New microbial origin compound capable of destruct T4 bacterial phage structure

M J Lee¹, ², B L Liu¹, J H Yen¹ and C A Chang¹, ²

¹ Department of Applied Chemistry, Chaoyang University of Technology, Taichung, Taiwan

² E-mail: cachang@cyut.edu.tw (CC Chang); mjlee@cyut.edu.tw (MJ Lee)

Abstract. Background: We have isolated culture of Streptomyces species that was able to degrade orchid virus capsid proteins and inhibiting virus infection. Odontoglossum ringspot virus infection could be greatly reduced by incubation with the bacterial culture. The infectivity to T4 was reduced after incubation of these culture filtrates, suggesting the activity of the culture filtrate could extend to non-plant viruses. Objectives: To further verify whether the reduction of the infectivity to T4 phage could result in visually measurable difference in the virus morphology after treatment of the bacterial culture. Methods: The T4 phage was grown in DH5α strain of E.coli, and collected in the culture medium. The phage containing medium was filtered with 0.45 μM filter. Two strains of Streptomyces spp, SML-1 and C5-6, were selected based on their optimal growth. The effects of culture filtrate of these two strains on destroying the T4 phage was tested. Morphology of T4 bacteriophage was inspected by atomic force microscopy (AFM). The culture filtrates of SML-1 and CA5-06 strains were diluted to 1/4, and mixed 1:1 to the T4 phage suspension, incubated for 30 minutes, before further 10x and 100x dilution. The mixture was dried on mica and observed using AFM. Results: The culture filtrate of CA5-06 strain is capable of reducing the fragment of the head of T4 phage from an average of 66.5nm to 53.3nm (1/8 dilution). The culture filtrate of SML-1 strain is capable of reducing the fragment of the head of T4 phage from an average of 66.5nm to 62.3nm (1/8 dilution). The data obtained helped to develop practical application methods of the culture filtrates to control virus spreading.

1. Introduction

Culture of Streptomyces species that was able to destroy orchid virus capsid proteins and reduce virus infection was isolated in Professor Ching An Chang’s lab [1, 2].

A bacterial culture was isolated. The culture filtrate was identified to degrade the coat protein of Odontoglossum ringspot virus (ORSV), as well as Cymbidium mosaic virus (CYmMV) [1]. The bacteria was identified to be Streptomyces species. It is discovered that the concentrated filtrate reduced the infection rate of both viruses to the orchid [1]. One of the strains tested for these experiments is the CA5-6. Additional strains of the genus were isolated from the soil around the Sun Moon Lake area in Taiwan for their anti-orchid virus activities [3]. The SML1 strain was among the strains isolated from the Sun Moon Lake area. The SML-1 and the CA5-6 strains were tested for their growth conditions, and were selected for further development and research based on their optimal growth (BL Liu, personal communications).

Next, to test whether the anti-viral effect could be extended to non-plant viruses, these two strains were tested to see whether the culture filtrate could reduce the infectivity to the T4 virus. The T4 virus was pre-incubated with the culture filtrates, and the infectivity was tested by the bacteria plate-based assay, to see if they reduced the rate of infection of T4 bacteriophage to the E. Coli [3, 4]. The plaque

[1] Content from this work may be used under the terms of the CreativeCommons Attribution 3.0 licence. Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.
Published under licence by IOP Publishing Ltd
forming unit (PFU) was used as an indication of the infectivity. It was demonstrated that when used one in eighth dilution, the culture filtrate decreased the PFU on the E. Coli layer. The virusbum, a proprietary anti-viral agent commercially available, further reduced the PFU when compared to the C5-6 treated group, but not to the SML-1 treated group[4].

In this project we aim to further verify whether the reduction of the infectivity could result in visually measurable difference in the virus morphology after treatment of the bacterial culture. Escherichia virus T4, or T4 phage, is a species of bacteriophages that infect Escherichia coli bacteria. It is a double-stranded DNA virus in the subfamily Teenviriniae from the family Myoviridae. We choose it as our test for non-plant virus because of several reasons: firstly, it is one of the best studied model organisms. Secondly, its size is the largest among the known viruses. The total length of the body of the phage is about 200nM, and the tails are another 200 nM. Third, they have a symmetrical while complex, almost rocket-like appearance and easy to identify morphologically. These make the virus an ideal target to use for the AFM study.

2. Material and methods

2.1. Material
T4 bacteriophage (cat number 70041) was bought from a depository facility of the in Food Industry Research and Development Institute (FIRDI) in Taiwan, BCRC, Taiwan. Streptomyces species are a gift from Professor Ching An Chang. DH5-α Escherichia coli was obtained from regrowing the competent cells of a commercial kit (Thermo Fisher Scientific). American bacteriological agar powder was bought from Laboratorios CONDA (Madrid, Spain). Difco™ Luria-Bertani (LB), catalog number 244620 was purchased from Becton and Dickinson (DB). Bacto™ trypsic soy broth (TSB) powder, catalog number 211825, was bought from DB.

