

Characterization of a cold-active bacteriophage on two psychrophilic marine hosts

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ABSTRACT: A cold-active bacteriophage designated 9A was isolated against *Colwellia psychrerythraea* Strain 34H at near *in situ* temperature (−1°C) by enrichment of seawater from an Arctic nepheloid layer, using a newly developed isothermal overlay technique. Phage 9A is classified as a Siphoviridae with a genome size of 80 to 90 kb. In addition to 34H, 9A infects *C. demingiae* ACAM 459^T; no other hosts (of 22 tested) were identified. In replete media, 9A formed plaques on 34H from −6 to 4°C and on *C. demingiae* from −6 to 8°C; the temperature range of plaque formation on 34H could be extended to 8°C by prior host starvation. An indirect plating method and microscopic evaluation also determined phage production at temperatures between −13 and −10°C. At −1°C, 34H had a broader salinity range of plaque formation than *C. demingiae*: 20 to 50 (but not 65) psu vs. 27 to 34 (but not 50) psu. As monitored by epifluorescence microscopy, phage production by 34H was observed at 1, 10, 100 and 200 atm (all at −1°C), but not at 400 or 600 atm. The 9A–34H system commonly had a low efficiency of plating (EOP; typically ~1%) which varied with culture age. Despite repeated attempts, no meaningful adsorption rate could be determined at −1 or 8°C. This result, the low EOP, and the effect of starvation on plaque formation suggest that fluctuating host phenotypes may play an important role in the dynamics of this system. One-step growth curves (using 34H as host) revealed a longer latent period (4 to 5 vs. 2.5 to 3 h) and greater burst size (55 vs. 5) at −1 than 8°C; at temperatures between −10 and −12°C, the estimated latent period was 5–10 d and the burst size 5. At both −1 and 8°C, rise times were comparable to latent periods. Although the cycle from infection to burst at −1°C required only 10 to 20% of the generation time of 34H at this temperature, the amount of viral DNA synthesized was comparable to the size of the host genome, suggesting very efficient and cold-active virus-encoded enzymes.

KEY WORDS: Virus · Phage 9A · *Colwellia psychrerythraea* 34H · *Colwellia demingiae* · Arctic · Adsorption · One-step growth curve · Pressure · Salinity

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INTRODUCTION

Early viral research by Spencer (1955, 1960, 1963) and later electron microscopic observations of high numbers of virus-like particles in seawater samples (Torella & Morita 1979, Bergh et al. 1989) helped to dispel the presumption that bacteriophage were of little ecological significance in aquatic environments. Spencer (1955, 1960, 1963), in particular, demonstrated phage infection of indigenous marine bacteria (rather than enteric hosts) specifically under conditions mimicking those *in situ* (3 to 4% salinity and 0°C). Later

workers have generally remained attentive to use of seawater salinities for marine phage–host cultivation but have overlooked Spencer's (1955, 1960, 1963) emphasis on temperature, despite the predominance of cold waters (≤4°C) at depth and high latitudes, as well as mounting evidence that viruses play active ecological roles in cold waters (Steward et al. 1996, Guixa-Boixereu et al. 2002, Wells & Deming 2006b) and sea ice (Wells & Deming 2006a).

Symptomatic of this oversight is the absence of well-defined terminology for viruses capable of infection at low temperature. While these are often called

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'psychrophilic' (e.g. Stamatina 1963, Olsen 1967, Ackermann & DuBow 1987) or 'psychrotrophic' (Greer 1982, 1983, Patel & Jackman 1986), the terms are ambiguous or misleading, since they imply growth characteristics of the host, not features necessarily specific to the virus (e.g. the temperature range of infection by a given virus may change with different hosts). For the sake of clarity, we define as 'cold-active' those viruses capable of infection and production at $\leq 4^{\circ}\text{C}$. This upper limit accords with the sparse literature about phage infection at low temperature and encompasses the temperatures of the perennially cold regions of the ocean. By this definition, several phage or phage–host systems (PHS) termed 'psychrophilic' or 'psychrotrophic' have not been demonstrated to be cold-active (e.g. Stamatina 1963, Whitman & Marshall 1971a, Greer 1982, Patel & Jackman 1986).

Beginning with Elder & Tanner (1928), we have identified 29 cold-active phage from the literature, only a few of which have been investigated in any detail. Of these, the most extensively studied from a marine environment, Phage 27, was isolated from Boston seawater and formed plaques on its original host at 2 but not 20°C (Delisle & Levin 1969). Further characterization determined that phage 27 was unusually thermolabile, had different maximal temperatures of plaque formation depending on its host ($< 20^{\circ}\text{C}$ on *Pseudomonas putrefaciens* P19X and $< 26^{\circ}\text{C}$ on *P. putrefaciens* P10; Delisle & Levin 1972a) and highest efficiency of plating (EOP) on P19X at temperatures between -5 and 4°C , decreasing 5 orders of magnitude at or above 10°C (Delisle & Levin 1972b). Similar characteristics have been reported for cold-active phages from other environments (Olsen et al. 1968, Kulpa & Olsen 1971, Greer 1983). Delisle & Levin (1972b) also presented evidence that passage of the phage through different hosts at different temperatures altered its characteristics, including the temperature range of plaque formation.

Besides the work of Delisle & Levin (1969, 1972a,b), no similarly comprehensive characterization of cold-active marine phages at low temperature has been reported, although recent, more limited work has supported their findings. A study by Middelboe et al. (2002) of a pelagic Arctic *Pseudomonas* PHS revealed a burst size (~ 18) and latent period (~ 15 h) at 0°C similar to those of Phage 27 on P19X at 2°C (~ 10 viruses and 14 h, respectively; Delisle & Levin 1972b). Borriss et al. (2003) determined that the temperature range of plaque formation for 3 PHS from Arctic sea ice was even more restrictive than that described by Delisle & Levin (1972a), with no plaques observed at temperatures $\geq 10^{\circ}\text{C}$ in one case. In general and regardless of environmental provenance, the maximum temperature of plaque formation by a cold-active

phage tends to be lower than the maximum temperature for growth of the host (e.g. Spencer 1963, Wiebe & Liston 1968, Whitman & Marshall 1971b, Delisle & Levin 1972a, Borriss et al. 2003).

Several studies of cold-active (but not necessarily marine) phages have also reported slower phage production rates, reduced burst sizes and longer latent periods at 3.5 to 4°C than at 25 to 26°C (Olsen et al. 1968, Kulpa & Olsen 1971, Whitman & Marshall 1971b, Sillankorva et al. 2004a). Unfortunately, some confusion surrounds these results, in particular those of Olsen et al. (1968). Using one of the same PHS (*PX14-Pseudomonas geniculata*) as in Olsen et al. (1968), Olsen (1967) had reported that the highest burst size (300 to 350) occurred at 3.5°C , not 25°C , but only if the bacteria were grown at 3.5°C prior to infection. Later studies by other authors have also shown that the temperature of growth before infection can alter PHS characteristics at near-zero temperatures, including adsorption rate (Delisle & Levin 1972b) and latent period (Sillankorva et al. 2004a), presumably due to host phenotypic changes accompanying growth at or transition to low temperature. With respect to one-step growth curves, however, Olsen et al. (1968), like Kulpa & Olsen (1971) and even Delisle & Levin (1972b), did not specify the pre-infection growth temperature. Olsen's (1967) and Olsen et al.'s (1968) data are in general agreement if one assumes that the 1968 host was grown at 25°C prior to infection; if so, determined characteristics of 'psychrophilic' phages may in part represent host reactions to dramatic temperature shifts.

