

Original Research Article

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Optimization of Plaque Forming Conditions for an *Aeromonas hydrophila* Lytic Bacteriophage

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ABSTRACT

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Recent emergence of antibiotic resistant bacteria has led to renewed interest in the phage research for control of aquatic bacterial pathogens. Plaque assay is an important part of phage research. For some phages, the plaques could be very much turbid and small in size resulting in difficulties during phage isolation and enumeration. Present study investigated the effect of soft agar as well as Ca⁺⁺ and Mg⁺⁺ ion concentrations on plaque forming ability of a *Aeromonas hydrophila* lytic phage. At 0.2% soft agar, with 10 mM of CaCl₂ and MgSO₄, mean ± SD plaque size of 5.55 ± 0.52 mm and counts of (58 ± 4) were significantly higher the other concentrations. However, increase of agar concentration by value of just 0.1% reduced the plaque diameters to less than one millimeter. No plaques could be observed at soft agar concentration of ≥4%. High CaCl₂ and MgSO₄ concentrations also affected plaque morphology, as plaques were found to be very much uniform and clear up to 25 mM, but turned to turbid and irregular size at 100 mM of CaCl₂ and MgSO₄. This study provides important insights to optimize plaque assay for phages forming turbid and micro sizes plaques leading to their efficient utilization in phage therapy in aquaculture.

Introduction

Propagation of lytic bacteriophage in a confluent bacterial lawn results in the formation of a circular zone of clearing (plaque) due to multiple rounds of phage infection, multiplication and release events in its close vicinity. Plaque formation is important for initial confirmation of lytic activity of phage, phage purification, quantification, and mutant detection. Classical

double agar overlay plaque assay (also known as double layer assay) involves mixing of appropriate phage dilutions, host bacterium and molten soft agar ("top agar") followed by pouring on a standard agar ("bottom agar") plate.

After solidification and incubation, plaques in the confluent bacterial lawn in top agar can be visualized by the naked eye (Kropinski *et al.*, 2009).

However, some phages may produce very much turbid and difficult to detect micro plaques during double layer assay (Mullan, 2002). Lack of visible plaque formation by phages can make their purification and quantification a very challenging task (Santos *et al.*, 2009). The present study was carried out to standardize the plaque forming conditions for a *Aeromonas hydrophila* lytic phage.

Materials and Methods

Initial phage enrichment and isolation

Initial enrichment of *A. hydrophila* specific phage from water samples was performed as per previously described protocols (Vinod *et al.*, 2006). After confirmation of phage lytic activity by spotting a 10 µl aliquot on *A. hydrophila* lawn, 100 µl of filtered phage lysate was serially diluted in phage buffer (10 mM Tris-Cl pH 7.5, 10 mM MgSO₄, 68 mM NaCl, 1 mM CaCl₂), and mixed with 900 µl of young *A. hydrophila* culture (OD₆₀₀ 0.5 - 0.6) for double layer assay.

The phage-bacterial mixture was incubated at room temperature for 10 min, and further mixed with 4 ml of molten top soft agar (LB with 0.4 % agar). The entire mixture was overlaid on the bottom agar plate (LB with 1.5% agar). After overnight incubation at 30 °C, a single plaque (representing a single type of phage) was picked up, further enriched in *A. hydrophila* host culture and designated as D9 phage.

Optimization of plaque forming conditions

Though D9 phage isolated by above protocol was able to show lytic activity against *A. hydrophila* in broth cultures as well as during lawn assay, post isolation it stopped forming visible plaques during subsequent rounds of double layer assay.

Thus, we tried to optimize the plaque forming conditions for D9 phage by experimenting with different agar and ionic concentrations in top soft agar. For this purpose, LB broth was supplemented with 0.2%, 0.3%, 0.4% and 0.5% agar concentrations for preparation of top soft agars.

At each concentration of top soft agar, CaCl₂ and MgSO₄ at 0 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM were also used. LB broth supplemented with 1.5% agar and 10 mM each of CaCl₂ and MgSO₄ was used as bottom agar for all the assays. Basic steps of double layer assay were carried out as described by Kropinski and colleagues (Kropinski *et al.*, 2009).

