Streptomyces spp. culture filtrates reduced T4 infectivity to E. coli

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Abstract. A Streptomyces spp. culture was isolated and identified. Its filtrate was found to be able to destroy ORSV and CYmMV virus coat protein, and could be used to reduce the transmission of plant virus disease in vivo, in our previous study. We also tested and found that it can destroy the coat proteins of 13 other of plant viruses, suggesting that the mechanism of action is not specific to a few plant viruses. Aim: To test if this culture filtrate can destroy or affect the infectivity of non-plant viruses. Materials and methods: Streptomyces spp. were cultured in soybean-based culture medium for 14 days, and the culture was collected and filtered. T4 phage infection of E. coli was used as a model. E. coli was cultured on 0.75% TSA agar plates. The T4 phage was incubated with various concentrations of Streptomyces spp culture filtrate for 30 minutes, before adding to the E. coli lawn. Culture filtrates of two strains, the C5-6 and the SML-1, were used. T4 phage incubated with 300 ppm virusbom (a known anti-viral agent) was used as a positive control. The formation of T4 lysis colony was calculated for the plaque-forming unit (PFU). Results: The dilution of 1 in 8 of the culture filtrates reduced the number of phage colony on E. coli lawn. The infectivity was significantly reduced when T4 was incubated with the culture filtrate at 1/8 dilution when compared to the non-treated groups. The virusbom treated PFU was significantly reduced, in comparison with C5-6 treated group (P=0.013), and with SML-1 treated group (P=0.028) as per ANOVA test followed by Tukey post-hoc comparison. Summary: These data demonstrated that culture filtrates of Streptomyces spp. reduced T4 to E. coli infectivity and suggested that the anti-viral compounds in the filtrate is not specific to plant viruses. The application of the culture filtrate and its content might have broader applications.

1. Introduction

Antiviral agents are chemicals that inhibit the spread of virus, by blocking replication of the genome, preventing entry to host cells, or reducing viral protein synthesis or viral assembly. Although there are small synthetic molecules or biotech engineered molecules available for popular, specific viral disease such as HIV/AIDS or HPV/cervical cancer, amongst many others [1-3], broad range antiviral agents are still in demands. They were not used for specific virus but rather a general anti-viral, environmental cleansing reagent that would prevent the spreading of the virus. Traditionally chemicals that denature proteins are often used for this purpose. For example, for prevention of influenza...
spreading, the hands were washed by detergent such as soap, or and the floor was mopped by diluted bleach. However, in certain area of application there is still needs for different chemicals with variety of working mechanism. For example, in a laboratory working bench, the sterilizing reagent of hands and gloves is 70% alcohol, which might not be ideal for technician with sensitive skin. Further, methods that apply heat, bleach, or alkaline solution are detrimental to the environment and were impossible to use on plants or human skin. A broad range antiviral agent could be used in cases like this.

It has been discovered that a sterilizing material of Streptomyces spp. culture filtrate was able to destroy Odontoglossum ringspot virus (ORSV) and Cymbidium mosaic virus (CYmMV) virus coat protein, and potentially act as an anti-viral agent [4,5]. Previous tests found that the concentrated filtrate could destroy the infectivity of both viruses to the orchid within one minute of incubation in culture filtrates [4]. Therefore, it is possible to us for immediate disinfection of equipment, bench surface, or even technician’s skin.

On a broader scale, some of the plant derived anti-viral reagents were reported to target more than just plant viruses [6]. Viral proteases, themselves are protein in nature, are target for protease [6-8]. In this sense, proteases are good candidate for anti-viral agents against specific viruses. Furthermore, bacteria of the Streptomyces genus were known to secrete various enzymes against biological big molecules [9-11]. We are interested to know whether our culture filtrate could target viruses other than plant viruses. In our case, the active ingredient was possibly a protein, as it was sensitive to treatment temperature higher than 70°C [12]. Proteases has been used to act against viral spreading or replication, as many of the enzymes that were vital to viral survival are proteins [7]. For example, the addition of serine protease inhibitors improves the treatment outcome of patients infected with hepatitis C virus genotype [13]. The activity of our product was stable for up to 6-months’ storage at 4°C.

We have previously found that the virus eradication of our culture filtrate was not restricted to ORSV and CymMV, as other 13 viruses were tested and found effective [4,12]. In this project, we aim to test if the application of this anti-viral agent could be extended to viruses other than plant viruses.

2. Materials and methods

2.1. Preparation of the test agent

Streptomyces spp. was cultured in a soybean based medium for 14 days, and the medium collected, filtered through 0.22 μm membrane (Amicon). Escherichia coli DH5-α was regrown from a commercial kit of competent cells and T4 bacteriophage (BCRC no. 70041) was purchased from Bioresource Collection and Research Center (BCRC) Taiwan. Difco™ Luria-Bertani (LB), Bacto™ trypsic soy broth (TSB) were purchased as powders from Becton, Dickinson and Company (DB) (LB broth, 244620, TSB, 211825). American bacteriological agar was purchased from Laboratorios CONDA (Madrid, Spain).