In this paper, we focus attention on environmentally relevant but neglected variables affecting PHS, including temperature, salinity and pressure, by describing characteristics of a cold-active phage grown on 2 psychrophilic species of *Colwellia*, with most of our work focusing on host *C. psychrerythraea* Strain 34H (hereafter '34H'). The phage, 9A, was isolated from an Arctic nepheloid layer (a particle-rich region of the water column) against 34H, itself originally cultured from Arctic shelf sediments (depth of 305 m; Huston 2003). Although little is known about the quantitative ecology of *Colwellia*, they are routinely isolated from cold, particle- or surface-rich environments, including flounder eggs (D'Aoust & Kushner 1972), sea ice (Junge et al. 2002, Borriss et al. 2003, Brinkmeyer et al. 2003) and nepheloid layers (Wells 2006); association with particles or surfaces is also supported by molecular approaches (DeLong et al. 1993, Gillan et al. 1998, Eilers et al. 2000, Bowman & McCuaig 2003). With a growth temperature optimum among the coldest of cultivated psychrophiles (8 to 9°C ; Huston 2003) and its genome sequenced (Méthé et al. 2005), 34H is an attractive organism with which to explore host and viral features enabling cold activity and their ecological consequences.

MATERIALS AND METHODS

Field sampling and shipboard phage enrichment.

Initial enrichment was conducted in Franklin Bay of the Canadian Arctic in October of 2002, aboard the NGCC 'Pierre Radisson' as part of the international Canadian Arctic Shelf Exchange Study (CASES). During our sampling period, Franklin Bay had a ~40 m surface layer of brackish (23 to 24 psu) water, high concentrations of suspended particulate matter (maximum of 9.3 mg l^{-1} at the surface), and in places, a complicated nepheloid layer structure (Wells et al. 2006). For phage isolation, 40 l of water were collected in rosette-mounted Niskin bottles from Stn 12 (69.8° N, 126.1° W) on 12 October from a near-bottom nepheloid layer at 128 m depth and with an *in situ* temperature of approximately -1°C ; the corresponding water column profile (including transmissivity trace) is shown in Fig. 2B of Wells et al. (2006), where further sampling and site details are given. Once aboard ship, water was consolidated into two 20 l carboys previously liberally rinsed with sample seawater, before transport to a -1°C laboratory for processing. There the water was gravity-filtered through a $0.2 \mu\text{m}$ HT Tuffryn membrane capsule filter (effective filtration area of 500 cm^2 ; Pall Gelman) to remove bacteria but not viruses. After filtration, which took ~36 h due to the large volume, high particle load and small filter area, the water was divided into two carboys and enriched with 4 to 8 ml each of turbid cultures of 34H, *Colwellia psychrerythraea* NRC 1004, and sea-ice isolates 6M3 and 11B5 (putative *Colwellia* and *Shewanella*, respectively; Junge et al. 2002). Multiple hosts were used for enrichment in an attempt to avoid bias toward phage monovalence (Jensen et al. 1998). Yeast extract was also added to a final concentration of 1%. After incubating at -1°C for 4 d, the seawater was again $0.2 \mu\text{m}$ filtered before being concentrated to ~90 ml by ultrafiltration over a 30 kDa cutoff, low-protein-binding, regenerated cellulose membrane (Prep/Scale TFF cartridge, nominal 0.23 m^2 filter area; Millipore). This concentrate was $0.2 \mu\text{m}$ filtered, diluted 1:1 in sterile phage buffer (0.1 M NaCl, 50 mM Tris-HCl pH 8.0, 8 mM MgSO_4 , 2 mM CaCl_2 , 5% glycerol), stored and returned cold ($\leq 4^\circ\text{C}$) to the University of Washington to test against 34H.

Phage isolation. To avoid exposure to high temperature, a modified isothermal version of the agar overlay technique for plaque assay was developed. A bottom layer of approximately 20 to 30 ml of 50% marine broth 2216 (Difco) solidified with 1.5% bacto-agar (Difco) was overlaid with 3.6 ml of a silica sol (generated as described by Dietz & Yayanos 1978) enriched with a tryptone, glucose and yeast extract solution (TGY, where 10× TGY is 5 g tryptone, 2.5 g yeast extract, 1 g glucose in a total volume of 100 ml distilled water), seeded with

bacteria and phage, and gelled by the addition of salt (where 10× artificial sea water [ASW] is 24 g NaCl, 0.7 g KCl, 5.3 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 7 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in a total volume of 100 ml distilled water). For convenience, we refer to this technique as a 'silica-gel overlay.' In a typical preparation, 2.7 ml of silica sol (at 2°C) were normalized to a pH of ~8.7 with a few μl of 5 N NaOH; then, in the following sequence and at 2°C (or lower), 0.3 ml of cold $0.2 \mu\text{m}$ filtered 10× TGY, 0.3 ml of cold $0.2 \mu\text{m}$ filtered 10× ASW, and 0.3 ml of a phage–bacteria suspension were added, with gentle head-over-tail mixing between each addition. Deviation from this sequence sometimes resulted in failed plates; moreover, the salt must be added before the phage–bacteria suspension to avoid osmotic shock. The final pH was 7.4 to 7.6. In side-by-side comparisons using cold-active viruses and a psychrophilic host, approximately five times more plaques, of generally larger size, were observed in silica-gel overlays than more traditional overlays with low melting point agarose.

For initial detection of infective phages, 100 μl of concentrate were added to 300 μl of a turbid bacterial suspension, plated as above and incubated at 2°C . Within 3 d, confluent lysis was detected. A dilution series was performed, with the result that plaques ~0.5 cm in diameter had formed (again within 3 d at 2°C) on the 10^{-4} dilution plate. Several plaques were picked by removing a small plug of gel and underlying agar with a sterile pipette tip and resuspending it in phage buffer. Thereafter a phage designated '9A' was plaque-purified by 3 successive platings. After the third plating, a high-titer phage stock was generated by incubating a nearly confluent lysed plate with 5 ml of phage buffer at 2°C with gentle shaking. After 2 d, the overlying buffer was removed with a sterile pipette, a few drops of chloroform were added to it and, after vigorous shaking, the chloroform was allowed to settle. Chloroform and cellular debris were removed by centrifugation for 20 min at $6000 \times g$ and 2°C . The resultant bacteria-free stock was stored at 2 to 4°C . This procedure typically yielded phage concentrations of 10^{10} to 10^{11} ml^{-1} , determined by staining with SYBR Gold and epifluorescence microscopy (see below).

Electron microscopy. Morphological characterization of Phage 9A was performed by transmission electron microscopy (TEM). Carbon-stabilized, formvar-coated, 300-mesh grids (Ted Pella) first rendered hydrophilic with 0.1% poly-lysine were floated for 10 min atop a 100 μl drop of fresh lysate. After gently wicking the grid dry, it was stained for 60 s on a drop of 1% uranyl acetate, before again being wicked dry. Grids were viewed within 24 h on a Philips CM-100 TEM at an 80 kV acceleration voltage and magnification of 130 to 180 K.

