Effects of temperature, salinity and clay particles on inactivation and decay of cold-active marine Bacteriophage 9A

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ABSTRACT: The effects of temperature, salinity and clay particles on the inactivation and decay of the cold-active Bacteriophage 9A, isolated from particle-rich Arctic seawater, were examined using a plating technique to evaluate infectivity (inactivation) and epifluorescence microscopy to measure phage concentrations (decay). Phage 9A was rapidly inactivated over a temperature test range of 25 to 55°C in marine broth (salinity of 36 psu), with half-lives ranging from <10 min at 25°C to ~1 min at 32.5°C and too rapid to measure at ≥35°C, making it among the most thermolabile phages. When salinity was varied at 30°C, the inactivation rates in brackish (21 psu) and briny (161 psu) broth were indistinguishable from that in marine broth (p > 0.20). At the environmentally relevant temperature of –1°C, however, loss of infectivity in briny broth was 3 to 4 times greater than in marine or brackish broth. As commonly observed, viral decay determined microscopically often substantially underestimated loss of infectivity: at 30°C, loss of infectivity exceeded the viral decay rate by approximately 1000-fold, while at –1°C, microscopic counts did not detect any of the losses observed by plaque assay. Under conditions comparable to a winter sea-ice brine inclusion (–12°C and 161 psu), however, plating and microscopy were in substantive agreement, indicating relatively minor losses of 16 to 34 % losses over a 5 wk period. Illite, kaolinite or montmorillonite clays had no statistically significant effect on phage inactivation as a function of temperature or salinity, although rates tended to be slower in the presence of the clays. In general, our results emphasize the importance of working with cold-active phages under environmentally-relevant conditions of temperature and salinity. They also imply decay processes that involve viral proteins rather than nucleic acids; as a result, affected viruses may be recalcitrant to reactivation by known host-based repair mechanisms.

KEY WORDS: Virus · Phage 9A · *Colwellia psychrerythraea* 34H · Temperature · Salinity · Clay · Illite · Mackenzie Shelf

INTRODUCTION

Cold-active viruses (i.e. those capable of infection and production at ≤4°C) await systematic study despite the volumetric dominance of the cold ocean. Field investigations in polar environments indicate that viruses can remain active at low temperature (e.g. Maranger et al. 1994, Gowing et al. 2002, Middelboe et al. 2002), with bacterial losses due to viruses sometimes equaling or exceeding those due to grazers (Steward et al. 1996, Guixa-Boixereu et al. 2002, Wells & Deming 2006b). Virus production has been demon-

strated even under conditions characteristic of brine inclusions in winter sea ice (–12°C and a salinity of 160 psu; Wells & Deming 2006a). High viral concentrations in the deep ocean (e.g. Hara et al. 1996, Weinbauer et al. 2003, Ortmann & Suttle 2005) also imply viral activity at low temperatures, but *in situ* or laboratory research pertinent to viral processes there has been rare (Wiebe & Liston 1968, Weinbauer et al. 2003, Wells & Deming 2006c, this issue).

Information on the decay of cold-active viruses derives from largely qualitative analyses conducted more than 30 yr ago (reviewed by Wells 2006). These studies

show that cold-active viruses are typically more thermolabile than viruses not capable of cold activity. While the latter tend to survive long exposure (60 min or greater) at 60°C with minimal loss of titer (Olsen et al. 1968), a dramatic reduction in titer of ≥99% is common for cold-active viruses at this or cooler temperatures (reported down to 45°C) within 10 min (Spencer 1955, 1963, Olsen et al. 1968, Wiebe & Liston 1968, Kulpa & Olsen 1971, Whitman & Marshall 1971, Delisle & Levin 1972). If associated with greater flexibility in viral proteins, as seen in cold-active enzymes (Feller & Gerday 2003), this increased lability could be an adaptive trait in environments of low thermal energy, for example, facilitating viral interaction with cell-surface receptor(s) or nucleic acid injection.

By further analogy to cold-active enzymes, we hypothesized that particles or organic material sorbed to particles could stabilize cold-active viruses at low temperature, just as various surfaces (Gianfreda & Scarfi 1991, Meyer-Reil 1991) and extracellular polymeric substances (Huston et al. 2004) appear to stabilize extracellular enzymes. Several viral studies, although not of cold-active viruses, point to this possibility. Hurst et al. (1989) found that the strongest correlates of the inactivation rate of enteroviruses in freshwater at 1°C were turbidity and suspended solid concentration; the correlations were negative, implying a preservative effect. Stotzky et al. (1981) reported that the presence of clays enhanced the lifetimes of different bacterial viruses in Tris buffer at both low (4°C) and high (37°C) temperatures, frequently by at least a factor of 2, depending on the virus and the clay. Survival of Type-1 poliovirus in seawater at 25°C was also enhanced in the presence of montmorillonite (tested at very high concentrations of 500 mg l^{-1}), from which the virus could desorb and be recovered (Gantzer et al. 1994). In contrast, lower montmorillonite concentrations did not effect poliovirus inactivation in deionized water between 19 and 35°C (Quignon & Schwartzbrod 1995).