2.2. Treatment groups

Control treatment was prepared by using the bacterial culture medium incubated at 37°C for 14 days, without inoculation with bacteria. The C5-6 strain was isolated by Chen and Chang [14] and SML-1 by Chien and Chang, 2018. Cultured medium was collected and filtered at 0.22 μm and used. T4 phage incubated with virusbom was used as a positive control for anti-viral effect.

2.3. Zymography

The zymography was performed by a method modified from Michaud [15]. Samples were separated, using 10% SDS-gel with 0.1% gelatin (Invitrogen, Carlsbad, CA). After separation, the gel was renatured by washing in 2.5% Triton X-100 and incubated in substrate buffer (50 mMTris-HCl, pH 7.5, 10 mM CaCl₂, and 0.01% Sodium Azide) for 48 hr at 37°C to enable the possible enzyme to cleave the gelatin. Each gel was then stained with Coomassie Blue for 1 hr and destained in a solution
of 30% methanol and 10% acetic acid. Negative staining indicated the locations of active protease bands.

2.4. Plaque forming unit (PFU)
This is performed by an adoption to Adams [16] and Andersons et al [17]. Briefly, LB agar in Petri dishes were streaked with DH5-α strain of E. coli and incubated overnight at 37°C. A 50mL aliquots of sterile LB broth was inoculated with a single colony and incubated overnight with shaking, until the optical density reached an OD600 >1. The petri-dish was lined with 10 ml of 1.5% TSA. The overlaying agar was prepared by mixing 3 mL of overnight culture of DH5-α E. coli and 50mL of 0.75% warm TSA. 5mL mixture was used for 1 dish. The T4 phage was incubated with treatment material for 30 minutes, followed by serial dilution by 1% peptone. 20 μl of the diluted T4 phage was spread evenly on the surface of 0.75% TSA by glass beads and incubated at 37°C overnight. Petri dishes, exhibiting between 20 to 200 well isolated plaques, were used to calculate the phage titers and for subsequent statistical analyses. The PFU was calculated as: pfu/m=number of colony * dilution * (1ml/phage volume used)

2.5. Treatment groups
T4 phage was passed through 0.22 μm filter, and 10 μl of T4 phage and 10 μl of the treatment liquid were mixed and incubated for 30 minutes, before 10 μl of mixture was spread on DH5-α E. coli-containing top agar layer. The treatments were: 1/8 of culture filtrate of SML-1 strain of the Streptomyces spp., 1/8 of culture filtrate of C5-6 strain of the Streptomyces spp., 1/8 of 300 ppm virusbom (commercial product line from Money Marketing Communication Ltd), or LB broth only.

3. Results
3.1. Biochemical characteristics
The anti-ORSV or anti-CYmMV activities were lost when incubated at temperature higher than 70°C, and these activities were sustained when kept at 4°C for 6 months. This suggested that at least one of the active ingredients in the culture filtrate was protein. We went on to test whether the active ingredient was a protease. In the zymography experiments, the culture filtrate was concentrated by DEAE sepharose column, and was separated on the 10% SDS-gel with 0.1% gelatin. The gel was renatured for 48 hours for the possible enzyme to activate. The commassie blue staining revealed a negative staining band, indicating the presence of protease in the culture filtrate (figure 1).
Figure 1. A Commassie Blue staining for a SDS-gel containing 1% gelatin which has been digested by the proteinase activity within the culture filtrate of C5-6 strain. The proteinase activity was located to roughly 37 kDa area of the gel, suggesting there may be a proteinase of this size in the culture filtrate. It is not known whether this is the same activity that affected the E. coli infectivity.

3.2. Bacteria infectivity
Three treatments (SML1, C5-6, virusbom) were diluted 1/2, 1/4 and 1/8 and tested for E. coli infectivity. The results were similar among different dilutions, suggesting that further dilution may be needed. In this paper we only listed the results for the 1/8 dilutions. The phage was mixed with treatment agents for 30 minutes before it was serial diluted $10^5$ to $10^{10}$ for plating. In the 4 tests, the phage colonies were about 20 for the phage diluted at $10^6$, and 100-200 colonies for the phage diluted at $10^5$ as shown in figure 2.

The T4 phage was incubated with treatment material (see following) for 30 minutes, followed by serial dilution by 1% peptone. 20uL of the diluted T4 phage was spread evenly on the surface of 0.75% TSA by glass beads and incubated at 37°C overnight. Plates that has $10^5$ diluted phage mixture typically yield about 200 colonies and were chosen to calculate phage forming unit (PFU).
Figure 2. Row A: T4 phage without treatment. Row B: T4 phage treated with 1/8 SML-1 strain filtrate. Row C: T4 phage treated with 1/8 C5-6 strain filtrate. D: T4 phage treated with 1/8 300ppm virusbom. N=4.

