Morphological characterization of putative phages infecting soil *Streptomyces* from Woods Hole, Massachusetts

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Limited information is available on phages infecting soil *Streptomyces* from Woods Hole, Massachusetts. I report here the result of preliminary attempts to isolate and to morphologically characterize putative phages infecting soil *Streptomyces* from the aforementioned area. Plaque assay using Nutrient Agar and AGS solid media yielded in putative plaques formation, with sizes ranging from 1 to 6 mm, in only one of seven isolates of *Streptomyces* sp. used in this study. Enumeration using an epifluorescence microscope indicated a higher number (8.9 x 10^9 cell/ml) of virus-like particles (VLP) in phage enrichment of soil compared to the number counted from the extract of soil collected from the courtyard of Loeb Laboratory (LC) (4.43 x 10^8 cell/ml). There were at least three types of negatively stained putative phage of *Streptomyces* observed with Transparent Electron Microscopy (TEM), and their morphology resembled those reported in prior publications. All three were linear double stranded (ds)-DNA phage. The first type had a polyhedral head (diameter approximately 84 nm) with collar, and non-contractile tail (about 150 nm long), whereas the second type’s head was smaller (about 53 nm) with contractile tail (around 73 nm). The third type had a slightly smaller head diameter (around 74 nm) with no apparent collar and shorter tail (126 nm). This last type formed a semi-rosette assembly with their tails. Further works will be necessary to completely characterize these phages.

Introduction

*Streptomyces* phages, are one of the most studied phages. Information regarding bacteriophages infecting other groups of bacteria from Woods Hole Massachusetts was available (e.g. Ling 2010, Sharon 2010, Lee 2008), but no study has ever been done on those infecting *Streptomyces*. This subject of research was chosen because it could mark the beginning of integral research about this type of phage from Woods Hole.

The oldest report published about the actinophages was from 1936 by Wiebols and Wieringa (Woodruff et al 1947), and the existence of phages attacking *Streptomyces* strains were reported in 1947 after their appearance in commercial fermentations (Alstyne et al. 1955). According to Lomovskaya et al. (1980) and Diaz et al. (1989) *Streptomyces* phages are classified into Bradley group B or Siphoviridae/Myoviridae family, with polyhedral heads ranging from 50-79 nm and tail length from 100 to 327 nm. They are easily isolated from soil with pH levels more than 5, and they will form plaque after overnight incubation at 28 to 30°C. It is estimated that there are 10 viruses for every bacteria. However, not every *Streptomyces* can be infected by a phage, and this is due to host specificity and host resistance phenomena (see for example Gilmour & Ingalsbe [1959] and Thieman et al [1964] about this subject).

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The objectives of this work were to isolate and morphologically characterize phage infecting soil *Streptomyces* from Woods Hole Massachusetts, and to identify any isolate that is sensitive to this phage infection.

**Material and methods**

*Plaque Assay*

Two approaches were used to assess the lytic capacity of phage to potential hosts. Seven *Streptomyces* isolates were used in both, and they were previously isolated from soil collected in the courtyard of Loeb Laboratory (LC), Woods Hole Massachusetts (Fig 1). For the first approach, 2 g of LC soil was mixed with autoclaved 20 ml of distilled water and vortexed. The mix was subsequently filtered using a 0.2 μ syringe filter. The filtrate obtained (1 ml) was then mixed with a 0.5 ml suspension of spore and mycelia and incubated for 15 minutes at room temperature to allow the phages to attack the potential hosts. The spore and mycelia suspension was prepared by scraping the spore and mycelia from isolate lawn on AGS media previously flooded with 1 to 3 ml autoclaved Tween 80. The suspension was transferred by sterile pipette onto sterile 15 ml centrifuge tubes and then filtered using autoclaved glass wool or cheese cloth and stocked at 4°C until use. The mix was then incubated at 42°C for 1 minute, and was subsequently used to make the overlay agar on 15 g/L Nutrient Agar (NA). The Nutrient Agar (3%) used for the overlay mix was enriched with Ca(NO3)2.4H2O and MgSO4.7H2O (4 mM each) to facilitate the attachment of phage to their potential host. Overlay agar plates were incubated at 30°C until the formation of the putative plaques.