While not testing temperature per se, additional work has shown that association with particles (especially clays) can substantially prolong viral survival in seawater (e.g. Bitton & Mitchell 1974, Smith et al. 1978, LaBelle & Gerba 1979, 1980, Schaiberger et al. 1982). Most of these studies, as well as those examining temperature effects explicitly, addressed viruses infecting animals or enteric bacteria; the effects of mineralogical or sedimentary particles on indigenous marine viruses have been little studied (in contrast to organic or uncharacterized particles, which appear to enhance decay; Suttle & Chen 1992, Noble & Fuhrman 1997). Hewson & Fuhrman (2003) reported rapid sorptive scavenging of marine viruses by mineralogically uncharacterized suspended sediments, depending on sediment concentration, size and source,

but no data regarding infectivity of the sorbed viruses were presented. Carlson et al. (1968) likewise observed sorption of bacteriophage T2 to a natural assemblage of river particles, with sorption enhanced as $CaCl_2$ concentration increased; in the case of additional experiments with kaolinite, sorption was reversible with no loss in titer. The fact that viruses infecting cyanobacteria and *Heterosigma akashiwo* have been isolated from sediments to depths of 30 to 40 cm, with a corresponding Pb^{210} age of ~100 yr in one case, also suggests that at least some particles act to preserve viruses in marine environments (Lawrence et al. 2002).

Isolated from an Arctic nepheloid layer, a distinct particle-rich region of the water column often associated with the bottom, and characterized by unprecedented cold activity to at least –10°C (Wells & Deming 2006c), Phage 9A is an obvious candidate for studies that explore whether, as in enzymes, cold activity is associated with greater lability in viruses and, if so, whether particles offset that lability. For this work, we focused on clay particles, which (along with silts) dominate the particle load of the Mackenzie River (Carson et al. 1998), the major sediment source for the Mackenzie Shelf area (Macdonald et al. 1998), whence 9A was isolated. Two of the clays we chose, kaolinite and illite, are of known regional significance, with illite composing up to 60% of total clays in the area (Mowatt & Naidu 1987). We also considered montmorillonite, present regionally only in trace amounts (Emeis 1985, Mowatt & Naidu 1987) but better evaluated in the microbial literature. Since the site from which 9A was isolated is notable for its wide salinity range, from brackish surface waters due to input from rivers and melting sea ice (see Fig. 2B of Wells et al. 2006) to the brines of winter sea ice, we also considered the effects of salinity on phage decay at both elevated (30°C) and *in situ* (–1 and –12°C) temperatures, including in the presence of clays.

MATERIALS AND METHODS

Media, reagents and viral stock. Most experiments were conducted in 50% marine broth 2216 (Difco), hereafter called marine broth, prepared by mixing equal volumes of full strength 2216 and artificial seawater (ASW), where ASW is 24 g NaCl, 0.7 g KCl, 5.3 g $MgCl_2 \cdot 6H_2O$, and 7 g $MgSO_4 \cdot 7H_2O$ in 1 l deionized water. The final salinity was 33 to 34 psu as measured by refractometer. Brackish and briny broths were made as above except that full strength 2216 was diluted in an equal volume of deionized water or 10× ASW, resulting in measured salinities of ~17 and ~172 psu, respectively. For 1 set of experiments, ASW rather than marine broth was used. In

this experiment, the ASW was amended with 6 mM CaCl$_2$·2H$_2$0 and buffered to pH 7.5–7.9 with 1.3 g l^{-1} TAPSO(3-[N-tris(hydroxymethyl)methylamino]-2-hydroxy-propanesulfonic acid), to correspond to 50% 2216. All media were pre-filtered (0.2 µm) and sterilized by autoclaving.

