RESEARCH ARTICLE

Virus-Induced Dormancy in the Archaeon *Sulfolobus islandicus*

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**ABSTRACT**  We investigated the interaction between *Sulfolobus* spindle-shaped virus (SSV9) and its native archaeal host *Sulfolobus islandicus*. We show that upon exposure to SSV9, *S. islandicus* strain RJW002 has a significant growth delay where the majority of cells are dormant (viable but not growing) for 24 to 48 hours postinfection (hpi) compared to the growth of controls without virus. We demonstrate that in this system, dormancy (i) is induced by both active and inactive virus particles at a low multiplicity of infection (MOI), (ii) is reversible in strains with active CRISPR-Cas immunity that prevents the establishment of productive infections, and (iii) results in dramatic and rapid host death if virus persists in the culture even at low levels. Our results add a new dimension to evolutionary models of virus-host interactions, showing that the mere presence of a virus induces host cell stasis and death independent of infection. This novel, highly sensitive, and risky bet-hedging antiviral response must be integrated into models of virus-host interactions in this system so that the true ecological impact of viruses can be predicted and understood.

**IMPORTANCE**  Viruses of microbes play key roles in microbial ecology; however, our understanding of viral impact on host physiology is based on a few model bacteria that represent a small fraction of the life history strategies employed by hosts or viruses across the three domains that encompass the microbial world. We have demonstrated that rare and even inactive viruses induce dormancy in the model archaeon *S. islandicus*. Similar virus-induced dormancy strategies in other microbial systems may help to explain several confounding observations in other systems, including the surprising abundance of dormant cell types found in many microbial environments, the difficulty of culturing microorganisms in the laboratory, and the paradoxical virus-to-host abundances that do not match model predictions. A more accurate grasp of virus-host interactions will expand our understanding of the impact of viruses in microbial ecology.

To date, models of coevolutionary dynamics between microbes and their viruses have been based primarily on tradeoffs between resistance and competitive fitness resulting from modification of cell surface receptors measured in a few model bacteria (1). Dynamics of the coevolutionary arms race have been demonstrated through experimental evolution and characterized at a molecular level (2, 3). Together, empirical data and theoretical models predict that variation in the tradeoff between resistance in the presence of the virus and competitive fitness without the virus result in the generation and maintenance of diversity in microbial populations (4, 5, 6) and may result in the majority of microbial cells exhibiting low activity due to resource limitation imposed as a tradeoff with resistance (7). However, whether these coevolutionary dynamics apply to the diversity of interactions between microbes and viruses across the three domains of life is not known. A broader understanding of microbe-virus interactions in model systems is needed in order to accurately infer the impact that viruses have on microbial ecology (8–11).

To augment our view of microbe-virus interactions occurring in natural systems, we examined the interaction between the double-stranded DNA (dsDNA) fusellovirus *Sulfolobus* spindle-shaped virus 9 (SSV9) (formerly *Sulfolobus* spindle-shaped virus Kamchatka-1) (12) and its crenarchael host *Sulfolobus islandicus* RJW002 (13). SSV9 was isolated from the Valley of the Geysers in Kamchatka, Russia, from an infected *S. islandicus* host (strain GV.10.6) (see Table S1 in the supplemental material). SSVs belong to the *Fuselloviridae* family, have dsDNA genomes, and can integrate site specifically into a host tRNA (14). Two SSVs have been studied in detail, SSV1 and SSV2, isolated from Japan and Iceland, respectively, from different *Sulfolobus* hosts (15–18), and neither of these has been shown to cause cell death in the nonnative host strain *Sulfolobus solfataricus* (15, 17).

*S. islandicus* (19) is a model system for investigating coevolutionary dynamics because it is becoming increasingly genetically tractable (20, 21) and natural variation of strains has been well characterized over time and space (22–24, 59). *S. islandicus*, like most *Archaea*, maintains a CRISPR-Cas immune system through which it targets and degrades invading genetic elements, guided by specific DNA spacer sequences in the CRISPR repeat-spacer arrays within its genome (25). *S. islandicus* strains have type I and type III CRISPR-Cas immune systems (23, 26, 27) and two or more CRISPR repeat-spacer arrays containing, on average, 180 spacers per individual (28, 29). In a population of *S. islandicus* cells from Kamchatka, Russia (23, 28), many of these spacers match to sequenced SSVs (28), suggesting frequent interactions with these viruses in nature and a selective benefit for cells to have immunity.
to viruses. Strain RJW002 has a single 100% CRISPR spacer match to SSV9, with an associated protospacer-associated motif (PAM) (30, 31).

