C for 10min followed by 35 cycles of denaturation at 94°C for 30s, annealing at 57°C for 30s, and extension at 72°C for 1 min/kb. After verified the band, the PCR product directly sent to the company for 16S rDNA gene sequence analysis. Obtained sequence data of 16S rDNA were searched through BLAST using the NCBI database to search the homology nucleotide sequence of bacteria. To determine the phylogenetic relationship, the highly homologous sequences of typical bacteria were obtained from EzBioCloud (https://www.ezbiocloud.net/). A phylogenetic tree was built using the neighbour-joining method in the MEGA 5.0 program. Bootstrap replication (1000 replications) was used as statistical support for the nodes in the phylogenetic trees.

Isolation of bacteriophages

To isolate the bacteriophage, diseased samples were used alongside strains preserved in the laboratory which served as a potential host. The bacteria were isolated from the diseased leaves as described before. After centrifugation, the peptone water suspension was filtered through a 0.22 µm filter. The phage, overnight culture of liquid bacteria, and 3-fold LB medium were mixed in the ratio of 2:1:10 respectively which were shaken at 180 RPM at 30oC for 10 hrs. After centrifugation (at 4,000g for 15 min at 4oC), the supernatants were filtered through a 0.22 um syringe filter to remove the residual bacterial cells. To confirm the presence of bacteriophage infecting pathogen Ao, 2 µl of phage filtrate was spotted on the top agar layer of their host bacterial lawn and incubated overnight at 30ºC to get the plaque (Sambrook *et al.,* 1989). Following the appearance of a transparent zone (plaque), the individual plaques with different plaque types were selected to purify the isolated phage. The individual plaques were picked with sterile pipette tips and soaked in 1 ml of SM (Saline Magnesium) buffer.

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Purification of bacteriophage

In order to purify, the selected phage suspension was filtered again through a 0.22 µm filter and spotted onto a bacterial lawn for plaque formation through a dilution plate technique (Sambrook *et al.,* 1989). In brief, the phage stock (100 µl) at the optimal multiplicity of infection (MOI) was mixed into a fresh bacterial culture (100 ml) of the representative bacterial strain at OD600 nm =0.2-0.3 and incubated at 30°C for 1 h followed by shaken until it became clear. The DNase I, RNase, and NaCl (5.84 g) was added to the cell lysate and incubated for 1 h and then centrifuged at 11,000 g at 4°C for 10 min. The supernatant was transferred to a new Erlenmeyer flask; solid polyethylene glycol (PEG 8000) was added to a final concentration of 10% (w/v) by slow stirring. After centrifuging the ice-cooled mixture, the phage pellet was suspended in SM buffer and chloroform mixture. The mixture was centrifuged at 5,000 g for 10 min at 4°C. The upper phase was collected and mixed with an equal volume of chloroform by vortexing and was collected again after centrifugation and stored at 4°C.

Determination of host ranges of phages

The host ranges of the isolated Ao phages were determined by examining their infection on the different strains of their respective bacteria Ao, respectively in this study based on the soft agar overlay method described above. The phage stock was diluted to about 1 × 108 plaque-forming units (PFU)/ml and spotted (2 µl) onto the top layer of the bacterial lawn and incubated at 30°C. The appearance of clear zones in the plates, resulting from the lysis of host cells, indicated the phage's ability to infect the tested bacteria (Lu *et al.,* 2003).

Determination of phage titers

Plaque-forming assays using the soft agar overlay method will be applied to determine the phage titers. The phage titers for three Ao phages were determined separately on Ao strain TGLAo-01 as host, respectively, by serial dilution method using the double-layered agar plate technique (or overlay method), as described above. The phage stock was diluted with the SM buffer to 10-8 ten-fold dilution. After the top agar had hardened, 2 µl of phage filtrate were spotted on the top agar layer of their respective plate. After the drops of phages had dried, the plates were incubated overnight at 30oC in an inverted position. The

numbers of plaques were counted, and the titers of phages (PFU/ml=plaque numbers × dilution fold × 10) were calculated.

