PSU McNair Scholars Online Journal

Volume 11
Issue 1 *Without Borders: Original Contributions in Undergraduate Research*

Article 5

2017

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Citation: Nader, S.M., Stedman, K. Sulfolobus Spindle-Shaped Virus 1 Growth Kinetics. Portland State University McNair Scholars Online Journal, Vol. 11, Year: 2017

Abstract

Geothermal and hypersaline environments are rich in viral particles, among which spindle-shaped morphologies predominate. Currently, viruses with spindle- or lemon-shaped virions are unique to Archaea and belong to two distinct viral families. The larger of the two families, the *Fuselloviridae*, encompasses spindle-shaped viruses with very short tails, which infect *Sulfolobus solfataricus* and close relatives. Sulfolobus spindle-shaped virus 1 (SSV1) is the best-known member of the family and was one of the first hyperthermophilic archaeal viruses to be isolated. However, our knowledge of fuselloviral life cycles and the relationships between these viruses and their hosts is still limited. As a result, to establish one-step growth curves to study virus replication kinetics and to investigate the mechanism of host binding, the adsorption kinetics of wild-type SSV1 to its Sulfolobus host needed to be determined.

Keywords: SSV1, adsorption kinetics, binding assay

Introduction

Prokaryotic-infecting viruses are the most predominant biological entities on Earth. Like all viruses, they are not able to replicate on their own, but require a host cell to release their genome and reprogram that host to produce more viruses. Viruses are thus intracellular parasites that have symbiotic relationship with all three domains of life (*Archaea*, *Bacteria* and *Eukarya*). Moreover, viruses often imply a co-evolution with their host and exert natural control of the dominant populations (Lobo et al., 2009; Sharp & Simmonds, 2011; Best & Kerr, 2000). In some ecosystems, viruses are known to serve as organic matter to lower parts of the food web and they are able to modify the fixation and cycling of key elements, such as carbon, phosphorus and nitrogen globally (Wilhelm & Suttle**,** 1999). Thus, viruses can have important ecological impacts. Fundamental studies of viruses are crucial in a broad range of biological sciences, such as biotechnology and molecular biology, health and medicine, ecology and evolution.

The viruses of Archaea in particular exhibit completely novel and unique morphologies and forms (Prangishvili and Garrett, 2005). Shortly after the recognition of the Archaea, research focused on isolation of viruses and viral particles from extremely hot, low pH or hyper saline ecosystems (Woese et al. 1978; Albers et al. 2013). In many geothermal hot springs around the world, members of the *Fuselloviridae* family of viruses are found to be most abundant. The archetype, *Sulfolobus* spindle-shaped virus 1 (SSV1), was isolated from a geothermal hot spring in Beppu, Japan and found to contain a 15.465 kb circular double-stranded DNA (dsDNA) genome, containing 35 open reading frames (ORFs), of which the products of only five have been assigned functions (Iverson and Stedman 2012; Quemin et al., 2015). The genomes can be found as episomes, integrated into the host genome, or packaged as enveloped virions. SSV virions are generally lemon or spindle-shaped, about 60nm by 90nm in size, with sticky tail-fibers at one end (Ceballos et al., 2012; Stedman et al., 2015).

The hosts for most *Fuselloviruses* are members of the *Sulfolobales*, a widespread genus of archaea. Brock et al. first described this hyperthermophilic and acidophilic sulfur-oxidizing Creanarchaeote in 1972. They isolated several *Sulfolobus* strains from thermic and acidic habitats in the Yellowstone National Park (USA), El Salvador, Italy and Dominica (Brock et al., 1972). The genome of *Sulfolobus solfataricus* from Pisciarelli, Italy, the main host for SSV1, is completely sequenced and its growth is optimal between 70°C and 80°C and at pH 2 to 3 (She et al., 2001). Sulfolobus have a proteinaceous Surface layer or S-layer with hexagonal symmetry which is strikingly similar to the geometry of the putative tail structure of SSV1 (Stedman et al., 2015).