2.2. Preparation of the bacterial culture filtrate and T4 phage
The medium for the bacterial growth is a soybean based medium, and the length for growing the bacteria is 14 days. The medium of Streptomyces spp. was filtered through a 0.22 μM membrane. DH5-α E. coli was purchased from Thermo Fisher Scientific and used as a phage host. DH5-α E. coli was grown in Tryptic Soy Broth (TSB). For the T4 bacteriophage, 100 mL liquid culture of E. coli obtained from single colony was grown in TSB at 37 °C overnight. The T4 phage was added to 1 mL of the E. coli culture, serial diluted, streaked on plate and grow overnight to obtain single viral plaques. 10 mL E. coli culture added to 10 mL of TSB media was inoculated with single viral plaque. After 6 hours of shaking, the solution was centrifuged at 1000 rpm for 25 minutes to sediment the cellular debris. The phage containing supernatant was collected and passed through filter; the solution generated was used for experiments.

2.3. Determination of T4 virus length using AFM
The mixture mounted on mica is observed by atomic force microscope (AFM) (Nanoview1000, FSM–Precision). Tapping mode was used, and 3000 nM x 3000 nM areas in channels including phase and amplitude were recorded. Images were obtained from 5 points on the mica for the same sample, which include the center, and four points in the periphery of the mica (as in figure 1). For measuring the virus size, zoomed-in pictures were captured and virus size estimated by the built-in software (Nanoview1000, FSM–Precision).

Figure 1: Location of 5 areas for sampling the viruses.
2.4. Effect of the bacterial culture filtrate on T4 virus morphology

The control for the experiment was the soybean based medium that we used for bacterial culture, incubated at 37°C for two weeks, without the bacteria. Filtrate of culture of test strains, the C5-6 and the SML-1, were isolated and passed through 0.22 μM before use [1]. The positive control was the T4 phage without the treatment of anti-viral agent. T4 phage was filtered using 0.45 μM filter, and equal amount of the T4 phage and the treatment liquid was blended well and incubated for 30 minutes, diluted with distilled water 100 time, before spotted on the mica and air dried overnight. The tests are: 1. 8 times diluted culture filtrate of SML-1 strain, 2. 8 times diluted culture filtrate of C5-6 strain, 3. 8 times 300ppm virusbum (proprietary product from National Taiwan University, commercial product distributed by Money Marketing Communication Ltd), or 4. LB broth only.

2.5. Statistical analysis

Six areas were selected, and head and tails of untreated individual viruses’ size were delineated and measured using AFM the built-in software. The average value was obtained for the size of the heads, and for the size of the tails, separately.

3. Results

3.1. Determination of T4 virus length using AFM

The head of the T4 virus is about 65 nM x 70 nM, the tail is about 120 nM, and the total of the length is about 235 nM. Therefore, it is predictable that most of the virus would be stuck by the pore of the 0.22 μM, unless the virus entered the pored vertically. Our results collaborated with this prediction that with 0.22 μM filter very few viruses was observed in the filtrate while 0.45 μM yielded many viruses (figure 2). The filtering of the virus was necessary, as the medium still contain bacterial fragments from the virus preparation, and without filtration, the filtrate contained gigantic particles that was fragment of lysed bacteria observed under the microscope.

![Figure 2](image-url)

**Figure 2:** Number of virus in the filtrate after.
A: 0.22 μM filter with the height channel;
B: 0.45 μM filter with the height channel;
C: 0.22 μM filter with the phase channel;
D: 0.45 μM filter with the phase channel

The T4 virus was selected from 5 area of the mica as described in the Material and Method section. Area of oval shaped, pale yellow to white, and about 70 nM in length, was delineated as the head of the virus (red arrow, figure 3). Rod like shape attached to the head that was about 120 nM was delineated...
as the tail of the virus (white arrow, figure 3). All discernible viruses were measured and averaged. The average length of the head was 66.5 nM, and the average length of the tail was 106.4 nM (figure 3).

Figure 3: Determination of untreated individual viruses size using AFM built-in software.

3.2. Effect of bacterial culture filtrate of CA5-6 strain on T4 virus morphology
When the culture filtrate of CA5-6 was tested, the T4 virus was incubated with 1/8 dilution of the filtrate of CA5-6 for 30 minutes, before diluted 100 time with water, and dried on mica for observation. Area of pale yellow to white in color was delineated as the head of the virus (figure 4). There was no rod like shape attached to the head (figure 4). Virus fragments in all 6 pictures were measured and averaged. The average length of the head was 53.3 nM.