Estimation of genome size. Genome size was estimated by restriction fragment length polymorphism, as in Middelboe et al. (2002). DNA was extracted using a Lambda Midi Kit (Qiagen), following the manufacturer's instructions. Aliquots of 100 to 400 ng phage DNA were digested for 1 to 2 h at 37°C with 5 U of one of the following 10 enzymes (from Promega: *Hind*III, *Dra*I, *Xba*I, *Rsa*I; from Stratagene: *Eco*RI, *Bam*HI, *Hae*III; from New England Biolabs: *Msp*I, *Bst*UI; from Gibco BRL: *Pvu*II), using manufacturer-supplied buffers and acylated BSA when indicated. Digests were visualized by electrophoresis on 0.8% agarose gels post-stained for 30 min with 0.01% buffered SYBR Green I (Molecular Probes).

Efficiency of plating (EOP). Efficiency of plating was determined by simultaneous quantification of phage by plaque assay, using the silica-gel overlay technique, and by epifluorescence microscopy. For the latter, the method of Noble & Fuhrman (1998) as modified by Chen et al. (2001) was used. Briefly, a suitable volume of phage was diluted in cold 0.2 µm filtered ASW amended with 1.5% formaldehyde, then filtered onto 25-mm, 0.02 µm Anodisc filters (Whatman). The filtered ASW contained negligible viral numbers. Filters were stained with 100 µl of 0.1% SYBR Gold (Molecular Probes) for 15 to 20 min, dried by gently brushing the back of the filter against a kimwipe, and mounted on a glass slide with 30 µl of medium containing 50% glycerol, 50% phosphate-buffered saline and 0.1% *p*-phenylenediamine. Slides were counted on a Zeiss Universal microscope at 1563× magnification.

Preliminary experiments to assess phage titers determined by plaque count versus microscopy found considerable variability, despite standardizing bacterial growth and phage infection conditions. To examine this variability further, an experiment was performed to measure changes in EOP at –1°C over short periods of time (less than 1 doubling period). A volume of 90 ml of mid-exponential 34H, grown at –1°C, was passed through a Type A/E glass fiber filter (Gelman, nominal pore size of 1.0 µm, sterilized by autoclaving) to remove large cells, including those on the verge of dividing. The filtrate of small cells was then incubated at –1°C until again reaching mid-exponential phase, as monitored by absorbance at 600 nm. The culture was filtered a second time as before and distributed in 30 ml aliquots to three 50 ml sterile polypropylene tubes. These tubes were incubated at –1°C without addition of phage 9A. Subsamples from each replicate were removed daily for 3 d and challenged with a dilution series of phage, plated using the silica-gel overlay technique and incubated at 2°C. The same phage stock was used for all time points and, to be consistent with the rest of the experiment, was stored at –1°C in the interim. At each time point, bacterial concentration

and the frequency of dividing cells were determined from 0.1-ml subsamples using SYBR-staining and epifluorescence microscopy. At the start of the experiment, the phage stock was quantified by SYBR-staining; no appreciable decay of viruses was observed over these time periods at –1°C (Wells & Deming 2006c, this issue).

Host range determined by plaque formation. Plaque formation by 9A was assayed against 21 bacterial isolates of marine origin besides 34H (see Table 1) using the silica-gel overlay technique and 2 temperatures, –1 and 8°C, representing the *in situ* and optimal growth temperatures of 34H. Prior to infection prospective hosts were grown in 50% marine broth 2216 to early or mid-exponential phase at the same temperature as the plaque assay (including phage adsorption). They were then concentrated 5-fold by centrifugation and plated against 9A, employing a dilution series if no lawns developed. All experiments included negative (no phage added) and positive (34H + 9A) controls. In 2 cases (*Colwellia rossensis*, grown at –1°C, and *C. hornerae*, grown at 8°C), starved bacteria were tested for altered susceptibility to phage infection. For starvation, the bacteria were pelleted, rinsed with cold ASW, resuspended in ASW and held on ice for 6 h. Following addition of 9A, plates were poured as usual and incubated at 8°C.

Temperature and salinity ranges of plaque formation. The temperature range of plaque formation was tested using the silica-gel overlay technique at a salinity of 34 psu. The temperatures examined were –6, –3, –1, 2, 4, 8, 13 and 18°C ($\pm 1^\circ\text{C}$ for each), corresponding to the known range of growth by 34H under these conditions (Huston 2003). For each experiment, bacteria were grown at –1°C prior to infection; to check for effects due to temperature shift, a few experiments were also conducted with 34H grown at 8°C. In all cases, early to mid-exponential cells (600 nm absorbance typically at 0.3) were concentrated 5-fold by centrifugation in 50% marine broth 2216 and infected with a suitable dilution of phage. Approximately 30 min were allotted for adsorption before plating, followed by incubation at the given temperature until plaques or a lawn were observed (typically 1 to 2 wk, depending on temperature). At –1, 8 and 13°C, we tested whether prior starvation in ASW (as already described) for 4, 29 or 53 h at –1°C affected the temperature range. Because of indications of phage production without plaque formation, we also examined viral production at 8 and 13°C by SYBR staining and microscopy.

The salinity range was assessed at –1°C following Huston (2003). A basal medium consisting (l^{-1} of distilled water) of 3 g peptone, 1.2 g yeast extract, 6 mg FePO_4 and 3 mM TAPSO (3-[N-tris(hydroxymethyl)

methylamino]-2-hydroxy-propanesulfonic acid) buffer, pH 7.2 was modified with 10× ASW to obtain salinities from 50 to 200% of normal seawater salinity (in the terms reported by Huston 2003), equivalent to 20–65 psu as determined by refractometer. These salinities correspond to the salinity range of growth by 34H at -1°C under these conditions (Huston 2003) and represent an environmentally realistic range from brackish water (as seen at Stn 12 due to riverine input and ice melt; Fig. 2B in Wells et al. 2006) to the mild brines of relatively warm (-3°C) sea ice. Prior to infection, 34H was grown at the experimental salinity through at least 1 transfer, then concentrated and infected as described above. For the silica-gel overlay plates, both the agar bottom layer and the silica-gel overlay were adjusted to the experimental salinity. At the highest salinity (200%, or 65 psu), the silica-gel overlays gelled poorly, so phage production was assayed in broth instead and evaluated by SYBR-staining and epifluorescence microscopy.

Finally, because 9A also infects *Colwellia demingiae* ACAM 459^T, we determined the temperature and salinity ranges of plaque formation on it, as above. All experiments with either host included negative (no phage added) and positive (34H + 9A under conditions permissive of phage production) controls.

Phage production between -10 and -13°C . Two experiments were conducted in an incubator maintaining a temperature between -10 and -12°C (first experiment) or -12 and -13°C (second experiment), as validated by both a low temperature thermometer and a thermocouple probe. Because plates froze at $<-6^{\circ}\text{C}$, the procedure outlined above was not viable. Instead, for the first experiment, changes in the concentration of plaque-forming units (PFU) were assayed indirectly as follows: 40 ml of early-exponential 34H (pre-grown at -1°C) were concentrated 2-fold in fresh, cold and $0.2\ \mu\text{m}$ filtered, 50% marine broth 2216. Phage 9A was added at a multiplicity of infection (MOI) of 2 (determined by SYBR-staining and epifluorescence microscopy) and allowed to adsorb for 10 min on ice. The cells were then pelleted again, the virus-containing supernatant was poured off, and the visible pellet was gently washed three times with 5 ml of the broth. After the last wash, the bacteria were resuspended in 15 ml of broth and 1 ml aliquots were dispensed into sterile Eppendorf microfuge tubes for incubation at -10°C . Three were sacrificed to measure concentrations of PFU at time $t = 0$, by plating on uninfected bacteria at 2°C . Thereafter, the remaining tubes were sacrificed in groups of three (chosen randomly and regardless of whether the samples had frozen) after 5, 10 and 15 d and plated as above. Frozen samples were thawed and processed as soon as the last ice crystal melted. An uninfected 34H control was also incubated at -10°C

and periodically assayed for plaque formation. Because of variability in EOP over such an extended time period (see 'Results' subsection; 'Efficiency of plating' below), a sample of phage with no added bacteria was set aside (at -1°C) and plated at each time point to serve as a standard. The stability of phage stocks for >6 mo at temperatures $\leq 4^{\circ}\text{C}$ indicated that decay of the plating standard over the 2 wk experiment at -1°C should be negligible.