Determination of the optimal multiplicity of infection (MOI)

MOI, the ratio of the number of phage particles to the number of the bacterial host cells, was determined as described by (Birge, 2000) with slight modification. In brief, the phage stock was adjusted to a titer of about 1 ×1010PFU/ml and then was 10-fold diluted to 10-8 with SM buffer. One microliter of each of the dilutions alongside 1 µl and 10 µl without dilution was taken and added to 100 µl of the host bacteria Ao (4 ×107 CFU/ml) with 1 ml of LB medium. All the mixtures were shaken for 4-5 h at 180 RPM, 30 oC after incubating for 30 mins. The cultures were then centrifuged at 5,000 g for 10 min. One microliter of the centrifuged supernatant was taken and assayed for phage titer as described above. The highest titer was regarded as the optimal MOI. The experiment was repeated three times and the results were averaged.

Quantitative analyses of bacterial biofilms

The capability of each phage to inhibit biofilm formation of the TGLAo-01 strain of Ao was studied according to (Liu *et al.,* 2012) with a little modification. A 200 µl of overnight bacterial culture (OD600 = 0.8): LB (1:50, v/v) was incubated in the 96 well-plate at 30 oC for 12 h while liquid LB medium served as a blank. Each phage (100 µl) at the optimal MOI, or SM buffer without phages as a control, was added to their respective tubes and incubated at 30ºC for 12 h. All non-attached cells were removed by discarding the culture media and each well in the plate was air-dried after gently rinsing it twice with double distilled water. Each well was stained with 150 µl of 1 % crystal violet for 30 mins at room temperature, and the plate was washed twice with double-distilled water. After air-drying the plate, 150 µl of 33 % acetic acid was added to each well and the optical density was measured at 570 nm. The experiment was repeated three times.

Quantitative analyses of exopolysaccharide (EPS)

EPS in bacterial culture supernatants was determined as described by (Patel *et al.,* 2013) with slight modification. In 100 ml LB, 1 ml of the bacterial grown strain was added (OD=0.2- 0.3) with 100 µl of each phage which was mixed and incubated for 15 mins to absorb the bacteria. The culture was centrifuged at 6,000 g for 30 min at 4oC after shaking in 180 RMP, 30oC for 12-16 h, and filtered through a 0.22 um syringe filter. One milliliter of trichloromethane: butanol (4:1, v/v) was added to 4 ml of the filtrate, followed by 30 min shaking at 180 RPM, then centrifuged at 6,000 g for 10 mins. The supernatant was mixed with three volumes of 95 % ethanol, and the mixture was kept at 4ºC for 24 h. To determine the dry weights of EPS, the precipitated EPS was collected by centrifugation at 6,000 g, 4ºC for 30 min and dried at 65ºC oven overnight before measurement. Three replicates were used for each phage and the test was repeated three times.

Data analysis

Experimental data will be analyzed with the SPSS software package SPSS 16.0 (IBM SPSS Statistics, Georgia, USA). The differences between mean values will be separated by LSD (least significant difference) posthoc statistic. The final results will be represented as the means± standard error of at least three independent experiments.

Results and Discussion

Isolation and identification of rice-pathogenic bacteria

In total, 10 bacterial isolates were obtained from infected rice plants grown in Gazipur (6) and Tangail (4), which are representative of the rice-growing regions of Bangladesh. The 16S rDNA genes of each isolate of selected bacteria were amplified using the universal primers 16S-F and 16S-R and they were verified in gel with the expected product size of approximately 1.5 kb (Fig. 1). Ten isolates of Ao in this study showed 100% similarities with Acidovorax avenae subsp. avenae ATCC 19860T and Acidovorax oryzae ATCC 19882T based on a blast search analysis using the NCBI BLAST search program (www.ncbi.nlm.nih.gov/BLAST.cgi). Furthermore, the studied sequences were further verified with the EzBio Cloud database (https://www.ezbiocloud.net/) and the homologous nucleotide sequences varied from 97.1% to 100% were obtained to construct the neighbor-joining tree. In addition, a phylogenetic tree showed that all the isolates of Ao were grouped into one group with Acidovorax oryzae ATCC 19882T, which has been previously demonstrated by recent studies (Song *et al.,* 2004).