Briefly, 200 µl of young bacterial cultures (OD₆₀₀ 0.5 - 0.6) were mixed with 100 µl diluted phage suspensions followed by incubation for 10 min to facilitate the attachment of phage particles to bacterial cells. The bacteria-phage suspension was further mixed with 3 ml of top soft agar and poured on the bottom agar plate.

After overnight incubation at 30°C, Plaque counts from 10 plates at each soft agar and ionic concentration were determined. Moreover, diameters of 10 numbers of the largest plaques from each plate were also measured. One-way ANOVA and t-test ($p < 0.05$) were used to determine the statistical significance of the difference in plaque sizes and counts under various experimental conditions.

Results and Discussion

The concentration of soft top agar had considerable effects on plaque forming capabilities of D9 phage. At 0.2% agar concentration, D9 formed largest plaques with overall mean±SD value of 4.01±1.13 mm just after 10 h of incubation (Fig. 1, Table 1).

However, increase of agar concentration by value of just 0.1% had a drastic effect as plaque diameters reduced to less than one mm at soft agar concentration of 0.3%, and no increase in plaque size was detected even after 24 h of incubation.

At soft agar concentrations of 0.4% and 0.5%, no visible plaques were observed even after 24 h of incubation. The concentration of soft agar also had a considerable effect on plaque forming unit (PFU) counts, as overall PFUs of 45 ± 11 (mean \pm SD) at 0.2% agar concentration were the significantly higher than the corresponding value of 19 ± 11 at 0.3% agar concentration (t-test, $p < 0.05$).

In addition to inherent characteristics of phages such as latent period and burst size etc., physiological factors such as phage diffusivity and adsorption of phage on the bacterial host may also affect plaque formation (Abedon and Yin, 2009).

It has also been proposed that at lower agar concentrations, high diffusivity enables the phage to infect and lyse more numbers of host cells in the surrounding area resulting in larger size plaques (Mullan, 2002).

Serwer and colleagues reported that after initial isolation, a *Bacillus thuringiensis* phage 0305 ϕ 8-36 was able to form visible plaques only at agarose concentration of 0.25% or less in the top layer (Serwer *et al.*, 2007).

In our study, the largest plaques were observed at agar concentration of 0.2%, and plaque size reduced drastically (to < 1 mm) at agar concentration of 0.3%.

We also investigated the effect of these Ca^{++} and Mg^{++} ions on plaque formation by supplementing each level of soft agar with 0mM, 10 mM, 25 mM, 50 mM, 75 mM and

100 mM each of CaCl_2 and MgSO_4 . In case of 0.2% soft agar, average plaque size of 5.55 ± 0.52 mm (mean \pm SD) at 10 mM CaCl_2 and MgSO_4 concentrations was significantly higher than corresponding values at 0 mM, 25 mM, 50 mM, 75 mM and 100 mM of CaCl_2 and MgSO_4 (ANOVA and Duncan's multiple range test, $p < 0.05$) (Table 1).

Moreover, the addition of CaCl_2 and MgSO_4 also affected plaque morphology, as plaques were found to be very much uniform and clear up to 25 mM, but turned to turbid and irregular size at higher concentrations (Fig. 1).

No statistical test to understand the effect of Ca^{++} and Mg^{++} concentrations at 0.3% soft agar level could be performed, all the plaques were < 1.0 mm in size and could not be measured accurately. In addition to effect on plaque sizes, Ca^{++} and Mg^{++} ions also had significant effect on plaque counts, as PFU counts at 10 mM CaCl_2 and MgSO_4 concentration were significantly (ANOVA and Duncan's multiple range test, $p < 0.05$) higher than the others at both 0.2% and 0.3% agar levels.

Moreover, a declining trend in PFU counts was observed with an increase of ionic concentrations at both 0.2% and 0.3% agar levels (Table 1). Studies have reported that the presence of metal ions enhance the efficiency of phage to kill its bacterial host (Ma *et al.*, 2018), and it supports our observation of large plaque formation in presence of Ca^{++} and Mg^{++} ions. Similar to our findings, Foddai and colleagues reported that phage counts increased up to 2 mM CaCl_2 , and showed declining trends at higher concentrations (Foddai *et al.*, 2009). These results indicated that though the addition of divalent metal ions may improve plaque formation, higher concentrations may be detrimental to plaque formation.