3.3. Effect of bacterial culture filtrate of SML-1 strain on T4 virus morphology

When the culture filtrate of SML-1 was tested, the T4 virus was incubated with 1/8 dilution of the filtrate of SML-1 for 30 minutes, before diluted 100 time with water, and dried on mica for observation. Area of pale yellow to white was delineated was the head of the virus (figure 5). There was no rod like shape attached to the head (figure 5). All virus fragments in the 6 pictures were measured and averaged. The average length of the head was 62.3 nM.

4. Discussion and Conclusion

The bacterial culture affected the morphology of T4 virus in several ways. Firstly, the head and the tail fragment were unattached. Secondly, the diameter of the head of the virus was reduced. The base plate and long tail fibers were too narrow for AFM to clearly detected. The head, the tail and the long tail fibers (LTFs) of T4 are assembled independently before they are joined together to produce a mature phage [5], therefore they should be the first to dissociate from each other, and so did we observed.

There are some early studies using T4 phage as a model system to test the feasibility of AFM to measure the size and delineate the shape of a virus [6-9]. There are some study using AFM for the study of T4 adhesin, or protein needle, for developing T4 protein as nano-material [10, 11]. However, there were no research done using AFM to study the effect of an anti-viral agent on T4 phage. Our study therefore is, if not the first, among the first to describe such study.

In our previous study, we have demonstrated that the bacterial culture contain proteinase [3]. In other words, part of the action we observed for the bacterial culture filtrate might be mediated by its proteinase activity. After incubation with the culture filtrate, the diameter of the T4 head was reduced. The T4 phage head capsid consists of 155 hexamers of the major protein, gene product 23. A similar protein, gp24*, forms pentamers at 11 of the 12 pentameric vertices. There are two accessory proteins, Hoc (highly antigenic outer capsid protein) and Soc (small outer capsid protein) attach to the capsid surface [12]. Of these proteins, the breakdown of gp23 or gp24 would results in large area of destruction, or even demolition of the whole capsid and exposure of DNA. Therefore, it is more possible that the proteinase, if indeed is the active ingredient, act on the accessory proteins to reduce the diabeter of the head.

For the product to be developed and used on human, the culture filtrate need to be tested for its safety. Our next project would be to test the toxicity of the culture filtrate. Also, we are currently isolating the proteins that would be responsible for this activity.

Both the 1/8 dilution of the CA5-6 and SML-1 strains could change the morphology of the T4 viruses. The CA5-6 was more effective in reducing the size of the head of the virus, while the SML-1 was not as effective. However, both were successful at destructing the tails of the viruses.

References

[1] Chang C A, Chen H R and Chiu C H 2010 Disinfection of odontoglossum ringspot virus and cymbidium mosaic virus from tools used during orchid cultivation. International Society for Horticultural Science (ISHS), Leuven, Belgium.

[2] Chen H R 2009 Production and application of an orchid virus degrading protease secreting by an Actinomycetes, in Graduate institute of biochemical science and technology. Chaoyang University of Technology Taiwan. p. 91.

[3] Lee M J, Liu B L, Chen C J, Chang K M, Huang S D and Chang C A 2019 Streptomyces spp. culture filtrates deconstruct Odontoglossum ringspot virus (ORSV) and Cymbidium mosaic virus (CymMV) virus coat. IOP Conf. Series: Earth Environ. Sci. 346
Lee M J, Liu B L, Kuo H R, Chen C J, Chang K M and Chang C A 2019 Streptomyces spp. culture filtrates reduced T4 infectivity to E. coli IOP Conf. Ser.: Earth Environ. Sci., 346.

Rao V B and Black L W 2010 Structure and assembly of bacteriophage T4 head. Virol. J., 7: 356.

Firtel M and Beveridge T J 1995 Scanning probe microscopy in microbiology. Micron., 26(4): 347-62.

Ikai A et al. 1993 Atomic force microscopy of bacteriophage T4 and its tube-baseplate complex. FEBS Lett., 326(1-3): 39-41.

Kuznetsov Y G, Chang S C and McPherson A 2011 Investigation of bacteriophage T4 by atomic force microscopy. Bacteriophage, 1(3): 165-73.

Matsko N et al. 2001 Atomic force microscopy analysis of bacteriophages phiKZ and T4. J Electron Microsc (Tokyo), 50(5): 417-22.

Brzozowska E et al. 2018 Interactions of bacteriophage T4 adhesin with selected lipopolysaccharides studied using atomic force microscopy. Sci. Rep., 8(1): 10935.

Ueno T et al. 2020 Dynamic behavior of an artificial protein needle contacting a membrane observed by high-speed atomic force microscopy. Nanoscale, 12(15): 8166-73.

Yap M L and Rossmann M G 2014 Structure and function of bacteriophage T4. Future Microbiol., 9(12): 1319-27.