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Lysis activity of bacteriophages isolated from sewage against *Ralstonia solanacearum* and *Erwinia chrysanthemi*

(Aktiviti lisis bakteriofaj daripada air kumbahan terhadap *Ralstonia solanacearum* dan *Erwinia chrysanthemi*)

G.H. Tan*, M.S. Nordin*, A.R. Napsiah* and H. Rosnah*

Keywords: lysis activity, bacteriophage, *Ralstonia solanacearum*, *Erwinia chrysanthemi*

Abstract

Two groups of bacteriophages (phages), one forming a clear plaque, and the other forming turbid plaque with a small holo zone in the middle were isolated from sewage using three different strains of *Escherichia coli* (TG1, ER2738 and BL21) as a host. All the phages were subjected to lysis study against phytopathogen bacterial wilt *Ralstonia solanacearum* and heart rot *Erwinia chrysanthemi*. These phages have very restricted host range and the application as an alternative biocontrol agent has recently gained wide interest. Thus, the present study was aimed to isolate novel phages against *R. solanacearum* and *E. chrysanthemi*, and to further evaluate their lytic characteristics. A total of 132 isolates of phages were obtained from sewage samples. Three phages (P45, P71 and P631) showed potential to lyse *R. solanacearum* with clear zone ratios of 3.34, 3.00 and 3.06 respectively. Only P621 formed a clear zone ratio of 3.26 against *E. chrysanthemi*. The other phages did not show any significant lysis activity. Even though these phages showed some promising results, more studies are needed to investigate the effectiveness of phage on the crop plants.

Introduction

Ralstonia solanacearum (Yabuuchi et al. 1995) and *Erwinia chrysanthemi* (Burkholder et al. 1953) are soil borne gram negative bacteria that caused bacterial wilt in tomato, heart rot in pineapple and several other diseases of important crops in the world (Perombelon and Hyman 1987; Hayward 1991). The large scale cultivation of tomatoes and pineapples in Malaysia has been limited by the widespread incidence of these bacterial diseases. In addition, the extremely high temperature in the lowland areas further aggravates the disease situation where total crop loss is not uncommon (Hayward 2000).

The bacterial infection takes place through the roots and then exhibits strong tissue-specific tropism within the host, specifically invading and extensively multiplying in the xylem vessels. It further spreads throughout the plant and multiplies to a high population density. *Ralstonia solanacearum* spreads easily through the contaminated soil and irrigation water. It can survive for many years in association with alternation host (Yamada et al. 2007).

The initial symptom in mature plants under natural conditions is the wilting of upper leaves during the hottest part of the day, followed by recovery during the evening and early hours of the morning. The

*Strategic Resources Research Centre, MARDI Headquarters, P.O. Box 12301, Serdang, 50774 Kuala Lumpur, Malaysia
Authors' full names: Tan Geok Hun, Mohd Shukor Nordin, Napsiah A. Rahim and Rosnah Hassan
E-mail: tangh@mardi.gov.my

wilted leaves maintain their green colour and do not fall off as disease progresses. However, the complete wilt may occur under condition favourable to the disease and the vascular tissues of the stem will show a brown discolouration (Momol et al. 2004).

Many research studies have been employed to control the bacterial wilt includes searching for resistant varieties of MT1 tomato (Ho 1988), cultural practices, compost (Masyitah 2004), and biological and chemical control. However, none of them is found to be effective against bacterial wilt disease, except for pre-planting seed treatment.

In Malaysia, *E. chrysanthemi* is of economic importance for the pineapple industry since the Josapine pineapple cultivar released by MARDI in 1996 was susceptible to this disease (Sahilah et al. 2008). The pathogen enters the fruits via the open flowers and the main source of inoculum is from the freshly collaped fruits and heart rot tissues. In 2001, plants in Tanah Merah (14%) and Pasir Mas, Kelantan (12%) were infected by the disease. In the same year, the incidence also happened in Kuala Ketil, Kedah (39%), Perak (40%) and Pontian, Johor (15%) (data not published; Sahilah et al. 2008).

In the natural plant ecosystem, *E. chrysanthemi* causes plant maladies such as necrosis, blight and soft rot. The pectate lyases (enzymes) are the most virulence factors secreted by this pathogen (He et al. 1991; Salmond 1994). There are five major isoenzymes of pectate lyases which encoded by the pelA, pelB, pelC, pelD and pelE genes (Lojkowska et al. 1995) which are responsible for the degradation of pectic polymers in the primary cells walls and middle lamellae of the plant (Barras et al. 1994).

As bacteria start to develop resistance against antibiotic, the use of certain bacteriophages (phages) against bacterial infection has gained general interest (Merril et al. 2003). Phage only interacts with a specific set of bacteria that express specific binding sites. Thus, unlike antibiotic, dysbiosis and chances of developing secondary resistance can be avoided.