Here, we investigate the impact of SSV9 on the growth and survival of S. islandicus RJW002 isolated from Kamchatka, Russia (see Table S1 in the supplemental material). Using a low multiplicity of infection (MOI), we uncover the fact that SSV9 induces a population-wide dormancy response from which only cultures with CRISPR immunity against the virus recover.

RESULTS

We began by identifying the effect of SSV9 on the growth of RJW002 in liquid cultures over time. The virus was added to RJW002 cultures for 5 h at an MOI calculated as 0.01 (by enumerating PFU), or 0.1 viral genomes/cell (using quantitative PCR [qPCR]) before unadsorbed virus was washed away and cells resuspended in virus-free medium. Surprisingly, as shown by the results in Fig. 1a, the addition of SSV9 at this low MOI to liquid cultures of RJW002 elicited a significant, 24- to 48-h growth delay compared to the growth of controls without virus. We observed a very low adsorption constant in these cultures of approximately $8.39 \times 10^{-11} \text{ ml/min}$ (see Fig. S1 in the supplemental material). Using a low multiplicity of infection (MOI), we uncover the fact that SSV9 induces a population-wide dormancy response from which only cultures with CRISPR immunity against the virus recover.

To test for cell viability during this population-wide growth delay, we removed RJW002 cells from the flask after the initial viral challenge and plated them on solid medium. The results in Fig. 1b show that RJW002 cells challenged with SSV9 were viable but not growing, with a constant colony count, from 12 to 24 hours postinfection (hpi), in contrast to the virus-free controls, which underwent two doubling events. These data indicate that challenge of RJW002 with SSV9 induced a population-wide stasis or dormancy response, where the majority of cells are viable but not actively growing.

Visualized by transmission electron microscopy (TEM) at 24 hpi, approximately 95% of RJW002 cells challenged with SSV9 appeared devoid of cellular contents and exhibit a spherical morphology, suggesting possible disruption of membrane integrity (Fig. 1c). Although the number of empty cells increased dramatically, from 40% to 95% (Fig. 1d), between 12 hpi and 24 hpi, the colony counts on plates (CFU/ml) (Fig. 1b) remained constant, demonstrating that these empty, dormant cells were able to recover and grow.

The genome of S. islandicus strain RJW002 has a single 100% match to SSV9 in the A1 repeat-spacer array (Fig. 2a). We hypoth-
esized that CRISPR-Cas immunity functioned to prevent the virus from establishing a productive infection, allowing the removal of the virus from culture and the rapid recovery of dormant *S. islandicus* cells. To test this hypothesis, we constructed mutants in the RJW002 background with in-frame deletion mutations of CRISPR-Cas components essential for CRISPR RNA processing and interference and of several components involved in other aspects of the CRISPR-Cas system (32) as controls (see Table S1 in the supplemental material). Virus quantification after viral challenge showed that no infectious particles were produced in RJW002 or the ΔA2 and Δcas1 mutants (controls whose deletions are predicted not to disrupt CRISPR immunity against SSV9) (Fig. 2b). SSV9 DNA was below the limit of detection by qPCR after 48 hpi (see Fig. S2a in the supplemental material) and decreased at a rate similar to the rate at which the virus degrades in controls without cells added (see Fig. S2b and S2c). In contrast, in cultures of mutants that had deletions of the spacer match against SSV9 (ΔA1 mutant) or CRISPR components involved in CRISPR RNA (crRNA) processing (Δcas6 mutant) or interference (Δcas3 + 3′ mutant, in which the helicase domain known as Cas3’ is fused with a nuclease domain known as Cas3′ [32]), newly produced infectious SSV9 particles were observed to increase by more than 2 orders of magnitude by 24 hpi (Fig. 2b; see Fig. S2a) and were maintained at a constant ratio (3 × 10^3 per ml ± 1 × 10^3 [average ± standard deviation] of approximately 0.01 infectious particles to cell. Whether new viral particles infect and replicate in dormant cells (33) or a small subset of nondormant host cells is not known. The fact that under these conditions, immune-deficient cells produced SSV9 while those with immunity did not supports our hypothesis that the type IA CRISPR-Cas system acts to prevent viral replication, allowing the virus to be eliminated from the culture and permitting host recovery.