![Figure 1](image.png)

**Figure 1.** Soil sample collection area in the courtyard of Loeb laboratory building, Woods Hole, MA (left). The white arrow showed the spot where the soil was collected (right).

The second approach was modified from a *Streptomyces* phage isolation protocol (Microarray website, 2013). Five grams of soil was mixed with 0.5 ml spore suspension and 9.5 ml Nutrient Broth enriched with Ca(NO3)2.4H2O, MgSO4.7H2O (4 mM each) and Glucose (0.5% final concentration). The mix was subsequently incubated overnight in a shaker (150 rpm, 30°C). About 5 ml from each of the seven phage enrichment with potential hosts were transferred to sterile 15 ml centrifuge tubes and were centrifuged
(4000 rpm, 40 min) using a Beckman Coulter Altegra to obtain clear supernatant. The supernatants were then filtered (0.45 µm) into clean 15 ml centrifuge tubes using a 10 ml syringe and 100 µl of chloroform was added. The filtrate was then used (100 µl) to make the overlay agar using 100 µl of spore suspensions in 2 ml top agar. The spore suspensions used were obtained using similar technique with the first approach, with careful separation from remaining mycelia. However, contrary to the first approach, AGS solid media was also used in a separate trial than the enriched NA, and the incubation was done at 37°C. Plaques formed were picked from the agar using autoclaved Pasteur pipette and transferred to an autoclaved microfuge tube and vortexed briefly. They were left in a closed bench drawer for 30 minutes, and used as phage filtrate for the subsequent agar overlay using similar media as the previous step. Plaque-forming units per ml (pfu/ml) was counted using equation (1):

\[
\text{pfu/ml} = \frac{\text{number of plaques/dilution factor} \times (1/0.1 \text{ ml plated})}{100}\]  

SYBR staining and viral count

Virus-like particles (VLP) were counted using epifluorescence microscopy according to Patel et al (2007) using 0.8 um nitrocellulose membrane support filters (Millipore) and 0.02 um Anodisc filters (Whatman). The total cell count was calculated using the equation (2) (Kleindienst, personal comm.):

\[
\text{TTC} = \frac{N \times A_{\text{Filter}} \times F_{\text{Dilution}}}{A_{\text{Grid}} \times V}
\]

Where, \(A_{\text{Grid}}\): Area of the Grid  
\(A_{\text{Filter}}\): Effective Filter Area (mm\(^2\))  
\(N\): Number of Cells per Grid (average from multiple Grids)  
\(F_{\text{Dilution}}\): Dilution Factor  
\(V\): Filtered volume (ml)  
\(TCC\): Total Cell Count (Cell/ml)

Transparent Electron Microscopy (TEM)

Prior to observation, the sample was negatively stained with a 2% uranyl acetate solution using the method proposed by Brum (2011). Morphological structure of Streptomyces phage and bacteriophage in the control (soil extract) were observed using the JEOL200CX analog TE microscope.

Host characterization

Macromorphological features of the potential host were identified according to Waksman (1959). The aerial mycelium type was observed using slide culture technique. The DNA was extracted using the phenol-chloroform method and amplified using universal bacterial 16S primers 8F/1492R. The PCR reaction includes 6.5 uL Nuclease-free water, 12.5 uL Promega Go-Taq Green 2X Mix, 2 uL of each primer, and 2 uL of template DNA per reaction. DNA was denatured at 95°C for 2 minutes followed by thirty-four cycles at 95°C for 30 seconds, 46°C for 30 seconds, and 72°C for 1.5 minutes. The final elongation step occurs at 72°C for 10 minutes. PCR product obtained was purified using Wizard PCR Preps DNA Purification System (Promega) and sent for sequencing. The sequencing result was analyzed online with BLAST in the GenBank database from NCBI.

If the potential host produced pigment soluble in solid media, the pigment was extracted from the agar by cutting or smashing the solid media into very fine particles, mixed with autoclaved distilled water and then filtered with Whatman filter paper. The remaining pigment in the agar was re-extracted until it became colorless. The water was evaporated from the crude extract using a water bath set at 60°C.
Results and discussion

Plaque assay

The plaque assay using the first approach yielded to spore growth in lawn without a clearing zone (plaque formation) (Fig 2a). The spore growth was observed in all plates after 48 hours of incubation. Interestingly, in one of the plates using spore and mycelia suspension of *Streptomyces* isolate YHT6, individual colonies grew (1-2 mm in diameter) and formed a clearing zone (Fig 1b). This could probably be interpreted as a reaction against other soil bacteria present in the soil extract on the overlay mix. However, further experiments will be needed to confirm this hypothesis.