Clay solutions were prepared using montmorillonite (bentonite clay K-10, Acros Organics), kaolinite (kaolin, Fisher Scientific) or illite (IMt-1, Illite Cambrianshale, Silver Hill, Montana, supplied by the Source Clays Repository, the Clay Minerals Society, Purdue University). The latter was crushed with a mortar and pestle and sieved to remove particles >125 µm in size (the other 2 clays were already powders). In each case, 2 to 5 g were added to a baked (>100°C), pre-weighed 250 ml glass bottle (the plastic rim of which had been removed) to which 100 ml of deionized water were added followed by vigorous shaking. The clays were allowed to settle for 80 to 85 min over a 4 cm pathlength before carefully removing the upper 80 ml as the ≤3 µm size fraction (calculated by solving for χ in the Stokes equation: ν = (χ^2 · g · [ρ$_s$ – ρ$_L$])/(18 · η), where ν is the settling velocity (4 cm in 80 to 85 min), χ is the estimated spherical diameter, ρ$_s$ – ρ$_L$ is the density difference of solid [clay] and liquid [deionized water] and η is the fluid viscosity). The bottle and remaining solution were again baked at >100°C and then weighed; this process was repeated until no further change in mass was observed (i.e. water had evaporated). The difference in weight at the start and end of this procedure enabled calculation of the concentration of clays ≤3 µm in the distilled water solution, which varied between 1 and 6 mg ml^{-1} depending on the clay. Clay solutions were autoclaved and aliquots of it added to broth (as above) to achieve a final concentration of 10 mg l^{-1}, which falls within the range of suspended particle concentrations measured in nepheloid layers from the Mackenzie Shelf region (2.2 to 18.4 mg l^{-1}; Wells et al. 2006). The clay–broth solutions were mixed and allowed to equilibrate for a minimum of 1 d prior to use.

A high titer phage lysate was prepared from silica overlay plates as described by Wells & Deming (2006c). The phage were stored at 4°C in phage buffer: 0.1 M NaCl, 50 mM Tris-HCl pH 8.0, 8 mM MgSO$_4$·7H$_2$0, 2 mM CaCl$_2$·2H$_2$0, 5% glycerol. As measured by refractometer, the apparent salinity of the phage buffer was ~60 psu, much of which was due to the 5% glycerol (which the refractometer registered as equivalent to 33 to 34 psu salinity).

Experiments on loss of phage infectivity at 25 to 55°C. To determine the inactivation rate of Phage 9A at temperatures of 25 to 55°C, 900 µl of media, with or without clays, were aliquoted into sterile, 1.5 ml microcentrifuge tubes and equilibrated to the experimental temperature in a precision oven for at least 1 h. To start

an experiment, 100 µl of Phage 9A (typically 10^9 to 10^{10} phage ml^{-1} in phage buffer) were added to the media and mixed gently. This addition brought the final salinity to 36 psu (for marine broth or amended ASW), 21 psu (brackish broth) and 161 psu (briny broth); when applicable, it brought the clay concentration to 9 mg l^{-1}. Following mixing and periodically thereafter, 50 µl samples were removed to sterile 0.5 ml microcentrifuge tubes on ice. When all samples had been collected (within 15 to 60 min, depending on the temperature), a dilution series of phage was plated using the silica-gel overlay technique (described by Wells & Deming 2006c) and *Colwellia psychrerythraea* strain 34H (henceforth 34H) first harvested in mid-exponential growth phase (A600 of 0.2 to 0.3) and concentrated 2 to 4-fold by centrifugation (6000 × g for 20 min at 2°C). Plates were incubated at 2°C; plaques took approximately 1 wk to form. Inactivation rate constants (k) were calculated as the slope of the linear regression of the natural logarithm of the abundance of plaque-forming units (PFU) against time.

Experiments on loss of phage infectivity at subzero temperatures. As discussed in Wells & Deming (2006c) and despite efforts to standardize 34H growth and infection conditions, the efficiency of plating (EOP) of Phage 9A on 34H was highly variable in a way that appears to reflect changing bacterial phenotypes. The exact cause of variability remains unknown. For experiments at temperatures below zero (which required days instead of minutes), variable EOP, rather than loss of infectivity, could account for changes in PFU numbers. To circumvent this ambiguity, a relative (rather than absolute) loss rate was determined as follows: triplicate 900 µl aliquots of marine, brackish or briny broth, with or without illite, were equilibrated for several hours to the experimental temperature before addition of 100 µl 9A. After gentle mixing, 5 µl were removed and diluted into 0.5 ml of ice-cold marine broth in a sterile microcentrifuge tube. This subsample was kept at –1°C until a second time point was sampled 3 wk later. The 2 time points were then plated as above on the same 34H stock to determine survival fractions relative to that in –1°C marine broth.