To address phage production specifically in liquid samples below -10°C , a second experiment was performed in which bacterial and viral concentrations were monitored by epifluorescence microscopy. Mid-exponential 34H was diluted 1:8 in cold 50% 2216 and infected with 9A to an MOI of 1.5. After gentle mixing, 1 ml aliquots of infected culture were dispensed into 18 sterile 1.5-ml microfuge tubes. At $t = 0$ and every 3 to 5 d for 1 mo, 100 μl were taken from a tube containing liquid sample and fixed into 3 ml ice-cold, $0.2\ \mu\text{m}$ filtered ASW amended with formaldehyde (1.5% final concentration). The samples were immediately filtered onto 0.02- μm Anodiscs, stained as above with SYBR Gold and counted within 24 h. During the first half of the experiment, a liquid-containing tube was selected at random for each time point. By Day 18, only a single tube remained unfrozen (sampled on Day 14). To conserve volume, 50 μl was subsampled repeatedly from this tube for the remaining time points.

Phage production under hydrostatic pressure. To determine the pressure range of phage production at -1°C , 34H was grown at 1 atm and -1°C to mid-exponential phase. The culture was then diluted 1:10 in fresh media and split into two 11 ml batches kept on ice. One batch was left untreated as a control; the other received 9A at a final concentration of $3 \times 10^7\ \text{ml}^{-1}$ (MOI of 3). After gentle head-over-tail mixing of each split, small aliquots were fixed in cold ASW amended with formaldehyde (1.5% final conc.) to measure initial abundances of bacteria and viruses by SYBR staining (as above). Sample volumes of 0.7 ml were then dispensed into sterile, 0.7 ml microfuge tubes with caps removed and sealed with parafilm (leaving no headspace). Two samples, 1 with phage and 1 without, were incubated at 1 atm and -1°C ; the remaining, in sets of two for control and treatment, were pressurized to 10, 100, 200, 400 or 600 atm, using stainless steel pressure vessels and distilled water as the hydraulic fluid (Deming 2001). At 8°C , 34H is known to grow at pressures of 1 to 400 atm, although no significant growth has been detected at 600 atm (R. E. Collins, J. G. Marx & J. W. Deming unpubl. data). From the time of phage addition, 1 h elapsed before all samples were pressurized and incubating at -1°C . For each end-point experiment at a given pressure, 2 vessels were used. One was decompressed after 24 h to estimate (by SYBR-

staining and epifluorescence microscopy) short-term bacterial and viral changes associated with the increased pressure and subsequent decompression. The other was incubated for 3 d (1 and 10 atm), 7 d (100 and 200 atm) or 14 d (400 and 600 atm) at -1°C before decompression and phage and bacterial enumeration. Incubation times were varied at different pressures to accommodate corresponding differences in 34H growth rate.

Adsorption experiments. Two methods were used to measure phage adsorption to 34H, the first relying upon PFU assays and the second on SYBR counts. In both, 9A was added to 3–5 ml of early exponential 34H culture ($\sim 10^8$ cells ml^{-1}) grown at -1 , 2 or 8°C , depending on the experiment. MOI ranging from ~ 0.1 to ~ 10 and incubation periods (on ice or at 8°C) from 5 to 180 min were tested. Periodically during the incubations, 0.1 ml aliquots were removed and diluted 1:100 in cold 50% marine broth 2216 to stop adsorption. After gentle mixing, cells were pelleted by centrifugation from a subsample of this dilution. In the PFU method, the supernatant was assayed for unadsorbed phage using the silica-gel overlay technique, while in the SYBR method, viruses were enumerated by epifluorescence microscopy as above; i.e., the sample was fixed in ASW amended with formaldehyde (1.5% final conc.) following the centrifugation step. For the PFU method, the sample was centrifuged at $3000 \times g$ for 5 min. For the SYBR method, it was centrifuged at higher speed and duration, $15000 \times g$ for 10 min, because the presence in the supernatant of even a small fraction of the total cells (which did not affect interpretation of the results of the PFU method) led to occlusion of the field of view for counting viruses. The MOI was determined by plating the phage stock (PFU method) or by counting viruses (SYBR method) and bacteria (both methods) using epifluorescence microscopy. Because of the low EOP (see below), MOI determined by plaque counts may have underestimated true MOI by as much as 100-fold.

One-step growth curves. One-step growth curves were determined at -1 and 8°C , using a protocol modified from Adams (1959); all steps were done at the experimental temperature, including growing the initial culture of 34H. Briefly, 1 ml of early exponential 34H was infected with 9A at a MOI of ~ 1 at 8°C (determined by SYBR-staining) or 3 at -1°C (by PFU). After 10 min for adsorption, the bacteria and phage were diluted 1:10 in 50% marine broth 2216 and immediately centrifuged to pellet cells. After removing the phage-containing supernatant, the cells were washed and resuspended in fresh growth media. This tube was called the 'experimental tube.' It was then diluted serially in increments of 1:10 to generate the 'first growth tube,' 'second growth tube,' etc., up to a 'fifth growth

tube.' Multiple growth tubes were used because the fraction of infected bacteria could not be calculated in advance. At $t = 0$, a dilution series of the experimental tube was plated by silica-gel overlay to determine the initial number of infected bacteria. Dilutions of each growth tube (1:10 and 1:100) were also plated at $t = 0$ and at regular intervals thereafter to measure phage production. The multiple growth tubes and different dilutions provided a series of redundant (but, strictly-speaking, not replicate) measurements at each time point. For both experiments, plates were incubated at 2°C .

RESULTS

Morphological characterization

Examination of 9A by TEM revealed it to be a tailed phage (Fig. 1). The capsid diameter is 90 nm in its longest dimension, with a tail length of ~ 200 nm. The tail does not appear to be contractile.

Genome size

The enzymes *EcoRI*, *PvuII* and *BstU1* yielded qualitatively similar digests, with 10 to 11 fragments of similar size, ranging from ~ 3 to 22 kb. The sum of fragments suggests a genome size of 80 to 90 kb. For the *DraI* and *HindIII* digests, the range of fragment sizes was < 2 to ~ 6 kb, implying a smaller genome of 40 kb; however, a large number of small fragments may have gone undetected. Phage DNA was not digested by *XbaI*, *BamHI*, *HaeIII*, *MspI* or *PvuII*.