Studies showed that upon infection by ϕRSS1 phage, the host *R. solanacearum* cells showed several abnormal behaviours including less turbidity in the liquid cultures, less colouration of the colonies on the plates, a decreased growth rate approximately 60% of the normal rate and increased sensitivity to ampicillin (uninfected cells were resistant to 150 μg/ml, while infected cells were sensitive to 15 μg/ml) (Yamada et al. 2007). Interestingly, ϕRSS1 infection also affects the pathogenic activities of the host cells (Yamada et al. 2007). In this study, the main objective was to explore the possibility of using phage as a biocontrol agent for the eradication of *R. solanacearum* and *E. chrysanthemi* in contaminated soil.

Materials and methods

Isolation and purification of phages

Sewage samples were collected from Indah Water Konsortium Sdn. Bhd., Puchong and Port Dickson. Three different types of *Escherichia coli* strains [TG1 (*supE, hsdΔ5, thiΔ(lac-pro AB), F'[traD36, proAB*$^{+}$ *lacI*^{q}*, LacZ, ΔM15*]), ER2738 (F$'$ *pro A*$^{+}$*B*$^{+}$$'$ *lac*$_{1}^{q}$ Δ (*lacz*) *m15 zzf*::*Tn*10 (*Tet*^{R})/*fhu Az gln* vΔ (*lac-proAB*) *thi*-1 Δ (*hsds-mcrB*) 5) and BL21 (*F-ompThsdSB (rB-mB-)galdcm*)] were used as a host for the amplification of the phages.

Each of the *E. coli* culture (5 ml) was added into the fresh Luria Bertani (LB) broth [tryptone (1%), yeast extract (0.5%), NaCl (1%); pH 7.5, 100 ml] containing tetracyclin (5 mg/ml, Sigma). The mixture was incubated with shaking until OD_{600} about 0.5. Sewage sample (50 ml) was added into *E. coli* culture which was prepared previously and incubated overnight at 37 °C at 250 rpm. The phage mixture was centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to the new conical flask.

The supernatant was filtered with membrane filter (0.45 μm, Whatman) to remove the unnecessary particles. The phage particles in the supernatant were precipitated by adding polyethylene glycol (20% PEG 8000) and NaCl (2.5 M). The suspension was kept at 4 °C for 1 h to overnight and centrifuged at 13,000 rpm for 30 min at 4 °C. Finally, phage pellet was resuspended in TBS (50 mM Tris; 150 mM NaCl, pH 7.5).

Phage titration

A single colony of each *E. coli* (TG1, ER2738 and BL21) was inoculated into 5 ml of LB broth and incubated with shaking until OD_{600} about 0.5. Top agarose (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% $MgCl_2.6H_2O$, 0.7% agarose) was aliquoted (3 ml) into tubes and equilibrated at 45 °C in a water bath. Phage (10 μl) and mid-log phase *E. coli* cells were added to the equilibrated top agarose, mixed and poured onto a LB agar plate.

The plates were allowed to cool, and incubated overnight at 37 °C. Plaques formed were counted and the amount of phage was determined as plaque forming unit (pfu). All assays were performed in triplicates. Different type of phage was categorized into different group based on the plaque morphology.

Virulence of Ralstonia solanacearum strains

The virulency of *R. solanacearum* (Code: MP11) was checked by streaking the bacteria suspension on tetrazolium chloride agar (TZCA) [Peptone (10 g/litre), Casein hydrolysate (1 g/litre), Glucose (5 g/litre), 2,3,5-triphenyl tetrazolium chloride (0.05 g/litre), agar (15 g/litre)] and incubated overnight at 28–30 °C. This technique is suitable to differentiate wild colony types (white with pink centres) from low virulence mutants or avirulent mutants (deep red colony) that could occur on subculturing (Kelman 1954).

Lysis activity using agar method

The overnight culture of *R. solanacearum* was mixed with top agarose (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% $MgCl_2.6H_2O$, 0.7% agarose) in tubes which were later incubated in the water bath at 45 °C for 15 min. The mixtures were quickly poured into LB agar and allowed to solidify for 30 min at room temperature (28 °C). The sterile disc (5 mm diameter) was then gently placed onto the surface of the agar. Isolated phage (10^{10} pfu/ml, 5 μl) was applied onto the disc and incubated overnight at 30 °C. The same method was also applied to *E. chrysanthemi* and complete plates were incubated at 35 °C. The test was repeated three times and the clear zone formation was recorded after 24–48 h.

