geneELISA for highthroughput examination of phage–host interactions

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Abstract

Bacterial viruses (phages) influence global biogeochemical cycles by modulating bacterial mortality, metabolic output and evolution. However, our understanding of phage infections is limited by few methods and environmentally-relevant model systems. Prior work showed that Cellulophaga baltica phage ϕ38:1 infects its original host lytically, and an alternative host either delayed lytically or lysogenically. Here we investigate these infections through traditional and marker-based approaches, and introduce geneELISA for highthroughput examination of phage–host interactions.

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Guidelines

Experimental procedures

Methods for phage ϕ38:1 and the C. baltica host strains have been adapted from previous work (Holmfeldt et al., 2007; Holmfeldt & Howard-Varona et al 2014).

Cell growth curves

Prior to the infection experiments (see below), growth curves for the *C. baltica* hosts were assayed in biological triplicates to determine optical density (OD, 595 nm) and cell abundance over time (via the agar plating technique, Sambrook and Russell, 2000), in order to determine growth rate. Growth curves started with the inoculation of a single colony (0 hours) and were sampled for OD (in technical duplicates using a microplate reader Appliskan, Thermo Scientific, USA) and colony forming units (CFU) mainly throughout exponential growth. Doubling time (t) was obtained from the exponential growth graph, with formula $G_t=G_0 \times a^t$, where G_t (the cell population at a given generation) = $2G_0$ (where G_0 is the initial cell density) and *a* is constant. This resulted in 94.8 minutes and 80 minutes of doubling time for the original and the alternative hosts, respectively. All infection experiments were done with cells in log/linear phase (i.e., 9-□□14 hours of growth), during which cells reached densities of ~$1.5x10^7$ - $3x10^8$ cells ml^{-1} (i.e., OD range after blank subtraction, 0.02-0.08).

One-step growth curves

One-step growth curves of ϕ38:1 infecting *C. baltica* hosts NN016038 and #18 were modified from Holmfeldt & Howard-Varona et al. (2014). For each phageâ□-host system, two biological replicates and the corresponding no-phage control were followed over time, measuring cell (see below) and phage abundance. Bacterial hosts in logarithmic phase, at densities of $3x10^7$ -10^8 cells ml^{-1} (i.e., OD

range 0.013-0.03), were separately mixed with phages at high multiplicity of infection (MOI 3 and 6 for the two short and the long infection experiments, respectively). All MOIs refer to infective phages. After 15 minutes of phage-□□bacteria adsorption time, the mixtures were diluted 10-fold (for experiments depicted in Figures 2-□□5) or 1000-fold in Marine Luria-Bertani (MLB) medium to reduce the likelihood of additional phage adsorptions. All times reported are after the phage-□□host dilution (i.e., a time reported as 70 minutes is 85 minutes after mixing and 70 minutes after the 10-fold dilution). Samples were collected periodically throughout each infection for downstream analyses.

Plaque assays and one-step growth curve parameters

To calculate the burst size, both free (extracellular) and total (extra- and intracellular) phages were enumerated as plaque forming units (PFUs) (Holmfeldt & Howard-Varona et al, 2014). At every assayed time point, free phages were obtained by diluting 100-fold 10 µL of the MLB-diluted original phage-□□host infection into 990 µL of MSM (Holmfeldt & Howard-Varona et al 2014), 0.2 µm filtering that mixture to remove the cells, and then diluting the flow-through for plaque assays. The total phages were sampled directly from the MLB-diluted original phageâ□□host infection without previous filtering. Plating was done by adding 100 µl from a diluted total or free phage tube to a Zobell plate (Holmfeldt & Howard-Varona et al 2014) followed by a mixture of 3.5 ml of 0.5% top MSM (i.e., low melting point agarose) warmed up to 35°C and 300 µl of host. Plates were left in the dark at room temperature (RT) until the plaques became visible, and plaque counts were averaged from two consecutive dilution series to obtain the number of PFUs ml^{-1} (i.e., phage abundance). The latent period was determined as the last time point in which total and free phage abundance did not increase significantly from the previous time, as determined by a twotailed t-test ($p<0.05$). The burst size (i.e., phages produced per infected cell) was calculated by subtracting the free phage values after the burst (i.e., point after which there was no significant increase in phage abundance) from those before the burst (averaged values from all the time points within the latent period), and then dividing that number by the number of infected cells, obtained from viable cell plating as explained below.

Estimates of infected cells by viable cell counts

Cells were diluted and plated in Zobell agar plates to obtain CFU estimates before phage addition and then at every time point that phage abundance was assayed (see above). The fraction of viable cells was obtained from the ratio of CFUs at that time and the CFUs before phage addition (divided by 10 to correct for the dilution), which subtracted from the initial 100% cells gave the percent of infected cells. The reported percent of infected cells was the average of all points that were within the host's doubling time (see above).