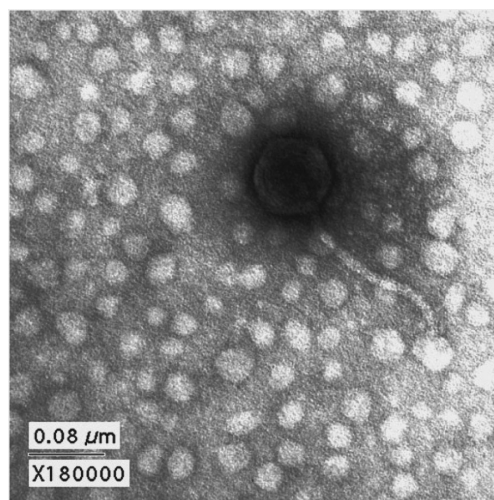


Fig. 1. Transmission electron micrograph of Phage 9A

Efficiency of plating

The EOP, routinely determined by comparing counts of SYBR-stained viruses and of PFU, was typically at or below 1% at -1°C . Because it occasionally varied more widely and once reached 74%, vacillating for a 2 wk period thereafter between 1.5 and 27% (data not shown), changes in EOP were evaluated over 3 d at -1°C . EOP was highest at the start of the experiment (1%) and declined linearly with time ($p < 0.009$) to 0.2% at the end of the experiment (Fig. 2). Over that time period, the 34H population increased by a factor of only 1.6 (Fig. 2). Although cell growth was not synchronized, the attempt to remove large and dividing cells by $1.0\ \mu\text{m}$ filtration was relatively successful, given the low concentration ($1.8 \times 10^6\ \text{ml}^{-1}$) and frequency of dividing cells (4%) at the start of the experiment (Fig. 2). Concentrations of dividing cells increased rapidly over the first day (by a factor of 7; Fig. 2), but then stabilized, with a relatively constant frequency over the final 2 d (15 to 20%).

Range of hosts supporting plaque formation

Of 21 bacterial cultures (besides 34H) against which Phage 9A was tested, no other characterized or probable strains of *Colwellia psychrerythraea* were susceptible (Table 1), although induction of a temperate phage from a probable strain (53A3) increased if 9A was pre-

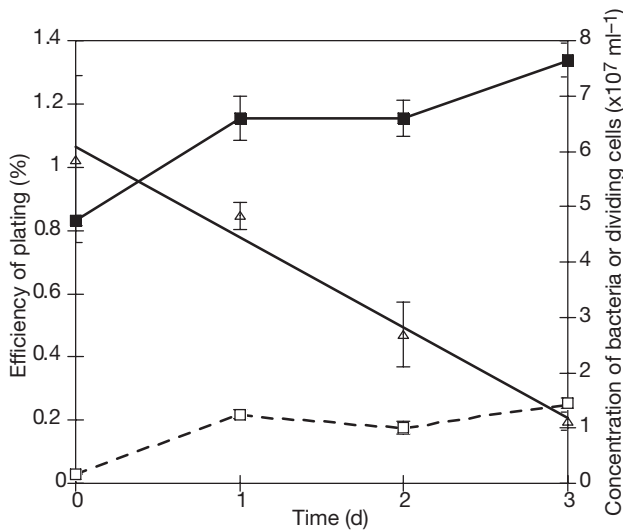


Fig. 2. Change in efficiency of plating (EOP) (Δ and regression line) and the concentrations of bacteria (\blacksquare) and dividing cells (\square) with time. Regression equation is $\text{EOP} (\%) = -0.29 \times \text{time} (\text{d}) + 1.1$; $p < 0.009$. Error bars represent $\pm \text{SE}$ of the mean of triplicate samples (where not visible, bars are smaller than the width of the symbol)

sent (see Wells 2006 for further details). Within the *Colwellia* genus, 9A could infect *C. demingiae* ACAM 459^T at both -1 and 8°C . No other hosts were identified among the other marine bacteria assayed, nor did prior starvation result in susceptibility to 9A for either *C. rossensis* ACAM 608^T or *C. hornerae* ACAM 607^T under the conditions tested.

Temperature range of plaque formation

When 34H was grown at -1°C prior to infection, 9A formed plaques on silica-gel plates from -6 to 4°C but not at 8°C (Table 2). Prior growth at 8°C did not change the temperature range, but the number of plaques was usually reduced considerably. The temperature range of plaque formation could be extended to 8°C by prior starvation of 34H (whether for 4, 29 or 53 h), although the plaques were smaller than usual. Given these results, SYBR-staining and epifluorescence microscopy were used to assess whether viral production persisted under non-starvation conditions at 8 and 13°C , despite the absence of plaque formation. Over 24 h, viral concentrations increased an order of magnitude at 8°C ; no increase was seen at 13°C .

Colwellia demingiae ACAM 459^T had a broader temperature range of plaque formation than 34H (without prior starvation), from -6 to at least 8°C (Table 2), despite the 2 bacteria having similar cardinal growth temperatures (Bowman et al. 1998). Weak growth in silica-gel overlays at $\geq 13^{\circ}\text{C}$ prevented determination of the upper temperature limit of plaque formation, however.

Salinity range of plaque formation

Over the salinity range for robust growth of 34H (50 to 150% seawater, or 20 to 50 psu), 9A also produced plaques on 34H (Table 3). Comparable numbers of plaques were observed at all salinities. At 200% seawater salinity (65 psu), growth of 34H was weak, as reported previously (Huston 2003). Silica-gel overlays were not effective at this salinity, but SYBR-staining and epifluorescence microscopy revealed no evidence of viral production over a 3 d period.

Colwellia demingiae ACAM 459^T appeared to have a narrower salinity range for plaque formation (but not growth) than 34H (Table 3). Plaques were observed at 75 and 100% seawater salinity (27 and 34 psu); a lawn was observed at 150% (50 psu). At 50% (20 psu), *C. demingiae* ACAM 459^T grew too weakly in silica-gel overlays to determine whether plaques had formed.

Table 1. Marine bacteria tested against Phage 9A for plaque formation on silica-gel overlay plates incubated at -1 or 8°C . Cultures were grown at the plaque assay temperature. +: plaques observed; -: no plaques observed (bacterial lawn); wg: bacterial growth too weak or variable for a definitive assay; ng: below the bacterium's minimum temperature for growth; nd: not determined; UT: University of Tasmania; UW: University of Washington; JCM: Japan Collection of Microorganisms; ATCC: American Type Culture Collection; IMB: Institut für Marine Biotechnologie

Culture	Source	Plaque formation	
		-1°C	8°C
Characterized <i>Colwellia</i> spp.			
<i>C. psychrerythraea</i> NRC 1004	ATCC	wg	-
<i>C. psychrotropica</i> ACAM 179 ^T	J. Bowman, UT	-	-
<i>C. demingiae</i> ACAM 459 ^T	J. Bowman, UT	+	+
<i>C. demingiae</i> ICP 10	J. Bowman, UT	-	-
<i>C. hornerae</i> ACAM 607 ^T	J. Bowman, UT	-	-
<i>C. maris</i> JCM 10085	JCM	-	-
<i>C. rossensis</i> ACAM 608 ^T	J. Staley, UW	-	-
Probable <i>Colwellia</i> (uncharacterized but for 16S rRNA analysis)			
Arctic surface-water isolate 53A3 ^a	This laboratory (UW)	_b	_b
Arctic nepheloid layer isolate 75C3 ^a	This laboratory (UW)	-	-
Arctic sea-ice isolate 21C ^c	M. Borriß & T. Schweder, IMB, Greifswald, Germany (Borriß et al. 2003)	-	-
Arctic sea-ice isolate 6M3	This laboratory (Junge et al. 2002)	-	-
Other characterized Bacteria			
<i>Idiomarina abyssalis</i> KMM 227	ATCC	-	-
<i>Idiomarina zobellii</i> KMM 231	ATCC	ng	-
<i>Halomonas pacifica</i>	J. Kaye & J. Baross, UW	ng	-
<i>Halomonas aquamarina</i>	J. Kaye & J. Baross, UW	ng	-
<i>Planomicrobium mcmeekinii</i>	This laboratory (UW)	-	-
Other uncharacterized Bacteria (but for 16S rRNA analysis)			
Marine isolate NP9 (putative <i>Vibrio</i>)	B. van Mooy & A. Devol, UW	ng	-
Arctic sea-ice isolate 11B5 (putative <i>Shewanella</i>)	This laboratory (UW) (Junge et al. 2002)	-	-
Arctic sea-ice isolate 4B3 (putative α - <i>Proteobacterium</i>)	This laboratory (UW) (Junge et al. 2002)	nd	-
Uncharacterized bacteria of uncertain affiliation			
Arctic sea-ice isolate 7W1	This laboratory (UW)	-	-
Arctic nepheloid layer isolate 12S3 (from same water sample as 9A)	This laboratory (UW)	nd	-
^a Based on 16S rRNA sequencing and phylogenetic analysis, most closely related to <i>C. psychrerythraea</i> (R. E. Collins, L.E.W. & J.W.D. unpubl. data); isolated during CASES 2002			
^b Isolate 53A3 is a known lysogen (Wells 2006): plaque counts increased in the presence of 9A, but the plaques were turbid			
^c Identified by Borriß et al. (2003) as most closely affiliated with <i>C. psychrerythraea</i> or <i>C. demingiae</i>			