A similar experiment was performed at –12°C in briny broth, since its salinity of 161 psu corresponded to the expected salinity of brine inclusions in sea ice at this temperature (~158 psu). A 900 µl sample of briny broth was equilibrated to –12°C for 6 h before addition of 100 µl Phage 9A as above, followed by gentle mixing. Subsamples of 50 to 100 µl for microscopic enumeration of phage (see next subsection) were taken and processed immediately after 0, 7, 18, 36 and 42 d. Subsamples for PFU were also collected after 7, 18 and 42 d by diluting 5 µl of sample into 0.5 ml of ice-cold marine broth. The t = 7 d diluted sample was then

stored at –1°C for 11 d in order to plate it against the same 34H stock as the $t = 18$ d sample and determine survival fraction as above. Likewise, at the end of the experiment, the stored $t = 7$ and 18 d samples were replated with the $t = 42$ d sample. Between Days 36 and 42, the incubator warmed to –7°C.

Microscopic evaluation of phage decay. For a subset of experiments at –12, –1 and 30°C, besides measuring loss of infectivity by plaque assay, changes in phage abundance were monitored by direct counts using SYBR gold staining and epifluorescence microscopy. Sample volumes of 5 to 50 μl were fixed in cold ASW amended with formaldehyde (1.5% final concentration), and processed to slide stage using a procedure modified from Noble and Fuhrman (1998) as described in Wells & Deming (2006c). Slides were usually counted immediately and always within 24 h, being stored at –20°C in the interim.

Adsorption of Phage 9A to illite at –1°C. To determine whether Phage 9A adsorbed to illite (the most environmentally relevant clay) in brackish, briny or marine broth, triplicate microcentrifuge tubes containing 900 μl of cold medium with 10 mg l^{-1} illite (prepared as described above) were inoculated with 100 μl Phage 9A and mixed gently. A subsample of 100 μl was removed and diluted in 900 μl of the same medium (i.e. brackish, briny or marine) but without illite. The diluted samples were immediately centrifuged for 4 min at $15\,600 \times g$ in a table-top centrifuge at room temperature to pellet clay and adsorbed viruses; 500 μl of overlying supernatant were then fixed in cold ASW containing 1.5% formaldehyde. A second sample was taken and prepared as above after 2 h. All samples were stained with SYBR gold and viewed by epifluorescence microscopy (as above).

RESULTS

Effects of temperature, salinity and clays on Phage 9A inactivation and decay at 25 to 55°C

As determined by plaque assay, Phage 9A was rapidly inactivated at temperatures between 25 and 32.5°C in marine broth, with half-lives declining from 7 to 10 min at 25°C to ~1 min at 32.5°C (Fig. 1). At higher temperatures (35 to 55°C), rates were too fast to determine accurately. Over the range of 25 to 32.5°C, phage inactivation rate constants declined linearly with increasing temperature in clay-free controls and all treatments (Table 1). While inactivation rates tended to be
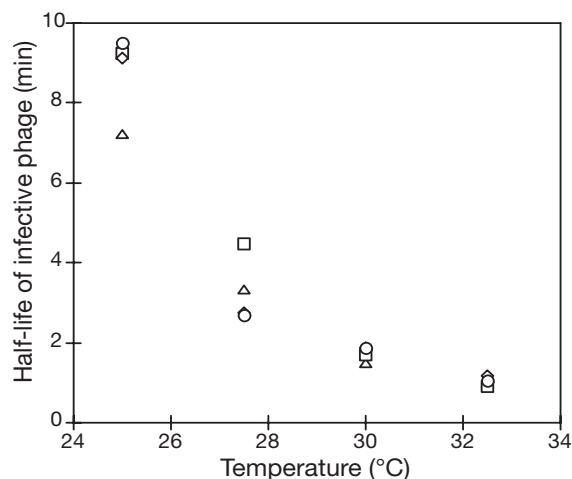

Fig. 1. Phage 9A. Effect of temperature on the half-life of infective phage (determined by plaque assay) in standard marine broth (△) or marine broth amended with 9 mg l^{-1} montmorillonite (□), kaolinite (◇) or illite (○). See Table 1 for rate constants and their confidence limits

Table 1. Phage 9A. Inactivation rate constants (k, min^{-1}) determined by plaque assay for Phage 9A incubated in marine broth with or without 9 mg l^{-1} clay; 95% confidence intervals in parentheses. $p < 0.05$ unless otherwise indicated; *: $p < 0.01$; **: $p < 0.001$. Significant ($p < 0.05$) regressions of k against temperature (T, °C) are given. Significance of linear regressions against time (to determine k) or temperature was tested by ANOVA