The growth of immune-deficient strains (Δcas6, Δcas3′ + 3′, and ΔA1 mutants) in liquid culture when challenged with SSV9 showed a very different trajectory than the growth of strains with immunity, with no significant increase in optical density (OD) for immune-deficient strains during the course of the experiment (Fig. 2c; see Fig. S3 in the supplemental material). In contrast to the immune strain RJW002, Δcas6 mutant colony counts decreased dramatically upon challenge with SSV9, such that only 1% of the cells were viable by 24 hpi (Fig. 3a). TEMs showed that 80% of immune-deficient (Δcas6) cultures had the same empty phenotype as RJW002 by 24 hpi (Fig. 3b) but they did not recover over the course of the experiment (Fig. 3c). Even in cells that were shown to be inviable, lysis (broken cells) was not observed (Fig. 3b). This may explain the constant, nondecreasing OD observed in immune-deficient cells. To test this hypothesis, we constructed mutants in the *S. islandicus* RJW002 background with in-frame deletion mutations of several components involved in other aspects of the CRISPR-Cas system (32) as controls (see Table S1 in the supplemental material). Virus quantification after viral challenge showed that no infectious particles were produced in RJW002 or the ΔA2 and Δcas1 mutants (controls whose deletions are predicted not to disrupt CRISPR immunity against SSV9) (Fig. 2b). SSV9 DNA was below the limit of detection by qPCR after 48 hpi (see Fig. S2a in the supplemental material) and decreased at a rate similar to the rate at which the virus degrades in controls without cells added (see Fig. S2b and S2c). In contrast, in cultures of mutants that had deletions of the spacer match against SSV9 (ΔA1 mutant) or CRISPR components involved in CRISPR RNA (crRNA) processing (Δcas6 mutant) or interference (Δcas3 + 3′ mutant, in which the helicase domain known as Cas3’ is fused with a nuclease domain known as Cas3′ [32]), newly produced infectious SSV9 particles were observed to increase by more than 2 orders of magnitude by 24 hpi (Fig. 2b; see Fig. S2a) and were maintained at a constant ratio (3 × 10^3 per ml ± 1 × 10^3 [average ± standard deviation] of approximately 0.01 infectious particles to cell. Whether new viral particles infect and replicate in dormant cells (33) or a small subset of nondormant host cells is not known. The fact that under these conditions, immune-deficient cells produced SSV9 while those with immunity did not supports our hypothesis that the type IA CRISPR-Cas system acts to prevent viral replication, allowing the virus to be eliminated from the culture and permitting host recovery.

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We hypothesized that death in these cultures resulted from a prolonged antiviral dormancy response induced by the continuous presence of SSV9 in immune-deficient cultures where the virus was produced. We tested this hypothesis by inactivating SSV9 with UV and observing the response of cultures when challenged with inactivated particles. As shown by the results in Fig. 4, challenge with virus particles that had been inactivated by UV irradiation resulted in the dormancy-recovery phenotype observed in RJW002. The same response was observed in the Δcas6 strain, where there was no virus production (Fig. 4a). We then subjected both strains to repeated addition of inactivated viral particles.
and found that in both immune and nonimmune strains, prolonged exposure by the addition of UV-inactivated viruses resulted in extensive cell death, and no colonies were recovered on solid medium from samples collected at 72 hpi (Fig. 4c). These results demonstrated that prolonged virus-induced host cell dormancy could lead to cell death with no viral replication or lysis.

DISCUSSION

We have shown that in the archaeon *S. islandicus*, exposure to the virus SSV9 induces cell dormancy and death. The population-wide effect of dormancy occurs even when viruses are rare or inactivated, indicating that it is an antiviral response that is independent of infection. The risky, bet-hedging strategy of dormancy is reversible, allowing host cells to recover and grow normally when virus particles are prevented from productive infection by CRISPR-Cas immunity.