Phage production between -13 and -10°C

The intention of the first experiment between -10 and -12°C was to measure phage production in a frozen sample, presumably within the brine inclusions formed during freezing (Junge et al. 2001, Wells & Deming 2006a). Surprisingly, only about half of the experimental samples froze, even after 15 d. Failure to freeze may reflect an absence of ice-nucleation events: in this case, neither 34H nor 9A has substantial ice-nucleating ability (K. Junge pers. comm.) and we were using $0.2\ \mu\text{m}$ filtered broth. In any event, these circumstances enabled us to compare phage production at

-10°C in liquid medium vs. ice. Considering average concentrations of PFU (scaled to the plating standard, as described in 'Materials and methods') for triplicate samples at each time increment, no change was observed after 5 d, but the concentrations had increased 5-fold after 10 d, declining by half thereafter (Fig. 3A). If these data are segregated according to liquid vs. frozen samples, the increases in PFU concentration were significant only in the tubes that remained liquid (Fig. 3A). Again, no change was observed during the first 5 d, but concentrations in liquid samples on Days 10 and 15 were 6- to 7-fold greater (and comparable to each other).

Table 2. Phage 9A. Range of temperatures at which 9A forms plaques on *Colwellia psychrerythraea* 34H or *C. demingiae* ACAM 459 in 50% marine broth 2216 (34 psu), including after starvation in ASW. Hosts were grown at -1°C prior to infection. +: plaques observed; -: no plaques observed (bacterial lawn); wg: host growth too weak or variable for a definitive assay. nd: not determined

Test temp. (°C)	34H	Test culture Starved 34H	459
-6	+	nd	+
-3	+	nd	+
-1	+	+	+
2	+	nd	+
4	+	nd	+
8	- ^a	+	+
13	- ^b	-	wg
18	wg	nd	nd

^aPhage production was detected microscopically by SYBR-staining
^bNo phage production was detected microscopically

Table 3. Phage 9A. Range of salinities at which 9A forms plaques on *Colwellia psychrerythraea* 34H or *C. demingiae* ACAM 459 at -1°C . +: plaques observed; -: no plaques observed (bacterial lawn); wg: bacterial growth was too weak or variable for a definitive assay. nd: not determined

Salinity		Test culture	
% seawater	psu	34H	459
50	20	+	wg
75	27	+	+
100	34	+	+
150	50	+	-
200	65	- ^a	nd

^aSilica-gel overlays gelled poorly at this salinity and could not be used. No phage production was detected microscopically

In the second experiment designed to consider changes in viral and bacterial concentrations in liquid samples only, the incubator maintained slightly colder temperatures of -12 to -13°C . As before, several samples remained liquid at this temperature for up to 2 wk and one remained so for the 1 mo experiment. Over the first 11 d, both viral and bacterial concentrations declined (Fig. 3B). Thereafter, the bacteria increased 6-fold over a 2 wk period, while viruses showed step-like increases of $\sim 4 \times 10^6$ viruses ml^{-1} and 7×10^6 viruses ml^{-1} on Days 14 and 27, respectively (Fig. 3B). On Day 32 bacteria but not viruses had begun to decline.

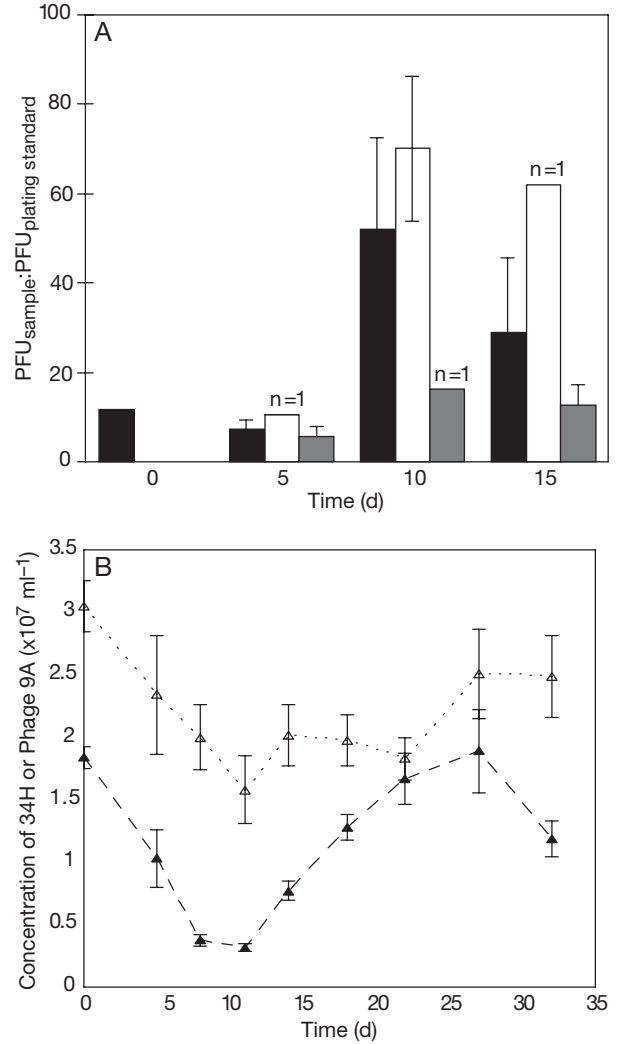


Fig. 3. (A) Phage 9A. Phage production during incubation at temperatures between -10 and -12°C , where phage abundance was assayed by ability to form PFU at 2°C and then normalized to a plating standard (see 'Materials and methods'), yielding the ratio $\text{PFU}_{\text{sample}}:\text{PFU}_{\text{plating standard}}$. Black bars: average ratio in triplicate samples (whether frozen or liquid); open bars: average ratio in liquid samples only; grey bars: in frozen samples only. Error bars are $\pm\text{SE}$ of the mean of triplicate samples (black bars) or range of 2 samples. At $t = 0$, error bar is too small to be visible. (B) Changes in the concentration of bacteria (\blacktriangle) or viruses (\triangle) monitored by epifluorescence microscopy in liquid samples at -12 to -13°C . Error bars represent $\pm\text{SE}$ of the mean of the microscopic count