Temperature (°C)	Incubation period (min)	No addition	Montmorillonite	Kaolinite	Illite
25.0	60	–0.096** (–0.103 to –0.089)	–0.075* (–0.107 to –0.043)	–0.076* (–0.103 to –0.050)	–0.073** (–0.079 to –0.066)
27.5	40	–0.208* (–0.224 to –0.191)	–0.155[a] (–0.486 to 0.176)	–0.252* (–0.308 to –0.196)	–0.259 (–0.409 to –0.109)
30.0	15 or 20[b]	–0.466* (–0.548 to –0.384)	–0.411* (–0.625 to –0.198)	–0.370* (–0.535 to –0.205)	–0.370** (–0.423 to –0.316)
32.5	15	–0.696 (–1.13 to –0.261)	–0.774* (–0.971 to –0.576)	–0.596 (–0.864 to –0.329)	–0.662* (–0.904 to –0.421)
Regression equations:		**$-0.082 \times T + 2.00$**	**$-0.094 \times T + 2.35$**	**$-0.067 \times T + 1.61$**	**$-0.075 \times T + 1.82$**

[a] $p < 0.12$
[b] 15 min for the experiments with clay and 20 min for the experiment without clay

slower if clays were present, especially at the lowest temperature (25°C; Fig. 1), rates were not statistically different between treatments (2-factor ANOVA without replication, $p > 0.71$ for presence or absence of the specific clay), as also implied by overlap of 95% confidence intervals (Table 1). Triplicate independent experiments at 30°C with or without illite determined slower inactivation rates in the presence of illite ($k \pm$ SE of the mean: -0.351 ± 0.043 min^{-1} vs. -0.416 ± 0.059 min^{-1} when illite was absent) but the difference was not statistically significant (1-factor ANOVA, $p > 0.45$). Illite had no effect on inactivation rates at 27.5°C in CaCl$_2$-amended ASW ($k = -0.164$ and -0.162 min^{-1} for the control and illite treatments, respectively); these rates did not differ significantly from those measured in marine broth at this temperature (2-tailed t-test, $0.05 < p < 0.10$).

A comparison of inactivation rate constants determined by plaque assay at 30°C in broths of differing salinities, with or without illite (Table 2 and Fig. 2), revealed that inactivation tended to be more rapid in briny broth and, at all salinities, if no clay were present. Treatments were not statistically different, however, whether in terms of salinity or the presence or absence of illite (2-factor ANOVA without replication, $p > 0.20$ for salinity and $p > 0.10$ for illite).

As for direct counts of phage at 30°C, no decline in number was detected in marine broth over a 15 min period ($p > 0.92$ for the slope of the regression against time; Fig. 2). The decline over a 24 h period was significant, however, yielding a decay constant of -0.025 h^{-1} (or -0.0004 min^{-1}, $p < 0.05$), approximately 3 orders of magnitude smaller than that observed when measuring loss of infectivity.

Phage 9A inactivation and decay at subzero temperatures

After incubation for 3 wk at -1°C, the relative survival fractions of infective viruses indicated only small ($\pm 20\%$) changes in brackish and marine broth, whether or not illite was present (Fig. 3). In contrast, the fraction fell to 34% of the initial value in briny broth, with a substantial but smaller decline in the presence of illite (to 61% of the initial value; Fig. 3). A 2-factor ANOVA determined that phage survival fractions differed significantly among the salinity treatments ($p < 0.005$) but not among the clay treatments ($p > 0.57$); interactions between salinity and clay treatments were not significant ($p > 0.67$). Accordingly, a post-hoc multiple comparison using the Tukey test was performed to ascertain the source of difference among salinity treatments. Relative survival fractions in briny broth were significantly lower than those in marine broth ($p < 0.005$) but were not different from those in

Table 2. Phage 9A. Inactivation rate constants (k, min^{-1}) for phage incubated at 30°C in brackish, briny or marine broth with or without 9 mg l^{-1} illite. 95% confidence intervals in parentheses. $p < 0.05$ unless otherwise indicated; *: $p < 0.01$; significance tested by ANOVA