Dormancy has been demonstrated in many nonmicrobial systems (34, 35) as a bet-hedging strategy in the face of environmental variation and predation (34) and in microorganisms challenged by other stresses (36). In these systems, the cost of remaining active (for example, selective predation of active individuals) allows the evolution of potentially costly bet-hedging strategies of dormancy (37, 38). By analogy, it is likely that if dormancy protects against infection with highly virulent lytic viruses known to exist in this system (39), it may be an adaptive antiviral strategy.

It is also possible that dormancy serves to facilitate immunity or other mechanisms of viral targeting. Recently, based on the conserved genomic association between CRISPR-Cas loci and putative toxin-antitoxin (TA) systems, Makarova et al. predicted that cells armed with CRISPRs should respond to the stress imposed by viral infection by going into a dormant state (viable but not growing) induced by TA systems while the cell is mounting an immune response (40). The predicted function of dormancy is to allow cells to acquire new CRISPR spacers before the virus is able to complete its life cycle. Although no novel spacer acquisition was observed in this system, this may result from an insertion within the putative regulator of spacer acquisition *csa3* (41). The molecular mechanism of dormancy and its consequence for CRISPR-spacer acquisition is yet to be determined.

The infection-independent induction of host cell response appears to be distinct from the few well characterized bacterial abortive infection systems which cause cell death through mechanisms.
that are triggered by virus replication, transcription, or translation (42). It remains to be determined whether the mechanism of dormancy in this system is host or virus associated or whether it is triggered by binding of SSV9 to the host or by some factor (e.g., host- or virus-derived proteins) packaged within the viral particles. It has been observed in mammalian cell cultures that host-derived proteins packaged within particles of Sindbis virus can modulate the production of type I interferon upon challenge with inactivated particles (43).

Rapid advances in molecular techniques have allowed high-throughput analysis of the vast diversity of uncultivable but ecologically important microbial virus and host abundance over time and space (44–49). Theoretical models are rapidly developing to explain these patterns (4, 5, 70) in order to relate phage-host dynamics to ecologically important factors, such as nutrient cycling in the ocean (51) or the human microbiome (52). Recent models of virus-host interactions predict that the majority of microorganisms in natural populations will have low activity due to the high cost of viral resistance, while abundant viruses will predominate in rare, highly active taxa (53). This prediction has been used to explain the relative virus-to-host ratio observed through culture-independent techniques (9) and is supported by the widespread observation of dormant cells in a diversity of environments (36). Here, we have shown that dormancy itself is an antiviral strategy in this archaeal system, as well as the dramatic impact rare or inactive viruses may have on a population of host cells even without infecting them. Could mechanisms like this explain the predominance of dormancy in many microbial ecosystems (54)? Could dormancy induced by the presence of viruses in an inoculum contribute to the difficulty of cultivating diverse microorganisms in culture? This type of antiviral response and others must be integrated into models of virus-host interactions before observations of relative virus-host abundances and their impacts on the ecology of natural systems can be well understood.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *Escherichia coli* was grown on Luria-Bertani medium at 37°C. Ampicillin (100 µg/ml) was added to the medium when needed. All *S. islandicus* strains were grown in dextrin-tryptone (DT) medium or DT medium supplemented with 20 µg/ml uracil (DTU) at pH 3.5 and incubated at approximately 75 to 78°C in a water bath. *D. salina* strains were grown in dextrin-tryptone medium when needed. All variants of natural systems can be well understood.

**Calculation of SSV9 adsorption constant.** To obtain SSV9, GV.10.6 (see Table S1 in the supplemental material) was grown in 300 ml of DT medium in 375-cm² culture flasks (BD Falcon) at 78°C until early stationary phase. Cultures were filtered through 0.22-µm pores (PES) filtered and freeze-dried. Virus preparation and quantification. To obtain SSV9, GV.10.6 (see Table S1 in the supplemental material) was grown in 300 ml of DT medium in 375-cm² culture flasks (BD Falcon) at 78°C until early stationary phase. Cultures were filtered through 0.22-µm pores (PES) filtered and freeze-dried.

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particles, 200 μl of a 10⁻³ dilution of the supernatant was mixed with 500 μl of mid-log-phase, 10-times-concentrated S. islandicus Y08.82.36. Cells mixed with virus dilutions were plated on overlays of SY medium and incubated at 78°C for 48 h. Dilutions were performed and plated in triplicate. Three independent experiments were performed. The SSV9 adsorption constant on RJW002 was calculated as previously described (58) using the formula \( k = [2.3/B \times r] \log (P_o/P), \) where \( k \) is the adsorption constant (ml/min), \( B \) is the concentration of cells (cells/ml), \( r \) is the time in interval in which the titer falls from \( P_o \) to \( P \) (min), \( P_o \) is the original titer, and \( P \) is the final titer.