Phage production under hydrostatic pressure

In the absence of 9A, after a 24 h incubation period under pressures of 10 to 200 atm, bacterial concentrations increased modestly (10 to 60%) at 10 and 100 atm and doubled at 200 atm. In contrast, viral concentrations increased 4-fold at 10 atm and marginally (15 to 20%) at 100 and 200 atm. At the highest pressures of

Table 4. Concentrations of bacteria and phages determined microscopically after incubation at -1°C and a range of hydrostatic pressures. $\text{Phage}_f/\text{Phage}_i$: final concentration of phages at the end of the incubation period divided by initial phage concentration. Initial concentrations were 9.3×10^6 bacteria ml^{-1} and 2.7×10^7 phage ml^{-1}

Pressure (atm)	Incubation period ^a (d)	34H (cells ml^{-1})		9A (phage ml^{-1})	$\text{Phage}_f/\text{Phage}_i$
		No-phage control	Phage added		
1	3	2.0×10^8	7.7×10^6	1.2×10^{10}	440
10	3	2.6×10^7	1.3×10^6	1.9×10^{10}	700
100	7	2.7×10^7	6.3×10^6	1.5×10^{10}	560
200	7	8.8×10^{6a}	3.9×10^6	1.9×10^8	7
400	14	3.8×10^{5b}	3.5×10^6	1.4×10^7	0.5
600	14	4.2×10^6	3.2×10^6	1.6×10^7	0.6

^aSee 'Results' for data after 24 h, including doubling of bacterial concentration at 200 atm
^bAnomalously low bacterial count possibly indicative of a leak

400 and 600 atm, concentrations of both viruses and bacteria were reduced by about 25 and 50 %, respectively, with noticeably deformed cells at 600 atm.

Over the full incubation periods of 3, 7 or 14 d, viral concentrations increased 440- to 700-fold at 1, 10 and 100 atm, more modestly at 200 atm (by a factor of 7; Table 4), and declined by half at higher pressures. In the absence of viruses, 2- to 22-fold increases in bacterial concentration demonstrated that 34H grew at pressures between 1 and 200 atm (Table 4). No evidence for growth was seen at 400 or 600 atm; the bacteria were visibly deformed at both pressures. Consistent with phage-induced mortality, bacterial concentrations were 2.3 to 26 times lower when phages were present than absent at pressures between 1 and 200 atm. Because of genomic evidence that 34H may be a lysogen (Methé et al. 2005, Wells 2006), we also examined the negative controls for evidence of lysogenic induction by pressure, but no phage were detected microscopically.

Adsorption experiments

Eighteen attempts were made to measure adsorption of 9A to 34H, varying the MOI, the growth temperature of 34H prior to infection, the adsorption temperature and the assay period. All experiments yielded results qualitatively similar to those shown in Fig. 4, whether using SYBR or PFU counts to determine the phage concentration and despite the difference in centrifugation conditions for the 2 methods. Although over a 2-h period concentrations of unadsorbed phage tended to decline, the decline was relatively small and could not be measured with precision. No marked difference was detected between adsorption at -1 or 8°C . Further purification of 9A by precipitation in polyethyl-

ene glycol 8000 (as in Sillankorva et al. 2004b) did not improve adsorption or increase EOP (data not shown).

One-step growth curves

The absence of a meaningful measure of adsorption rate necessitated plating multiple growth tubes for one-step growth curves, since the number of cells infected over the 10 min adsorption period could not be calculated *a priori*. Longer adsorption periods were undesirable to the extent that infections would not be synchronous. The fraction of viruses that infected cells was determined after the

fact, however, and in both the -1 and 8°C experiments, was very low: ~ 0.07 and $\sim 0.006\%$, respectively. The calculated fraction for -1°C is probably an overestimate, however, since phage concentration in the stock solution used for that experiment was measured only in terms of PFU; concentrations determined by SYBR staining were typically 10 to 100 times greater (as discussed with respect to EOP).

At -1°C , the 9A–34H PHS had a latent period of 4 to 5 h, a prolonged rise time of 4 to 5 h and an average burst size of ~ 55 viruses (Fig. 5). In comparison, the latent period, rise time and average burst size at 8°C were reduced, to 2.5–3 h, 2–2.5 h and ~ 5 viruses, respectively (Fig. 5).

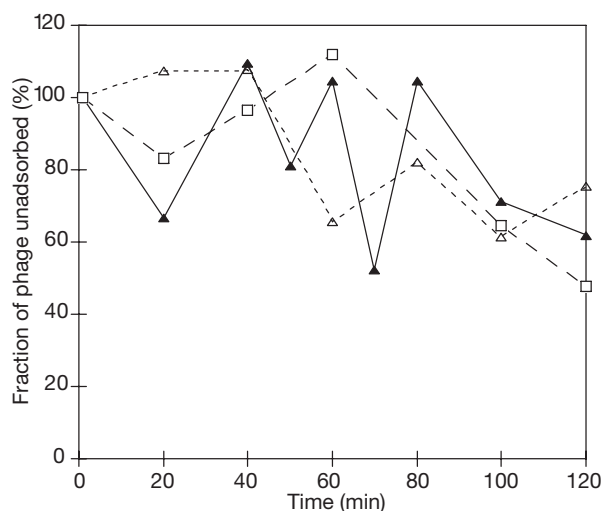


Fig. 4. Phage 9A. Adsorption to host *Colwellia psychrerythraea* Strain 34H at an MOI of ~ 0.1 in 3 independent experiments as determined from PFU (\blacktriangle) or SYBR-staining (\triangle) at -1°C , or by SYBR-staining at 8°C (\square). Bacteria were grown at 2°C (or 8°C) prior to infection for the -1°C (or 8°C) experiments

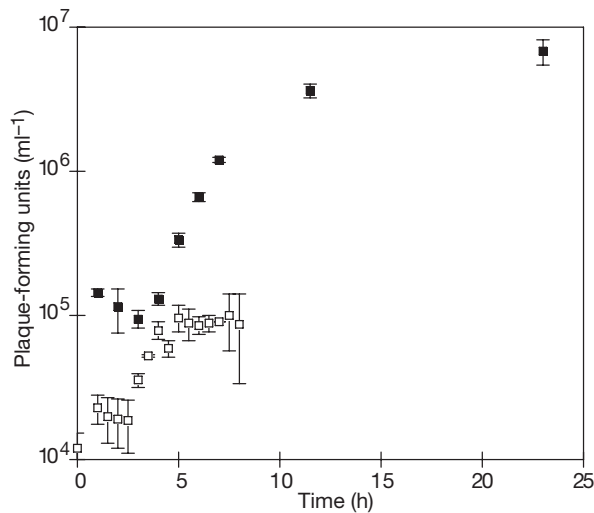


Fig. 5. Phage 9A. One-step growth curves for phage grown on *Colwellia psychrerythraea* 34H at -1°C (■) or 8°C (□). Error bars represent $\pm\text{SE}$ of the mean of redundant measurements (defined in 'Materials and methods')

DISCUSSION

Phage 9A, with a double-stranded DNA genome, Bacterial host and non-contractile tail, can be classified according to the 'Seventh Report of the International Committee on Taxonomy of Viruses' (van Regenmortel et al. 2000) as a Siphoviridae. The estimated capsid size and 80 to 90 kb genome are most similar to T5-like viruses, but no tail fibers were observed.