Results and discussion

A total of 132 phage isolates were isolated from the sewage samples (Tan et al. 2008). Each phage showed different morphology which was categorised as clear and turbid plaques with holo in the middle. Out of the 132 phage isolates, 107 (81.1%) were obtained from *E. coli* ER2738, 16 (12.1%) from *E. coli* TG1 and 9 (6.8%) from *E. coli* BL21. The diameter range of the plaque was about 1.0 ± 0.1 to 4.0 ± 0.2 mm. Some phages formed clear zones while the others formed turbid area with one small holo zone in the middle. However, most of these phages formed clear zones (129 isolates) as compared to turbid holo zones (3 isolates). Each of phage isolate was titred and the pfu/ml of phage isolates was ranged from 10^7 to 10^{10} pfu/ml. The titre of 10^{10} pfu/ml was subjected to lysis study against *R. solanacearum* and *E. chrysanthemi*.

From this study, the inhibition reaction could be considered as an initial stage for selection of phages as a potential antagonist agent. From the literature, the diameter of clear zone could be interpreted as (1) <10 mm – bacteria is resistant to the antagonist, (2) range between 11–14 mm – bacteria still can survive at the moderate

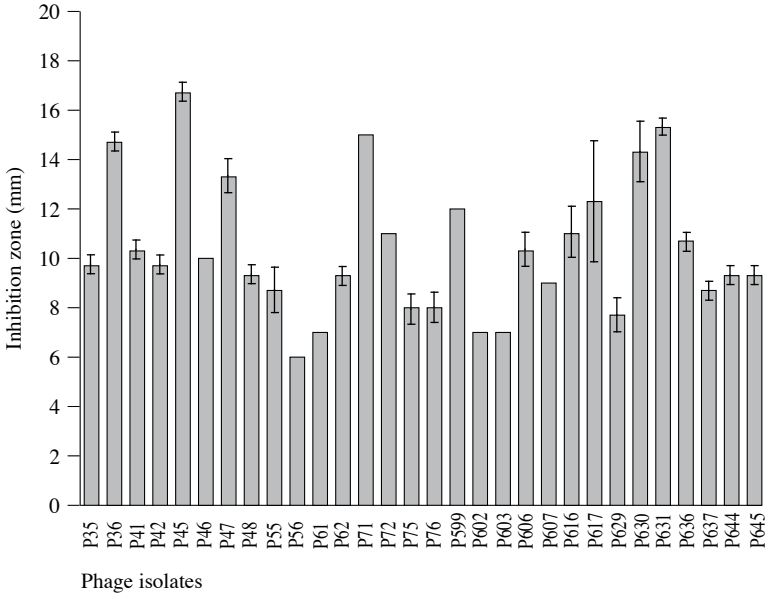

Figure 1. The inhibition zone (mm) formed by lysis activity of isolated phages from sewage against bacterial wilts ***Ralstonia solanacearum****. The test was repeated three times and the clear zone formation was recorded after 24–48 hours*

condition and (3) ≥15 mm – bacteria is susceptible/sensitive to the antagonist (Thomas and Michael 1991).

Out of 132 phage isolates, 30 showed inhibition zone against *R. solanacearum* (*Figure 1*), and 5 showed inhibition zone against *Erwinia chrysanthemi* (*Figure 2*). Three isolates (P45, P71 and P631) (*Plate 1*) caused the highest lysis reaction against *R. solanacearum* on agar diffusion assay (*Table 1)*. Meanwhile, for *E. chrysanthemi*, the highest clear zone ratio only obtained from phage P621 (*Table 2*).

Phages are bacteria predator which can persist within the bacteria cells. In order to grow and multiply, they need to colonize by injecting their DNA into the cell and take over the cell mechanism (Freifelder 1987). For successful initiation of infection, an incoming phage genome has to cross the barrier formed by the host cell wall. In some cases, phage particles are known to contain lytic enzymes that are capable of producing local openings into the murein sacculus (Rydman and Bamford 2002). Once they have colonized the bacteria cells,

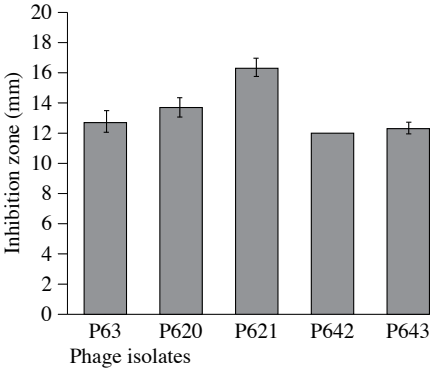

Figure 2. The inhibition zone (mm) formed by lysis activity of isolated phages from sewage against heart rot ***Erwinia chrysanthemi****. The test was repeated three times and the clear zone formation was recorded after 24–48 hours*

they will start producing their progeny by incorporating their DNA into the bacterial DNA and converted into "phage factory". After the process is completed, they will release lytic enzyme to hydrolyze the bacteria cell wall and release their progenies.