SSV9 challenge experiments. S. islandicus RJW002 and all of the CRISPR-Cas deletion mutants (see Table S1 in the supplemental material) were grown until mid-log phase (optical density at 600 nm [OD₆₀₀] between 0.08 and 0.15). Amounts of ~5.0 × 10⁶ cells were spun down for 15 min at 4,000 × g, and the supernatant was discarded. Cells were resuspended in 3 ml of DTU liquid medium. Two milliliters of the concentrated cell suspension were challenged with virus filtrate adjusted with DTU (if necessary) to a final multiplicity of infection (MOI) of ~0.01 in a final volume of 40 ml. The MOI was calculated based on the titer obtained on S. islandicus Y08.82.36. This MOI would be equivalent to ~0.1 if qPCR quantification was used instead. The remaining 1 ml of concentrated cell suspension was mixed with the equivalent volume of DTU without virus as a control. Uracil was added to the virus filtrate to a final concentration of 20 μg/ml prior to mixing. The mixtures were shaken at 60 rpm for 5 h at 78°C. Following virus challenge, the cells were washed twice with DTU to reduce background quantification of unadsorbed virus. Cell pellets of cultures challenged with virus were resuspended in 140 ml of DTU that was split into two 75-cm² tissue culture flasks and incubated at 78°C without shaking. Controls were resuspended in 70 ml of DTU. Cell growth was measured by the OD₆₀₀ for all strains, and SSV9 abundance was measured by qPCR to quantify virus in the entire culture and by using the formula

\[
V = \frac{k \times (B \times r) \times (P_o - P)}{\log (P_o/P)}
\]

where \( V \) is the virus concentration (PV cm⁻²), \( k \) is the adsorption constant (ml/min), \( B \) is the concentration of cells (cells/ml), \( r \) is the time in minutes in which the titer falls from \( P_o \) to \( P \) (min), \( P_o \) is the original titer, and \( P \) is the final titer.

Transmission electron microscopy. Cells from RJW002 and Δcas6 strain liquid infections were collected at 12, 24, 48, and 72 hpi by low-speed centrifugation. The cells were resuspended with Karnovsky’s fixative in phosphate-buffered (pH 7.2) 2% glutaraldehyde and 2.5% paraformaldehyde solution and stored at 4°C until processed. Samples were processed and visualized at the Frederick Seitz Materials Research Laboratory Central Research Facilities, University of Illinois at Urbana-Champaign, as follows. Microwave fixation was used with this primary fixative. Cells were then washed in cacoctyde buffer with no further additives. Microwave fixation was also used with the secondary 2% osmium tetroxide fixative, followed by the addition of 3% potassium ferricyanide for 30 min. After washing with water, saturated uranyl acetate was added for en bloc staining. The sample was dehydrated in a series of increasing concentrations of ethanol. Acetonitrile was used as the transition fluid between ethanol and the epoxy. The infiltration series was done with an epoxy mixture using the epoxy substitute Lx112. The resulting blocks were polymerized at 90°C overnight and trimmed, and ultrathin sections were cut with diamond knives. The sections were stained with uranyl acetate and lead citrate and examined and photographed with a Hitachi H600 transmission electron microscope.

Enumeration of dormant cells. Thin sections of control (no-virus) and SSV9-infected cells prepared for TEM were viewed under low magnification (×5,000) (see Fig. S4 in the supplemental material), and cell types were counted at each time point. At least 200 cells were counted at each time point from two independent experiments and assigned to the following categories: (i) empty or (ii) full.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl?doi=10.1128/mBio.02565-14/-/DCSupplemental.

Figure S1, PDF file, 0.6 MB.
Figure S2, PDF file, 0.7 MB.
Figure S3, PDF file, 0.7 MB.
Figure S4, JPG file, 0.3 MB.
Table S1, PDF file, 0.8 MB.
Table S2, PDF file, 0.7 MB.
Table S3, PDF file, 0.5 MB.
Table S4, PDF file, 0.6 MB.

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