Probe synthesis for phageFISH and geneELISA

Genomes of ϕ38:1 and *C. baltica* NN016038 and #18 were obtained from GenBank, accession numbers KC821614.1, CP009887 and CP009976, respectively. A total of 12 probes (300 base pairs each) achieved high gene detection efficiency within infected cells (i.e., ~100 %, Allers et al., 2013). Polynucleotide probes against phage DNA were synthesized and digoxigenin (DIG)-dUTP was incorporated using a PCR DIG-probe synthesis kit (Catalog No. 11636090910, Roche, USA). Annealing/melting temperatures were determined for these probes and their targets (Moraru et al., 2010) and using a SYTO 9 fluorescent nucleic acid stain (Catalog No. S-34854, Molecular Probes®, Life Technologies, USA).

PhageFISH assay and adjustment of infection parameters

Samples were prepared on positively-charged slides with a 10-minute incubation before sample drying and fixation, and phageFISH was performed as previously described (Allers et al., 2013). Briefly, after host cell permeabilization and inactivation of endogenous peroxidases, bacterial 16S rRNA was detected by horseradish peroxidase (HRP)- labelled oligonucleotide probes (EUB338; Amann et al., 1990) and a subsequent catalyzed reporter deposition (CARD) reaction of Alexa488-tyramides (Catalog No. T-20932, Molecular Probes®, Life Technologies, USA). Detection of phage ϕ□38:1 was accomplished by targeting 12 genomic regions (i.e., 39844-40143, 40144-40443, 43571-□□43870, 43871- 44161, 44784-□□45083, 45084-□□45392, 45760-□□46059, 46394-□□46693, 48221-□□48538, 48570-48861, 48870-□□49169, 49203-49507, in base pairs), which were simultaneously hybridized with the 12 DIG-containing probes. DIG was then detected by HRP-labelled antibodies, followed by CARD of the Alexa594-tyramides (Catalog No. T-20935, Molecular Probes®, Life Technologies, USA). Samples were counterstained with DAPI and analyzed by epifluorescence microscopy to determine the percent of infected cells (i.e., overlap of phage and bacteria signals) and phage-induced lysed cells (i.e., scattering of phage signal and reduction/loss of bacterial signal). A total of 200 infected cells per replicate were counted and the area of their phage signal was classified into three classes: <0.4 μm^2 (class I; early-lysis stage), 0.4-1.4 μm^2 (class II; mid-late stage) and >1.4 μm^2 (class III; late-lysis stage). The phage signal area was calculated as: πab where a and b are the semi-major and semi-minor axis of an ellipse, respectively.

PhageFISH estimates allowed adjusting the latent period and burst size calculations described above. The latent period ended with phageFISH-detected cell lysis, while the burst size incorporated the percent of infected cells during that revised latent period and the free phages after such burst.

GeneELISA

GeneELISA detected phage genes via a colorimetric reaction of 3,3′,5,5′- Tetramethylbenzidine (TMB) catalyzed by HRP directly or indirectly linked to the probes. Samples were pooled from replicate flasks, fixed in 1% formalin, stored at 4°C for <12 h, and aliquoted into quadruplicate wells (100 µl in each) of Corning™ BioCoat™ poly-D-lysine multiwell plates (Catalog No. 08-774-127, Fisher Scientific, USA). Plates were briefly centrifuged (500 g, 5 minutes) to allow cells to bind to the positively-charged surface of the wells. The unbound cells and free phages were removed after aspirating off the supernatant and washing twice with Phosphate Buffered Saline-Tween 20 (0.05%), allowing the total number of cells in the wells to remain constant over time. Fixed cells were immobilized, permeabilized, and treated with 0.01 M HCl at RT for 10 minutes to inactivate endogenous peroxidases, and with RNase I (0.5 U μl^{-1}) and RNase A (30 µg μl^{-1}) to digest mRNA. Phage genes were simultaneously hybridized with all 12 DIG-labelled probes, and HRP-labelled antibodies were introduced to detect the DIG molecules. Signal amplification was conducted by using the Tyramide signal amplification kit (Catalog No. T-20931, Molecular Probes®, Life Technologies, USA) to allow the binding of tyramide-biotin, followed by streptavidin-HRP. HRP molecules catalyzed the reaction of a chromogenic substrate 3,3′,5,5′-Tetramethylbenzidine (Catalog No. 54827-17-7, Sigma-Aldrich, USA), and the color development was measured via OD at 620 nm using a microplate reader (Appliskan, Thermo Scientific, USA).

References

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Materials

PCR DIG-probe synthesis kit 11636090910 by Sigma Aldrich

SYTO 9 fluorescent nucleic acid stain S-34854 by Life Technologies

Alexa488-tyramides T-20932 by Life Technologies

Corning™ BioCoat™ poly-D-lysine multiwell plates 08-774-127 by Fisher Scientific

Tyramide signal amplification kit T-20931 by Life Technologies

3,3′,5,5′-Tetramethylbenzidine 54827-17-7 by Sigma Aldrich

Protocol