3.3. PFU
We calculated the PFU from those of the $10^5$ dilutions of phage. The PFU was listed in the table 1 and figure 3. The standard deviation (SD) was between 0.05-0.29, which was typical of the experiments and was acceptable (table 1).

| treatments   | test 1 | test2 | test3 | test4 | mean | SD  |
|--------------|--------|-------|-------|-------|------|-----|
| T4           | 3.18   | 3.06  | 2.66  | 2.60  | 2.88 | 0.29|
| T4+SML1      | 2.24   | 2.16  | 1.76  | 1.74  | 1.98 | 0.26|
| T4+C5-6      | 2.08   | 2.1   | 1.8   | 1.84  | 1.96 | 0.16|
| T4+virusbom  | 1.52   | 1.58  | 1.66  | 1.58  | 1.59 | 0.05|
Figure 3. Distribution of PFU values for T4 with different treatments. N=4.

3.4. Statistics
ANOVA and Tukey HSD test were used to assess whether measurements were different between treatments and where the source of major variation (see supplementary file). Multiple one-tailed two-sample t tests were conducted to assess our major concerns: whether T4+virusbom, T4+C5-6, or T4+SML1 were better than T4, and whether T4+virusbom is better than T4+C5-6 or T4+SML1. The analyses were computed in R v3.5.

The results of 5 one-tailed t-tests indicated that the measurements of T4+C5-6, T4+SML1, and T4+virusbom are significantly less than the measurements of T4 (*, p value=0.006, 0.006 and 0.006), and the measurements of T4+C5-6 and T4+SML1 were significantly higher than the measurements of T4+virusbom (#, p value=0.013, 0.028) (figure 4).

Figure 4. Comparison of the treated with non-treated. SMLA-1 treated and C5-6 treated are significantly lower than T4 none-treated (*). Those treated with virusbom showed significantly smaller PFU than T4 treated with C5-6, as well as with SML1 treated (#). N=4, ANOVA and Tukey HSD followed by one tail test.
4. Discussion

4.1. Biochemical characteristics
A gelatinase activity was demonstrated by the C5-6 culture filtrate. The proteinase activity was located to roughly 37 kDa area of the gel, suggesting a proteinase of this size was present in the culture filtrate. Although the activity that eradicated the plant virus was lost when the concentrated culture filtrate was heated over 70°C, this only suggested that part of the activity could be contributed to a protein, and our present data do not support a hypothesis that this gelatinase activity is the major activity that reduced the *E. coli* infectivity. Further study is underway to search for the identify for the possible active molecule.

4.2. Information for further safety evaluation
The culture filtrate itself was a mixture of rich nutrient aimed for providing the growth of bacteria, and part of the activity was attributed to a protein. This conclusion provided information to the safety evaluation for further development for this product. One of the possible usage of this product would be application on the technician’s skin. We have done a separate project on the cytotoxicity and genotoxicity effects of the filtrate using cell culture model (manuscript submitted).

4.3. Extension of target to T4 phage merit further development for human disease related virus
Our data demonstrated that the culture filtrate from SMLA-1 treated and C5-6 treatment reduced the T4 to *E. coli* infectivity. Please note that these were only the culture filtrate without concentration or isolation. The way that un-concentrated filtrate could work on infectivity of a phage suggest that the concentration of the active ingredient is high enough in the culture filtrate to merit further development to make an anti-viral product. We have previously demonstrated that the targets of the concentrated culture filtrate extended to more than 13 plant viruses. In this project the extension of targets to the T4 phage suggested a broader application on viruses related to human disease could be explored.

4.4. Comparison to virusbom
Virusbom, a patented anti-viral product sold in Taiwan was used as a positive control. This chemicals collapsed viral envelop, change the structure of the bacteria and reduced bacteria growth [18]. The structure of the virusbom was not published, but it was reported in news to be a synthetic enzyme. 300-1000 ppm was the concentration reported to kill NDM positive Klebsiela Pneumonae [18]. In our tests, 300 ppm of virusbom, when diluted 1 in 8, was able to reduce the infectivity of T4 phage to the *E. coli*, and the effect was better than that of C5-6 and SML-1 filtrate. The C5-6 and SMLA-1 was not concentrated and the final concentration of usage was not determined. Hence this piece of information does not provide evidence to support the superiority of virusbom over our future product. It would be interesting to carry out side-by-side comparison when we further develop this into a more mature form of product.

5. Summary
These data demonstrated that the culture filtrates from both strains of the culture reduced the T4 to *E. coli* infectivity, and suggested that the anti-viral compounds in the filtrates was not specific to plant viruses. The application of the culture filtrate and its content might have broader application than we previously known.

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