Although spindle-shaped viruses represent one of the most prominent viral groups in Archaea, very little is known about the life cycle of SSV1 and its relationship with its host. The gold-standard for examining virus replication cycles is the so-called one-step growth curve, whereby host cells are simultaneously infected with virus, washed to remove free virus, and then virus production followed over time to determine the average kinetics of virus replication. The first step of such a one-step growth curve, and the very first step of infection is when viruses bind to cells. It is this step, the virus adsorption period, which is the focus of this study.

Knowledge of host-cell binding will allow one-step growth curve experiments to determine the kinetics of SSV1 replication. Moreover, how SSV1 interacts with its Sulfolobus host at high temperature (80°C) and low pH (3) is unknown. Determining binding kinetics will also allow imaging experiments where SSV bound to Sulfolobus at the very first step of the infection process can be imaged with transmission electron microscopy. The mechanism by which the positively supercoiled SSV1 genome is released into the host cell is also not known. Being able to isolate freshly bound SSV1 may allow insight into this critical step in virus infection.

Once wild-type (WT) SSV1 has been characterized, this will allow us to work with different SSV mutants and identify the growth characteristics of these mutant viruses. Optimization of binding may also allow the production of large numbers of SSV1 virus particles, which will be critical for the determination of a higher resolution structure than currently available and allow testing of the hypothesis that the capsid of the SSV1 virus is similar in overall morphology to that of the nucleocapsid of HIV-1 (Stedman et al., 2015).

Methods

Growth of Sulfolobus cultures:

Sulfolobus cultures were grown from small amounts of frozen cell stocks (~50 µL) and inoculated into 5 mL of Yeast-Sucrose (YS) medium at pH 3.0-3.2, then incubated in a 75º C shaking incubator (Gyrotory water bath shaker G76, Speed 4.2, New Brunswick Scientific) until turbid (48 – 96 hr) (Iverson & Stedman, 2012). Generally, cells were then transferred to a larger volume of YS medium (50 – 100 mL) in long-neck Erlenmeyer flasks and incubated until the desired OD_{600nm} was reached.

SSV1 production in stably-infected S441 cells:

S524 (*Sulfolobus* strain *S441* infected with wild-type SSV1) was cultured from frozen stock in 5 mL of YS medium at 75º C until turbid (~72 hr) (Iverson and Stedman, 2012). This culture was transferred to 50 mL fresh YS medium in a long neck flask and grown for 120 hours. The cell density (OD_{600nm}) of S524 was 0.854 when cultures were removed from high temperature. Cultures were centrifuged for 10 min at 6,000 x g (Eppendorf 5810R) at room temperature. The supernatant was collected and split into two aliquots of equal volume, and viral titer (PFU/mL) was measured (Schleper et al., 1996). The average viral titer at each time point was calculated from plaque assays done with cell-free supernatants.

Viral binding to host:

A culture of S441 was diluted with YS medium to OD_{600nm} of ~0.13 and allowed to recover at 75º C for 2 – 4 hours until the optical density reached 0.16, a value experimentally determined to equal 10^8 *Sulfolobus* cells/mL (Iverson & Stedman, 2012; Prangishvili et al., 1999). 40 mL of the S441 culture was added to 4 mL of SSV1 to yield the desired MOI of 0.1. Negative controls were prepared using an equal volume of YS medium in place of the host cells. Following addition of virus, cultures were incubated at 75º C and the viral titers were measured at various time points throughout the experiment. Samples (1 mL) were prepared for plaque assay by pelleting the cells via centrifugation (5 min) at 6,000 x g room temperature and carefully collecting the virus supernatant (~500 µL) by pipet.

Plaque Assays

Virus titers were measured via plaque assay as in Schleper et al. (1992) and Stedman et al. (1999). Briefly, an S441 culture was diluted with fresh YS medium to OD_{600nm} 0.35 – 0.38 and allowed to recover at 75º C until the cell density reached ~0.40 (2 – 4 hours). Cells were centrifuged at 3,000 rpm (Eppendorf 5810R) for 5 minutes and pellets were re-suspended in a volume of YS resulting in ten-fold concentration of the cells. Purified virus suspensions were serially-diluted in YS liquid medium. Virus dilutions were prepared by ten-fold serial dilution of a virus stock or the collected samples at different time points in YS medium. 100µL of each SSV1 dilution were added to 500 µL of 10X concentrated *Sulfolobus* cells and 5 mL of soft-layer was added and the mixture was quickly poured and evenly distributed on a warm YS Gel-rite plate. So-called "Soft-layers" were allowed to solidify at room temperature for 20 min and were then placed in a sealed plastic box with a moist paper towel and incubated for 48 hours at 27º C. Plaques began to appear after 24 hours but were most visible after 48 hours of incubation. Plaque Forming Units (PFUs) were counted and the titer of the original purified virus suspension (PFUs/mL) was calculated as follows:

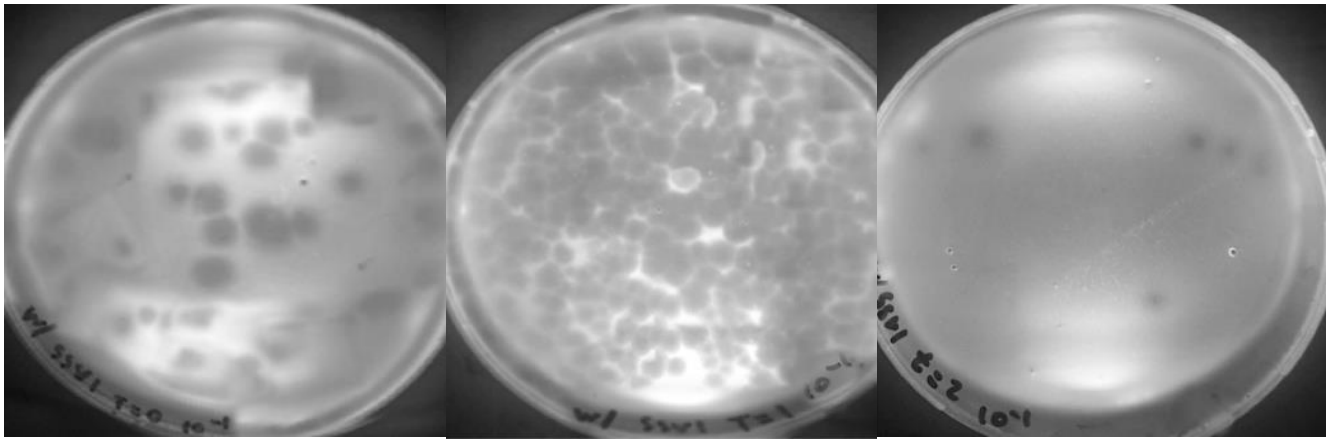
$$Virus\ titer = \frac{Plaques\ observed}{Volume\ added * \ Dilution\ Factor}$$

Results and Discussion

Infection of S441 cells with SSV1 at a low MOI was performed to measure the kinetics of viral adsorption. Briefly, 4 mL of SSV1 with a titer of 2.0 x 10^8 PFU/mL was isolated from the virus preparation made from culture of S524 (as above) and was mixed with 40 mL of S441 cells (OD_{600nm} = 0.16 = 10^8 CFU/mL) for an MOI of 0.2. An uninfected control was also prepared using an equal volume of YS in place of virus supernatant. The infected SSV1 cultures and the uninfected control were simultaneously grown at 75º C for 4 hours with shaking in long-neck flasks and samples (1 mL) were taken after every 30 minutes. The samples were centrifuged at high speed (6,000 x g; Eppendorf 5810R) to pellet cells and the virus-containing supernatant was collected to measure the SSV1 titer via plaque assay (typical plaques are shown in Figure 1). The initial titer of the infected sample (immediately after dilution) was low (~10^1 PFU/mL) and reached a maximum of 10^5 PFU/mL within 30 minutes of incubation. In stably infected cultures after several hours the titer significantly decreased (Figure 2). Repetitions of this experiment routinely resulted in viral titers in this range (10^5 – 10^7 PFU/mL) (Table 1). Ideally, a decline in virus titer would be expected upon virus adsorption, however since the contamination of the sulfolobus cultures, it resulted in unusual titers between points 0 and 0.5 and between 1.5 and 2 (Figure 2). Also, the majority of the uninfected cells produced good lawns mostly in the early time points indicating that the host cells were in good condition for the first half hour, after which cell debris were observed in the both infected and uninfected cultures. This correlated with the plaque results showing debris on the lawns after time point 1 (Figure 1B). Similar results were obtained for infected cultures, in which dramatic decrease in titer were observed after 30 minutes (Figure 2). Ideally it was expected that for infected cultures viral titer would decrease after incubation for longer than 1 hour. Data for the SSV1 adsorption constant has not been published, however, data from an undergraduate honors thesis reported that SSV1 at an initial titer of 10^5 HFU/mL (halo-forming units/mL) became undetectable within 5 hours following incubation with *S. solfataricus* G-theta at 23º C. The MOI of this incubation was 0.01 (Drummond thesis 2010). In our case, it is probable that the viral adsorption did not occur properly because the viral titers before infection were within a reasonable range (10^5 – 10^8 PFU/mL), however, after incubation of the host with SSV1 for several hours, there was a noticeable decrease in titers with visible cell debris. The presence of cell debris implies cell death, but it is unclear if this is due to external contamination or lysis by SSV1. In addition, incubating SSV1 with YS media did not result in cell debris after several hours of incubation, this indicates that the debris comes from S441 cells.