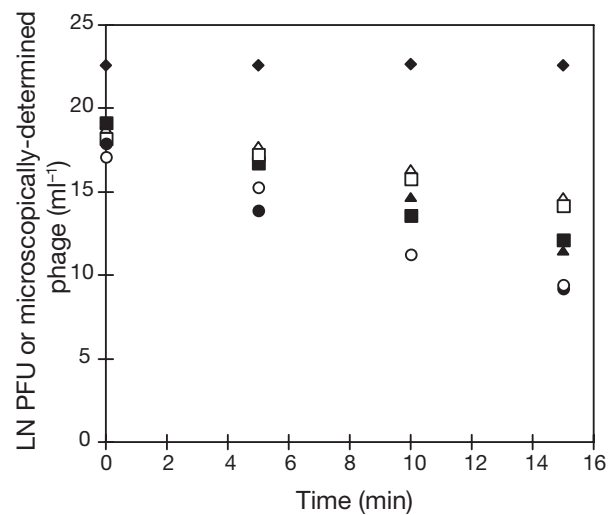| Salinity (psu)	No addition	Illite
21	-0.495* (-0.639 to -0.351)	-0.275* (-0.366 to -0.184)
36	-0.482* (-0.680 to -0.283)	-0.269* (-0.355 to -0.182)
161	-0.565^a (-1.61 to 0.482)	-0.540 (-0.810 to -0.270)
$^a p < 0.10$		

Fig. 2. Phage 9A. Inactivation at 30°C in broth of 21, 36 or 161 psu, with or without 9 mg l^{-1} illite. Triangles (21 psu), squares (36 psu) and circles (161 psu): data determined from plaque assay, in the absence (closed symbols) or presence (open symbols) of illite. Diamonds (36 psu): data determined by microscopic count of viruses in a separate experiment

brackish broth ($0.05 < p < 0.10$); survival fractions in brackish and marine broth could not be distinguished statistically ($0.20 < p < 0.50$). A corresponding experiment using epifluorescence microscopy did not detect phage decay at -1°C in any of the 3 broths after 3 wk incubation: the mean recovery percentage in triplicate samples at each salinity was 93 ± 3, 92 ± 7 and $101 \pm 5\%$ for brackish, marine and briny broths, respectively.

Finally, as determined by direct microscopic counts, no phage decay occurred in briny broth at -12°C ($p > 0.13$ for the regression over the 42 d incubation, sampled weekly; $p > 0.21$ if limited to the first 36 d, after which the incubator warmed to -7°C), although the concentrations had declined to 70–80% of initial
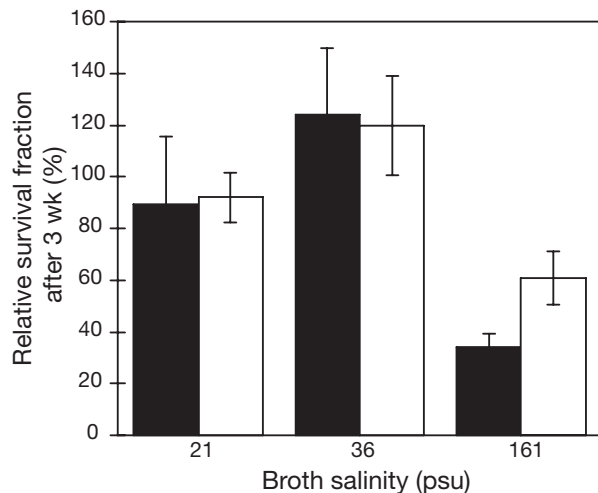
Fig. 3. Phage 9A. Inactivation (relative to that in –1°C marine broth) by plaque assay after 3 wk of incubation at –1°C in broth of 21, 36 or 161 psu, with (open bars) or without (black bars) 9 mg l^{-1} illite. Data are averages of independent triplicate experiments, with the standard error of the mean indicated

values by Days 36 or 42. As regards loss of infectivity over time (relative to that in –1°C marine broth), only fairly minor differences were observed between PFU counts for samples at t = 7 d and t = 18 d, whether plated on Days 18 or 42; the sample at t = 42 d had 16 to 34 % fewer PFU than the samples at the other 2 time points (Table 3).

Adsorption of Phage 9A to illite at –1°C

Adsorption of Phage 9A to illite was negligible regardless of salinity. The mean percentages of unadsorbed viruses after 2 h (± SE of the mean, N = 3) were 97 ± 5, 97 ± 3 and 98 ± 5%, for brackish, marine and briny broths, respectively.