Isolation of Ao phages

After enrichment of potential bacteriophages in bacterial host cultures, 3 lytic bacteriophages (able to kill the bacteria) were obtained from Ao strains. From each positive sample with similar plaque morphology but different sizes, the plaques were further purified 3 times to obtain pure phage. All the 3 phages were assayed for their ability to plaque on the 10 strains of Ao in this study based on the soft agar overlay method (Fig. 3). The three Ao phages AP-1, AP-2 and AP-3, infected respectively 6, 5 and 2 strains out of 10 Ao bacteria collected from two districts of Bangladesh. Two Ao bacterial strains (GAZ Ao-02 and TGLAo-01) were infected by all three respective phages (Table 1). Similarly, several studies have shown that bacteriophages have been isolated and applied as excellent biocontrol agents for the bacterial diseases of plants such as Pseudomonas syringae pv. glycinea which cause bacteria blight of soybean, and Acidovorax citrulli, the causal agent of bacterial fruit blotch of melon, respectively (Balogh *et al.,* 2003; Rahimi-Midani *et al.,* 2020).

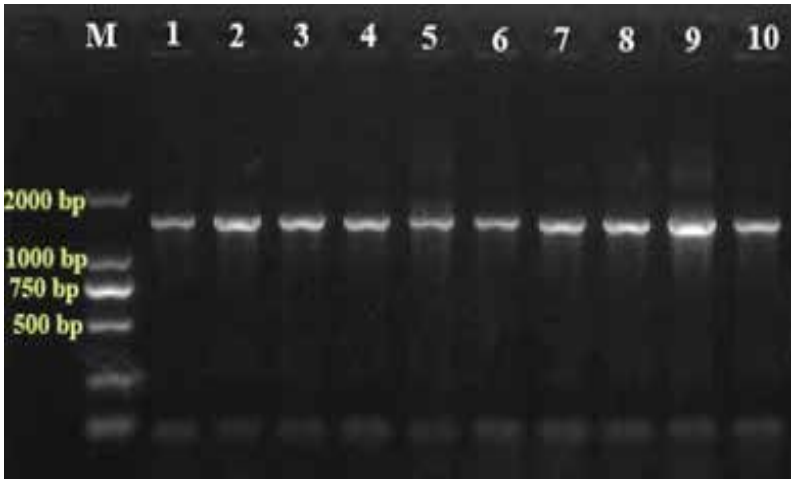

Fig. 1. PCR Validation of 16S genes for Acidovorax oryzae (M: Maker; Lane 1-10: GAZAo-01, GAZAo-02, GAZAo-03, GAZAo-04, GAZAo-05, GAZAo-06, TGLAo-01, TGLAo-02, TGLAo-03, and TGLAo-04 isolates, respectively).

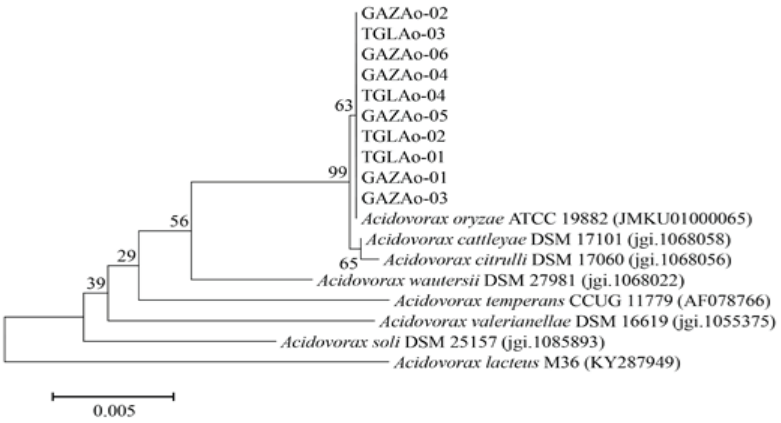

Fig. 2. Phylogenetic tree of 10 isolates of A. oryzae generated using 16S rRNA gene sequences. Bootstrap analysis (1000 replicates) for node values greater than 50% are given. Bar 0.005 substitutions per nucleotide position.