![Plates of plaque assay using the first approach](image)

**Figure 2.** Plates of plaque assay using the first approach (see material and methods for further details) after 48 hours of incubation at 30°C. Example of lawn in one of the plates (*Streptomyces* sp. isolate YHTS) without any identifiable plaque (a). Clearing zone observed on plate using *Streptomyces* sp. isolate YHT6 (6-14 mm in diameter).

The second approach of plaque assay resulted in plaque formations on both types of media (NA and AGS) (Fig 3.) after 48 hours of incubation only on those using *Streptomyces* sp. isolate YHT6 as inoculums. No plaque was observed on the remaining six isolates’ plates. This could be an indication that the aforementioned isolate was potentially more susceptible to lytic phage infection compared to the other six used in these experiments. Additionally, the phages infecting other isolates could only be from the lysogenic types and thus no formation of plaques could be observed due to the phage not provoking host cell lysis.

Plaques formed in NA plates (23.4 pfu/ml) were clear with the sizes ranging from 1 to 3 mm (Fig 3a). In contrast, plaques formed in AGS plates (7.65 pfu/ml) were turbid, with sizes ranging from 1 to 6 mm (Fig 3b). Plaques morphology can be influenced by the phage type, the host, and the growth conditions. The difference in plaque sizes could due to different time of infection during the bacteria growth phase (Maloy 2002). Pre-adsorption at low temperature would then be preferable should the work be carried out in the future, in order to ensure the development of all the phages at the same time. An attempt to isolate the phage was carried out. Unfortunately, when two plaques from each plate were picked and transferred to
another plate using the same plaque assay method, no growth was observed. This was most likely caused by the spore suspension in inoculum being used. Spore density was probably not high enough to form a lawn, and as a consequence, no growth was observed.

Figure 3. Plates of plaque assay using the second approach after 48 hours of incubation at 37°C with NA (a) and AGS (b) as solid media. Inoculum used was Streptomyces sp. isolate YHT6. Note that the irregular forms on these two plates were uneven distribution of top agar when poured onto the solid media.

SYBR staining and viral count
Due to technical constraints and time limitations, only phage filtrate with plaque formation was SYBR-stained and counted. Virus-like particles from phage filtrate of Streptomyces sp. isolate YHT6 were visibly denser than those observed in soil extract LC as control (Fig 4). This was confirmed by the count, where the phage filtrate VLP count was higher (8.9 x 10^9 cell/ml) compared to the one from soil extract (4.43 x 10^8 cell/ml). There was most likely a bias in the counting, because VLP signals tended to fade instantly when excited with fluorescence work. This could due to the degradation of anti-fade reagent (Patel et al 2007). SYBR green was made to stain double stranded DNA (Invitrogen, no date) so the probability that the VLP observed were eventually Streptomyces phage was higher. However, this is still need to be confirmed with morphological observation of the putative phages using TEM.
Figure 4. SYBR staining results of soil extract LC (a), phage filtrate with *Streptomyces* sp. isolate YHT6 (b) and filter-sterilized distilled water (c). White ellips: Virus-like particles; rectangular: Bacteria

**TEM**

Due to similar reason with the SYBR stain, TEM observation was also carried out only with the phage filtrate with *Streptomyces* sp. isolate YHT6. Soil extract LC (Fig 5) was used as control to increase the probability that the phages found in the filtrate were eventually the ones that attacking the *Streptomyces* sp. isolate YHT6 and not other type of bacteriophages. Some interesting structures were observed. There were at least three different types of Virus-like cells. One structure resembled a lot to lambdoid bacteriophage with head and tail, although the head was not very clear to be identified morphologically. Another two structures were similar morphologically with member of virus family with single strand DNA (filamentous and head without tail). One paramaecium-like cell was also observed on the grid, and one structure was not identifiable. There was not much of structure to be found due to the grid’s poor condition (torn).
There were at least three types of putative phages infecting *Streptomyces* sp. isolate YHT6. All of them resembled bacteriophage belonged to the family *Myoviridae*, which were characterized by a tail and polyhedral head containing linear double stranded DNA (Fig 6 and 7). The first type had a polyhedral head (about 84 nm in diameter), collar and non-contractile tail (around 150 nm) and tail fiber. The second type had smaller head (53 nm in diameter) and shorter tail that was contractile (73 nm long). A collar was also observed in this second structure. These structures resembled the previously published phages infecting *Streptomyces* strain (see for example in Diaz et al. 1980).