Table 3. Phage A. Effect of incubation for 7, 18 or 42 d at –12°C in briny broth on number of PFU, determined by plating after 18 or 42 d. tntc: too numerous to count

| Dilution | — Plated after 18 d[a] — | | Plated after 42 d[a] | | |
	t = 7 d	t = 18 d	t = 7 d	t = 18 d	t = 42 d[b]
1:10^5	tntc	tntc	308	338	259
1:10^6	125	124	40	50	33
1:10^7	16	11	–	–	–

[a]Because of the variable EOP, viruses sampled on different days were plated on same host stock by storing viruses in the interim in –1°C marine broth; inactivation in –12°C briny broth is thus relative to that in –1°C marine broth (see 'Materials and methods' for further details)
[b]Incubator warmed to –7°C between Days 36 and 42

DISCUSSION

As with other known cold-active viruses, Phage 9A is extremely sensitive to temperatures readily tolerated by other viruses, being among the most thermolabile of bacteriophages yet described. The linear regression against temperature (Table 1) implies that 1.5 to 3 min would suffice to reduce 9A titers to 1% of initial values at temperatures between 45 and 60°C (the temperature range in which other studies have been conducted), comparable to reported characteristics of cold-active Phages P/14 and P/SW1/b (Spencer 1963), ps$_1$ (Whitman & Marshall 1971), 23 and 27 (Delisle & Levin 1972) and an unnamed *Aeromonas* phage (Wiebe & Liston 1968) in this temperature range. The use of different media for determining inactivation rates, usually only at a single temperature, constrains rigorous comparison, however, since medium composition, including cation and organic concentrations, affects thermal inactivation of viruses, including cold-active viruses (Spencer 1955, 1963). At least among variables that we considered, such effects were minor for 9A, as indicated by the statistically indistinguishable rate constants determined in amended ASW (at 27.5°C), brackish or briny broth (at 30°C; Fig. 2) and in the presence of various clays (at 25 to 32.5°C; Table 1) when compared to the corresponding rate constants in marine broth.

At a temperature of 25°C, commonly used for laboratory work with viruses, inactivation of Phage 9A was still several orders of magnitude faster (–0.096 min^{-1}) than reported for other (non-cold-active) marine phage at 23 to 25°C (undetectable to –0.0002 min^{-1} in 0.2 µm-filtered seawater incubated without sunlight; Suttle & Chen 1992) as well as non-native viruses in seawater at these temperatures (Kapuscinski & Mitchell 1980). Below 24°C, unrealistic inactivation rate constants ($\geq$0) predicted from the linear regression of k versus temperature suggest that, at lower temperatures, thermal inactivation of 9A proceeds much more slowly and possibly by a different mechanism than at higher temperatures. In contrast to loss of infectivity, microscopically-determined decay of 9A at 30°C (–0.025 h^{-1}) was comparable to values reported in other studies at mesophilic temperatures: e.g. –0.016 to –0.031 h^{-1} at 18°C in Chesapeake Bay estuarine waters (Wommack et al. 1996); and –0.009 to –0.025 h^{-1} at summer temperatures (not specified) in Gulf of Mexico surface waters (Wilhelm et al. 1998a). These studies also emphasized the discordance between loss of infectivity and phage decay, as discussed earlier by Suttle & Chen (1992). While the loss of infectivity in Gulf of Mexico waters was 40 times greater than the decay rate of viral particles (Wilhelm et al. 1998a), for Phage 9A at 30°C it was approximately 1000 times greater (Fig. 2). A less

dramatic disjunction was also observed in briny broth at −1°C (Fig. 3). These results add to the importance of distinguishing loss of infectivity from phage decay determined by direct counts: at least under some circumstances, large fractions of microscopically observed viruses in cold waters and in sea ice may not be infectious.

The exact mechanism by which temperature affects phage inactivation is not clear; for example, some authors have suggested that it merely modulates other inactivation mechanisms (Kapuscinksi & Mitchell 1980). Our experimental conditions ruled out other common causes of inactivation (Suttle & Chen 1992, Wommack et al. 1996, Noble & Fuhrman 1997, Weinbauer et al. 1999), including sunlight, biological activity (the solutions were sterile) and adsorption to particles (the solutions were 0.2 µm-filtered, and added clays had no significant effect). Nor is there evidence that the nucleic acids of cold-active viruses are more thermolabile than those of other viruses or organisms; the only available data do not indicate a pronounced difference (Chen et al. 1966). Instead, the enhanced thermal sensitivity of these viruses may be due to structural changes in the virion, potentially involving proteins or enzymes utilized for host recognition, attachment or DNA injection. An important ecological implication is that, in contrast to loss of infectivity due to largely reversible UV-associated nucleic acid damage (Wilhelm et al. 1998b), loss of infectivity due to protein lability may prevent infection altogether and be irreversible. In general, this suggests that decay processes affecting viral nucleic acids should be distinguished from those affecting viral proteins, especially given known mechanisms of repair only for the former and the likelihood that light-mediated nucleic acid damage does not pertain to large volumes of the ocean.