Table 1. Host range assay of bacteriophages against the strains of Acidovorax oryzae.

Source	Isolate Name	Ao Phage strain*		
		AP-1	AP-2	AP-3
Gazipur	GAZAo -01	+	+	-
Gazipur	GAZ Ao-02	+	+	+
Gazipur	GAZAo -03	+	+	-
Gazipur	GAZ Ao-04	+	-	-
Gazipur	GAZAo -05	-	-	-
Gazipur	GAZ Ao-06	-	+	-
Tangail	TGLAo-01	+	+	+
Tangail	TGLAo-02	-	-	-
Tangail	TGLAo-03	+	-	-
Tangail	TGLAo-04	-	-	-

** Ao, Acidovorax oryzae; + symbol indicates positive result while (-) symbol indicates a negative result.*

Table 2. Characteristics of Ao phages on TGLAo-01 as host.

Ao Phages	Titer value (10^{10})	Diameter of plaque (cm)	MOI	Number of plaques
AP-1	3.28 ± 0.27 a	0.98 ± 0.10 b	10^1	$4x10^8$
AP-2	2.23 ± 0.20 b	1.21 ± 0.02 b	10^0	$2x10^7$
AP-3	1.19 ±0.27 c	1.27 ± 0.02 a	10^0	$4x10^6$

Values are the mean ± SD of three replicates. Values with different letters are significantly different in LSD tests ($P < 0.05$).

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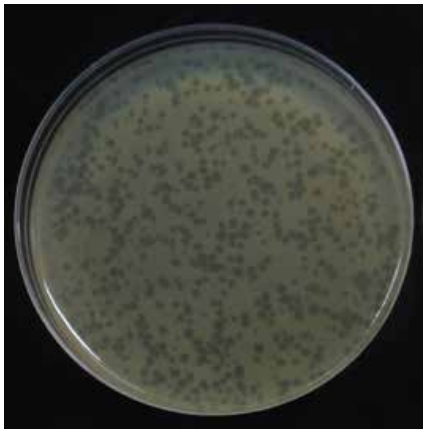

Fig. 3. *In vitro* infection of host bacterial strain TGLAo-0 bacteria with their respective phage AP-1 as representative.

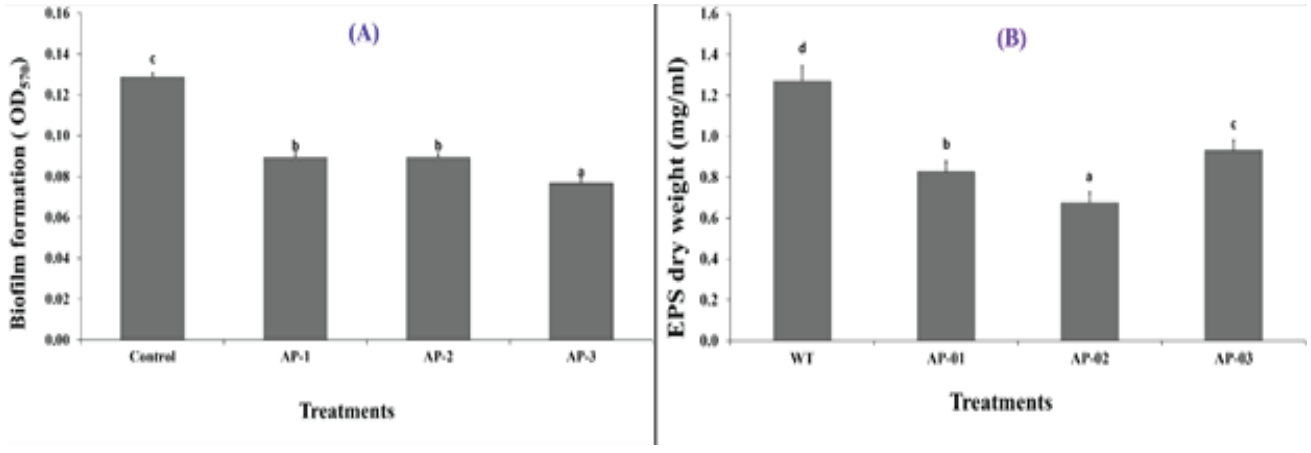

Fig. 4. Effect of bacteriophages AP-01, AP-02 and AP-3 on the biofilm formation (A), and dry weight of EPS (B) produced by the TGLAo-01 strain of Ao bacteria. Values represent the mean ± SD of three replicates and bars with the same letters are not significantly different on LSD test ($P < 0.05$).