Table.1 Effect of soft agar and Ca⁺⁺, Mg⁺⁺ ions concentrations on plaque sizes and counts of *Aeromonas hydrophila* lytic D9 phage

CaCl ₂ and MgSO ₄ concentrations	Plaque sizes (mm) and counts (PFU)	
	0.2 % soft agar	0.3% soft agar
0 mM	3.50 ^{cd} ± 0.52, 48 ^b ± 5	<1.0, 21 ^{bc} ± 4
10 mM	5.55 ^a ± 0.52, 58 ^a ± 4	<1.0, 33 ^a ± 3
25 mM	3.46 ^{cd} ± 0.78, 49 ^b ± 3	<1.0, 17 ^{bcd} ± 3
50 mM	4.38 ^b ± 0.87, 45 ^b ± 4	<1.0, 23 ^b ± 9
75 mM	4.14 ^{bc} ± 1.17, 46 ^b ± 3	<1.0, 13 ^{cd} ± 5
100 mM	3.23 ^d ± 1.01, 24 ^c ± 4	<1.0, 7 ^d ± 5
Overall	4.01 ± 1.13, 45¹ ± 11	<1.0, 19² ± 10

Superscripts a, b, c..... show a significant difference in plaque sizes and counts at different CaCl₂ and MgSO₄ concentrations (ANOVA and Duncan`s multiple range test, *p*<0.05).

Superscripts 1 and 2 show a significant difference in overall plaque counts at 0.2% and 0.3% soft agar levels (t-test, *p*<0.05); No statistical comparison for plaque sizes at 0.3% soft agar level could be performed, as plaque sizes were found to be less than 1.0 mm at all concentration of CaCl₂ and MgSO₄

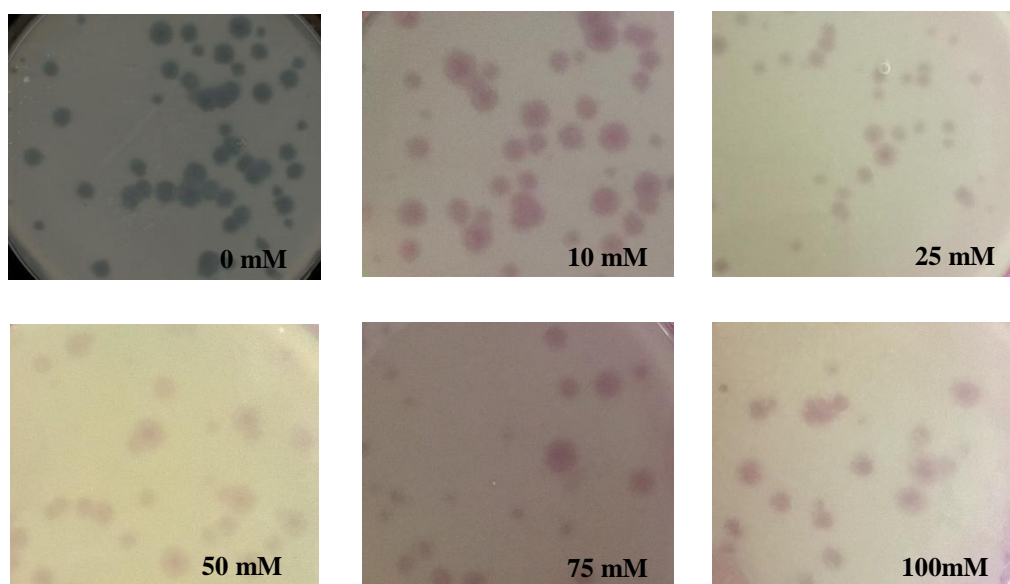


Fig.1 Plaque formation by *Aeromonas hydrophila* lytic D9 phage at 0.2% soft agar level with different concentrations of CaCl₂ and MgSO₄

In conclusion, this study determined the optimized plaque forming conditions for a *A. hydrophila* lytic phage. Addition of 10 mM CaCl₂ and MgSO₄ at 0.2% soft agar concentration resulted in optimum plaque sizes and counts. Optimization of plaque

forming conditions is very much crucial to continue phage research, and the availability of published studies could be a useful start point to improve the double layer assays for poor plaque forming phages.

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