Type 3 resembled a lot to type 1, with slightly smaller head (around 74 nm) and shorter tail (126 nm) (Fig 7). Interestingly, no apparent collar was observed, and it formed a semi-rosette tail assembly. Such
structure was never reported before in prior studies about phages infecting *Streptomyces*. However, similar structures, such as rosette tail assembly (Grahn *et al* 1999; Quiberoni *et al* 2010) and clustered tails (Brussow *et al* 1994) were reported on other bacteriophages. Quiberoni *et al* (2010) hypothesized that the formation was caused by the agglutination of the tail. However it was not clear whether this formation was derived from the sample treatment prior to mounting, or as a form of adaptation. Further studies will be necessary to know the exact cause of this formation on a putative *Streptomyces* phage.

![Semi-rosette tail assembly](image)

**Figure 7.** Transparent electron microscopy image of the third type of putative phages infecting *Streptomyces* sp. isolate YHT6. Note that two heads were empty and this could probably due to the injection of their genomes already into the host during the phage enrichment from soil (see material and methods for details regarding the incubation).

**Host characterization**

According to the identification guide written by Waksman and Lechevalier (1953), *Streptomyces* sp. isolate YHT6 resembled highly to *Streptomyces ipomoea* morphological characteristics. This strain was known as sweet potato pathogen. Individual pure colonies had white aerial mycelium, chalky, tough and leathery surface (Fig 8a). Soluble faint purple pigment was observed after seven days of incubation (Fig 8b). This pigment was soluble in water (Fig 8c) and in Acetone: Methanol 7:3 (v/v). It was not stable when exposed to light and temperature higher than 30°C.

Sporulation observed after five days of incubation, with purple microdroplets of secondary metabolites appeared after seven days of incubation (Fig 8a) and turned colorless two days after. Spore production in long chain was the retinaculapiaperti type (Fig 8d).

Sequencing result of 16S rRNA showed similarity of sequence (92%) with *Streptomyces* sp. SAP837.1, which was isolated from floral nectar bacterial communities of Mediterranean plants. However, no literatures were found about phages infecting this strain. The low similarity of sequence was due to the poor quality of 16S rRNA sequence obtained.
Figure 8. Morphological features of *Streptomyces* sp. isolate YHT6. Individual colony with microdroplets of secondary metabolites (a); Soluble pigment in AGS media after 7 days of incubation (b); Pigment crude extract in water (c); Structure of spore production in long chain (retinaculiperti) (d).

Conclusion

Despite time limitations and technical constraints, preliminary morphological characterization of putative phages infecting soil *Streptomyces* isolates from Woods Hole could be accomplished. Only one of seven isolates of *Streptomyces* sp. was susceptible to phage infection. Total cell count of VLP was 10 times higher than bacteria like particle. Three type of putative phages of *Streptomyces* sp. isolate YHT6 were identified and characterized morphologically. They belonged to Myoviridae family and characterized by polyhedral head and tail. One type showed formation similar to other non-*Streptomyces* phages, which was semi rosette.
**Perspective**

In order to obtain integral picture about phages infecting soil *Streptomyces*, more approaches needs to be done, should there be any project done in the future regarding this subject. Optimization of lytic phage isolation can be done using R2YE medium, a type of medium known to facilitate optimal growth of *Streptomyces* strains. DNA extraction and sequencing of phage genome should constitute the next step, as well as adsorption and one step growth experiments. Finally, it will be interesting to attempt the cross-infection with soil extract from other location to find out the host specificity of the putative phages. It will also be interesting to study the connection between *Streptomyces* isolates’ secondary metabolite production and their susceptibility to phage infection.

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**References**