Characterization of Ao phages

The phages showed different titers of 1.19x1010-3.28x1010 and plaque diameters ranging from 0.98 to 1.27 cm. In contrast, phage AP-1 showed the highest titre phage and AP-3 displayed the highest diameter of plaque (Table 2). The optimal multiplicity of infection was determined by finding the ratio of the optimal phage particles (PFU) and bacterial host cells (CFU). The Ao strain TGLAo-01 was used for the three Ao phages (AP-1, AP-2 and AP-3. The Ao phages AP-1, AP-2 and AP-3 showed 101, 100 and 100, respectively with the plaque numbers ranging from 4x108, 2x107, and 4x106 (Table 2). It is obvious that there is a complex interaction between phages and Ao strains as this resulted in a wide range of infection efficiency as seen in titer values, plaque diameters, and MOI when Ao strain TGLAo-0 was utilized as the host.

Effect of phages on biofilm formation and exopolysaccharide (EPS) production

Biofilm formation of the Ao strain TGLAo-01 was susceptible to infection by all three specific bacteriophages used in this study (Fig. 4A). The bacterial biofilm formation was determined after 12 h incubation by measuring the OD570 after crystal violet staining. The OD570 value was significantly reduced from the control value (0.129) by each of the 3 phages AP1, AP2, and AP3, with respective reductions of 28.5, 30.5 and 40.40% over the control (Fig. 4A). Additionally, the mean exopolysaccharide quantity produced by TGLAo-01 strain of Ao bacteria was 1.3 mg/ml, which was significantly reduced ($P<0.05$) by the phages AP-1, AP-2, and AP-3 by 36.46, 48.08 and 28.46% (Fig. 4B). Bacteria often reside as multicellular assemblies in a structured, protective biofilm matrix made of EPS and other elements (Ramey et al., 2004). In addition, the dynamics of biofilm formation and detachment are crucial for the survival and completion of disease cycles by bacterial phytopathogens (Rigano *et al.,* 2007; Zhang *et al.,* 2013), whereas interference with Worthington *et al.,* 2012. Previously, it has been shown that phages can destroy parts of the exopolymeric biofilm matrix, allowing them to enter into the core layers of biofilms and be utilized to destroy or control infectious biofilms (Azeredo & Sutherland, 2008). In this investigation, the formation of degradation halos surrounding plaques supports the effect of the Ao phages in reducing bacterial EPS, which may be brought on by the activity of phage polysaccharide depolymerases (Pires *et al.,* 2021).

Conclusions

Taken together, the findings of the present investigation identified the rice pathogenic bacteria *A. oryzae* based on molecular analyses, including 6S gene sequence analyses. This is the first work on the isolation and characterization of rice bacteriophages in Bangladesh. Moreover, this study showed the effect of bacteriophages on biofilm formation and exopolysaccharide production suggesting the potential application of phage-based biocontrol agents for bacterial disease control of rice plants. All the phages used in this study proved to be a good alternative to the chemical method, which is environmentally and health-wise safe to use. Therefore, we strongly suggest that all the phages need to be used as a cocktail to serve as a good biological agent that can be selected for phage therapy to effectively manage bacterial leaf blight and the bacterial brown stripe of rice.

However, host range tests showed that neither of all the phages used could infect strains of Ao collected from different rice growing areas in Bangladesh, therefore, suggesting that more virulent bacteriophages which can infect strains from this area need to be screened and collected and also the genes responsible for lysing of the bacteria in the phages used in this study need to be researched on and sequenced in future.

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Conflicts of interest

The authors declare no conflict of interest.

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