Phages have been reported as plant pathogen control agents in apple, pear tree

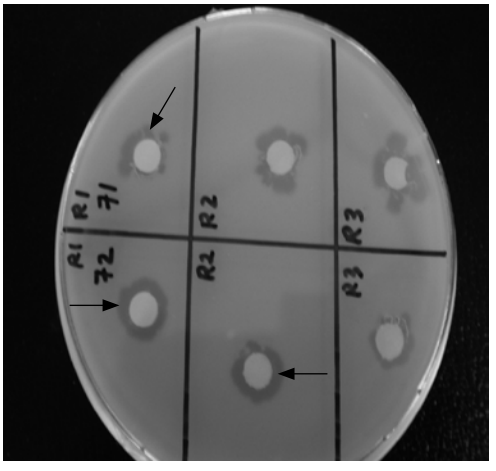

Plate 1. Example of clear zone produced from lysis activity of phage against ***Ralstonia solanacearum*** *(indicated by arrows)*

Table 1. Clear zone ratio produced from lysis activity of phage against *R. solanacearum*

Phage no.	Ratio of clear zones (mm) to disc diameter (mm)*
P36	2.94
P41	2.06
P45	3.34
P46	2.00
P47	2.66
P71	3.00
P72	2.20
P599	2.40
P606	2.06
P616	2.20
P617	2.46
P630	2.86
P631	3.06
P636	2.14

*Disc diameter = 5 mm

Table 2. Clear zone produced from lysis activity of phage with *E. chrysanthemi*

Phage no.	Ratio of clear zones (mm) to disc diameter (mm)*
P63	2.54
P620	2.74
P621	3.26
P642	2.40
P643	2.46

*Disc diameter = 5 mm

and raspberry. These phages effectively control the bacteria population by reducing the optical density to 96% ± 4%, below that of bacteria grown without phage (Schnabel and Jones 2001). The same observation was also reported by Yamada et al. (2007), whereby the growth rate of *R. solanacaerum* strain C319 in tobacco plants was decreased approximately by 60% after being infected by phage ϕRSS1.

Phage was also used against *Salmonella* associated with fresh cut fruit (Leverentz et al. 2001) to disinfest *Streptomyces scabies* that infect potato seed-tuber (McKenna et al. 2001); against bacterial leaf spot of mungbeans (in combination with *Streptomyces*) (Borah et al. 2000); against *Xanthomonas pruni* associated with bacterial spot of peaches (Chattopadhyay and Puls 2000); to control *R. solanacearum* in tobacco (Tanaka et al. 1990; Yamada et al. 2007) and to control soft root and fire blight associated with *Erwinia* sp. (Eayre et al. 1990). Phages are potential to be exploited and utilized as biocontrol agents for eradication and prevention of phytopathogenic problems in plants.

Conclusion

The *R. solanacearum* phages (P45, P71 and P631) and *E. chrysanthemi* phage P621 were found to be potential in reducing pathogenic activity of *R. solanacearum* and *E. chrysanthemi*. However, more studies need to be carried out to determine the effect of phage on plant in order to develop biocontrol agent for the prevention of bacterial wilt in tomato and heart rot in pineapple.

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Abstrak

Dua kumpulan faj, salah satu membentuk plak jernih dan satu lagi membentuk plak keruh dengan ruang kosong di bahagian tengah, telah diasingkan daripada air kumbahan dengan menggunakan tiga strain *Escherichia coli* (TG1, ER2738 dan BL21) sebagai perumah. Semua faj ini digunakan untuk kajian lisis terhadap patogen bakteria *Ralstonia solanacearum* dan *Erwinia chrysanthemi*. Faj-faj ini adalah sangat spesifik kepada perumah dan boleh digunakan sebagai agen pengawalan biologi terhadap penyakit tanaman. Dengan itu, kajian ini bertujuan untuk mengasingkan faj terhadap *R. solanacearum* dan *E. chrysanthemi*, dan seterusnya menilai sifat-sifat lisisnya. Sejumlah 132 isolat faj telah diasingkan daripada air kumbahan. Tiga daripadanya (P45, P71 dan P631) menunjukkan potensi untuk mengawal *R. solanacearum* dengan kadar zon jernih yang tinggi iaitu masing-masing 3.34, 3.00 dan 3.06. Bagi mengawal *E. chrysanthemi*, cuma P621 yang menunjukkan potensi kadar zon jernih 3.26. Faj yang lain tidak menunjukkan keputusan yang baik. Walaupun faj-faj ini menunjukkan potensi yang baik, namun kajian yang lebih terperinci perlu dijalankan untuk menghasilkan agen pengawalan penyakit tanaman yang efisien.

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