Another potential factor affecting rates of adsorption is motion within the adsorption medium, where too little motion (i.e., lack of mixing or agitation) or too much motion could have resulted in reduced rates of virus adsorption (Delbrück et al., 1940; Koch et al., 1960; Anderson, T.F. 1949). Thus, it would be worthwhile to further investigate this phenomenon, as the collected data is not sufficient to make an accurate determination of the true kinetics of viral infection. Multiple trials need to be conducted to eliminate plating and/or culture preparation errors that might have limited the precision of measurements, as sufficient data will help improve the accuracy of one-step growth curves and the information about SSV1 infections that they provide.

A.



B

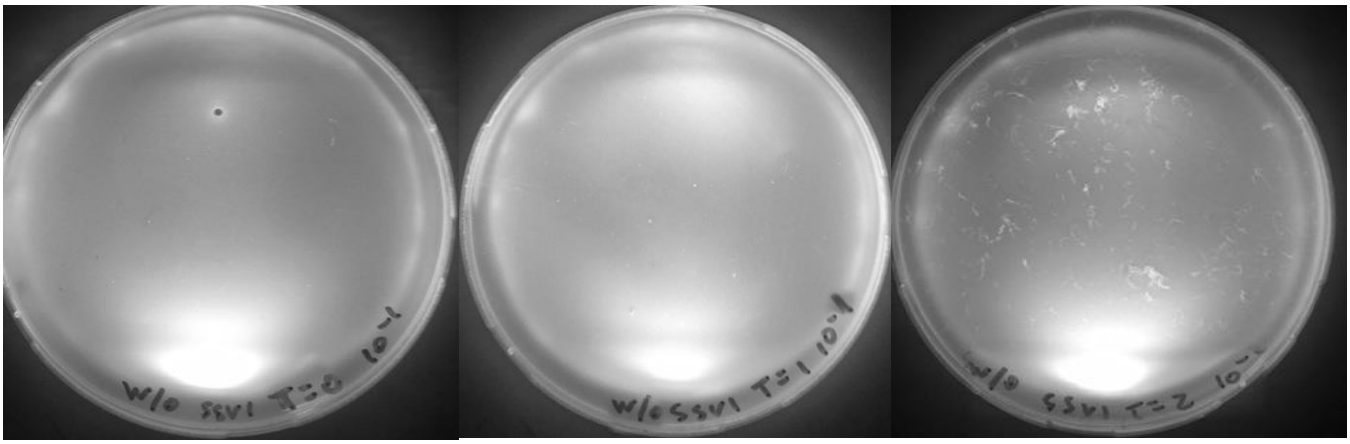


***Figure 1: A**. Plaque assay results for infected S441 with SSV1 immediately after mixing with SSV1 (10^{-1} dilution), after 1 and 2 hours of incubation at (10^{-1} dilution), respectively. **B**. Plaque assay results for uninfected control immediately after incubation (10^{-1} dilution), after 1 and 2 hours of incubation (10^{-1} dilution) respectively.*