Because of the variable EOP of the 9A–34H PHS (for detailed discussion, see Wells & Deming 2006c) and the long incubation periods required for meaningful measurements at low temperature, we were unable to estimate an absolute inactivation rate at −1°C. The viability of stocks at 4°C for >6 mo in phage buffer, with minimal loss of titer determined epifluorescently and only the expected range of vacillations (due to the variable EOP) measured by plating, argue that 9A is stable for long periods of time at low temperature. In support of this, no significant decline in phage was observed microscopically over a 3 wk period at −1°C, either in normal, brackish or briny broths. Likewise, the relative survival fractions in marine and brackish broths over this time period varied only by ±20% (Fig. 3), which is within the accuracy of our plaque-counting method. These results do not preclude some loss of infectivity, however, since the experiments were normalized to −1°C marine broth, in which the absolute inactivation

rate is unknown. The relative decline in briny broth also serves as a reminder that direct counts may underestimate loss of infectivity, as discussed above.

While salinity–temperature couples of 21 or 36 psu and −1°C are environmentally relevant in the Arctic region where Phage 9A was isolated, it is unlikely that Phage 9A would experience 161 psu salinity except at much lower temperatures characterizing winter sea-ice brine inclusions. Since previous work has shown that concentrations of winter sea-ice viruses persist or increase over prolonged (8 d) incubations with co-occurring bacteria in 160 psu brine at −12°C (the environmentally-relevant temperature; Wells & Deming 2006a), we investigated inactivation of Phage 9A at this salinity and temperature in a sterile solution relative to that in marine broth at −1°C. Over a 5 wk period, the number of both phage and PFU decreased by only 16 to 34% (Table 3), much less than the decrease in PFU observed at −1°C in briny broth over 3 wk (66%; Fig. 3). Thus, consistent with our previously reported field results, these data support possible long-term persistence, if not activity, of viruses in the extreme brines and low temperatures of winter sea ice, although other loss factors in natural brines (for example, instability after precipitation of a specific salt or degradation by cells or extracellular enzymes) require further consideration.

Finally, contrary to our hypothesis, none of the examined clays significantly enhanced the stability of Phage 9A either as a function of temperature or salinity, despite the tendency for the inactivation rates to be lower in the presence of clays (Figs. 1 & 3 and Tables 2 & 3). Consistent with this conclusion, we found no evidence at any of the salinities tested that Phage 9A adsorbed to illite, the best-represented clay along the Mackenzie Shelf. Using an experimental approach basically the same as that detailed here, but in a freshwater system, Carlson et al. (1968) measured considerable adsorption (typically >80%) of T2 to illite, kaolinite or montmorillonite, with greater adsorption observed at increasing NaCl concentrations. They used higher concentrations of clay (50 mg l^{-1}) than in our experiments, however, as also in another study reporting significant effects of clays on viruses (Gantzer et al. 1994). Such concentrations of suspended matter are only occasionally observed in the Mackenzie Shelf region (Hill & Nadeau 1989, Wells et al. 2006). A potential confounder in our experiments was the high concentration of organic matter in the broths we used, as both Carlson et al. (1968) and Stotzky et al. (1981) reported that organics could inhibit phage adsorption to clays, presumably by outcompeting phage for binding sites. On the other hand, organics did not affect adsorption of enteric viruses to sediments in the experiments of LaBelle & Gerba (1979) and are inevitably

present environmentally. That we saw no statistical difference between inactivation rate constants in marine broth or ASW (with or without illite) argues against the importance of this possible confounder. Instead, our data indicate that the clays we considered had little effect (either preservative or deleterious) on 9A, although we caution against indiscriminately extending these results to the diverse assemblages of particles and viruses found *in situ*. We also note that viruses not adsorbing to particles and thus possibly avoiding removal from the water column may be selectively favored, perhaps especially if their hosts are free-living.

CONCLUSIONS

Like the few other cold-active viruses examined to date, Phage 9A is extremely thermolabile, a fact that argues that considerable care should be taken to isolate and study cold-active viruses at low temperatures. In the range of 25 to 32.5°C, where infectivity was readily quantifiable, this lability was not affected by salinity or clay particles. At –1°C, relative inactivation was negligible over a 3 wk period except in high salinity (161 psu) broth. In the same briny solution dropped to –12°C, minor viral losses pointed to the possible persistence of phage in winter sea-ice brine inclusions on seasonal and possibly multi-year timescales. Future work could profitably examine the structural basis of thermolability in 9A, explore the proteomes of this and other cold-active phage for proteins and enzymes specifically adapted to operation at near-zero temperatures, and systematically investigate whether phage survival and persistence are optimized to the temperature range of their *in situ* environments.

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