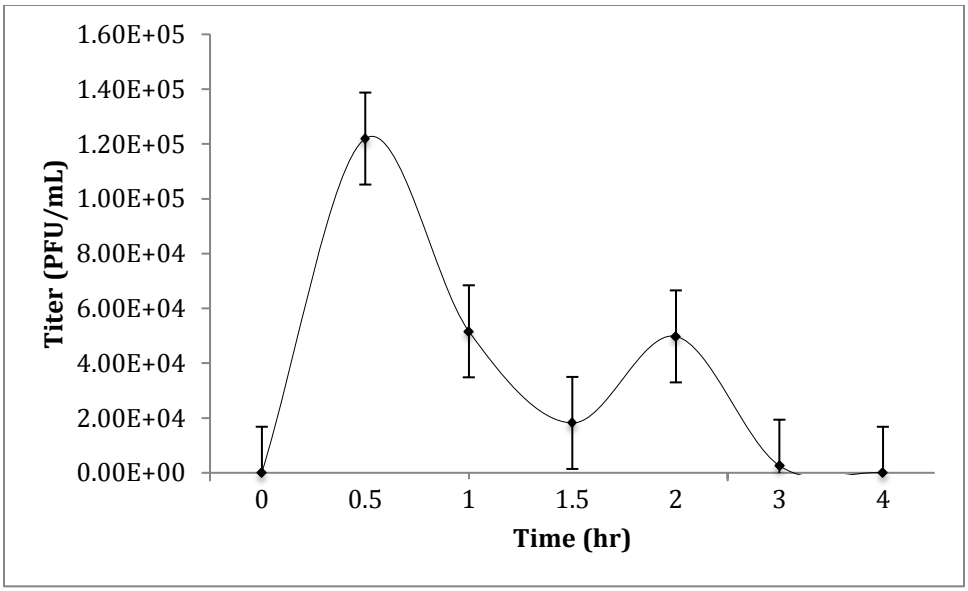


Figure 2: Titer of SSV1-infected S441 cells
Sulfolobus cells of strain S441 stably infected with SSV1 (diamonds) were diluted to 75 mL with fresh YS medium and incubated for 4 hours at 75º C. Samples (1 mL) were taken every half hour to measure the viral titer by plaque assay. The viral titer was measured in triplicate at each time point and the average viral titer (PFU/mL) of two independent cultures are presented with standard deviations.

Table 1: Titers from serial dilutions of the prepared SSV1 supernatant with 0.01 MOI for 3 replicates.

SSV1 dilutions	*Viral Titers for Trial 1 (PFU/mL)*	*Viral Titers for Trial 2 (PFU/mL)*	*Viral Titers for Trial 3 (PFU/mL)*
10^{-1}	1.32×10^{7}	2.05×10^{8}	1.28×10^{5}
10^{-2}	1.73×10^{5}	6.75×10^{5}	3.04×10^{6}
10^{-3}	1.71×10^{5}	1.70×10^{5}	4.00×10^{5}
10^{-4}	3.04×10^{5}	0	0
10^{-5}	2.00×10^{6}	0	0
Average	**$2.81 \pm 5.81 \times 10^{6}$**	**$4.12 \pm 9.16 \times 10^{7}$**	**$7.14 \pm 1.31 \times 10^{6}$**

Conclusion

This work focused on the hyperthermophilic virus from the family *Fuselloviridae*: SSV1. While in the process of establishing a platform for performing a one-step growth curves to study virus replication kinetics and to investigate the mechanism of host binding, we were unable to gather viable data to be able to draw conclusions about the adsorption kinetics of wild-type SSV1 to its Sulfolobus host. However, through this experiment we learned how to better handle and work with hyperthermophilic viruses. For instance, we were able to infer from the results that to better understand how the binding of the virus to its host occurs, infection protocols should be optimized in order to obtain high titers. In addition, it was found that titer values should be reported to the cell concentration, in order to obtain more comparable data and be able to perform one-step growth curve that would be equated to those performed in the past (Iverson thesis 2015). Thus, more work needs to be done to clarify and expand upon these ambiguous results and optimize the binding assay. In the future, this would also permit to observe how SSV bound to Sulofobus at the very first step of the infection process by transmission electron microscopy (TEM). In addition, being able to isolate freshly bound SSV1 may allow insight into this critical step in virus infection.

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