Under the conditions tested, 9A had 2 closely related hosts, *Colwellia psychrerythraea* 34H from the Arctic and *C. demingiae* ACAM 459^T from the Antarctic, thus qualifying, like many marine Siphoviridae, as a virus with a broad host range according to the criteria of Wichels et al. (2002). Conclusions reached about host range, however, depend critically on experimental conditions and type of assay. In our case, prior host starvation, temperature and salinity all affected the propensity of 9A to form plaques, with the 2 identified hosts having overlapping but distinct characteristics (Tables 2 & 3). Phage production also persisted under conditions not conducive to plaque formation (8°C ; cf. Table 2 with Fig. 5), as observed in other PHS (Adams 1959). An important ecological implication of these data is the suggestion of a mechanism by which high concentrations of bacteria and phage can coexist: host specificity may reflect adjustable bacterial phenotypes responsive to changing environmental conditions. These findings also provide a reminder that no current assay for host specificity is ecologically satisfying, being undertaken against a minuscule representation of microbial diversity and always under set environmental conditions.

The temperature range of plaque formation by 9A accords with our definition of a cold-active virus: on both hosts, plaques were observed at $\leq 4^{\circ}\text{C}$ and down to -6°C (below which plates froze). The latter data add to the report by Delisle & Levin (1972b) of plaque formation by *Pseudomonas putrefaciens* down to -5°C , to our knowledge the only other phage study to test sub-zero temperatures. We also found evidence by both plaque assay and epifluorescence microscopy that 34H continued to produce phage in liquid samples at temperatures between -10 and -13°C (Fig. 3), consistent with the theoretical minimum growth temperature of 34H (-14.5°C ; Huston 2003), prior observations that 34H remains motile at -10°C (Junge et al. 2003) and the current, minimum growth temperature of a bacterium in pure culture (-12°C for *Psychromonas ingrahamii*; Breezee et al. 2004). In fact, the observed increase in 34H concentrations after the first 11 d at temperatures between -12 and -13°C implies a generation time of 5 to 7 d (Fig. 3B) compared to ~ 10 d for *P. ingrahamii* at -12°C . These results parallel experimental evidence from winter sea-ice brines for bacterial metabolic activity at -2 to -20°C (Junge et al. 2004) and viral production and bacterial growth at -12°C (Wells & Deming 2006a). Significant production of 9A at -10°C was not observed (by PFU assay) in frozen samples, possibly due to impingement of host cells by ice crystals or to high salinities in liquid brine inclusions where cells and viruses presumably partitioned. Salt inhibition is supported by the failure to detect viral production at salinities > 50 psu on either host (albeit at -1°C ; Table 3). The possibility exists that phage were produced in frozen samples but were defective. Inactivation after production is unlikely, since only relatively small (16 to 34%) decreases in 9A infectivity or viral concentration were observed over 1 mo at -12°C in brine of 160 psu (Wells & Deming 2006c). Moreover, normalized phage concentrations in frozen samples did not decline appreciably from initial values (Fig. 3A).

Like other cold-active phages, 9A when grown on host 34H appears to have a more restricted temperature range than does the host itself, not forming plaques above 4°C (8°C if 34H is first starved) and not producing viruses at 13°C (Table 2; insufficient data are available for a similar analysis of 9A on *Colwellia demingiae*). Restricted temperature ranges may suggest a general feature of cold-active phage: adaptations to low thermal energy may be offset by higher temperatures seldom if ever experienced *in situ*. This possibility, the greater number of PFU observed using silica-gel rather than agar overlays, and the extreme thermolability of 9A (as well as other cold-active viruses; see Wells & Deming 2006c), argue for the importance of acquiring and studying phage–host

dynamics under experimental conditions mimicking as closely as possible those of interest environmentally.

A large fraction of the cold waters of the ocean are under significant pressure; i.e. ~75% of the ocean by volume lies below a depth of 1 km (calculated from Table J.6 in Pilson 1998; only ~5% is at or above 200 m). The presence in the deep ocean of high concentrations of viruses (e.g. 10^4 to 10^8 ml⁻¹; Hara et al. 1996, Weinbauer et al. 2003, Wommack et al. 2004, Ortmann & Suttle 2005) hints at a significant viral role in bacterial mortality there (Weinbauer et al. 2003). Yet, to our knowledge, apart from the work of Wiebe & Liston (1968) on a PHS functional at 200 atm (temperature unspecified), no laboratory-based studies of phage production under relevant hydrostatic pressures are available. In our work, 34H produced phage under pressures of at least 200 atm at -1°C (Table 4), simulating conditions on Arctic continental shelves. The 440- to 700-fold increase in viral concentration observed at low pressures of 1 to 100 atm implies that several infection cycles occurred. In contrast, the modest (7-fold) increase at 200 atm, most of it happening after the first day at pressure, may indicate that only a single infection cycle took place, whether because viruses could not infect cells under these conditions, or cell growth had diminished or ceased, or the incubation period (7 d) was too short. Deeper waters and sediments may yield PHS with greater tolerance of, or adaptation to, high pressures.

Under our experimental conditions, the 9A–34H PHS is notable for its low EOP and the absence of measurable phage adsorption. Low EOP may in part reflect the use of epifluorescence microscopy to determine total phage concentrations, since EOPs are normally standardized to more conservative, electron microscopic counts. Epifluorescent counts of viruses typically correlate with electron microscopic counts, however, even if generally being higher (e.g. Noble & Fuhrman 1998). Slow adsorption may also help to explain the low EOP, if only a small fraction of cells were infected prior to plating. The essentially undetectable adsorption is unlikely to be a methodological artifact, since it was supported by several different types of measurements, including assay of unadsorbed phage by both plating and microscopy (Fig. 4) and of adsorbed phage in the one-step growth experiments (<0.1% of phage adsorbed within 10 min), and the first experiment conducted between -10 and -12°C (<1% adsorbed). These results instead suggest 3 possible explanations.

First, only a small fraction of cells might be susceptible to phage 9A. This possibility can be excluded, since eventually 9A routinely lysed >95% of bacteria under permissive conditions (data not shown). Second, the vast majority of the viruses might not be infective. Yet, under some circumstances, the majority of viruses were

infective, with the highest measured EOP being 74%. Third (and most likely), a changeable cellular phenotype might account for the low adsorption rate. This explanation is consistent with the results in Fig. 2, which imply that the substantial drop in EOP could be due to a systematic bacterial adjustment. Note that Fig. 2 cannot be interpreted as selection of phage-resistant mutants, since the bacteria and phage were not cultured together. The importance of host phenotypic changes to phage dynamics is also implied by the extension of the temperature range of plaque formation following starvation and by the decrease in number of plaques commonly observed at different temperatures if 34H was first grown at 8°C rather than -1°C . Phenotypic variability within the bacterial population could also account for the long rise times. In the context of lysogeny and pseudolysogeny, other studies have implicated modified availability of phage receptors on the cell surface to explain similar results (Barksdale & Arden 1974, Williamson et al. 2001). While such modification is often interpreted in terms of genotypic changes, either through the selection of resistant 'adhesion-deficient' mutants or phage conversion (Williamson et al. 2001), phenotypic changes associated with the physiological state of the host (Delbrück 1940) or with cell-surface characteristics, including those responsive to temperature (Benedi et al. 1991), are also known to affect adsorption. These latter features could include the rare or transient expression of a receptor, removal of a receptor or receptor masking (Benedi et al. 1991), or release of competitive binding agents by the host (the suggested Factor X of Moebus 1997a,b, although Moebus believed that Factor X was probably phage-encoded). We considered the possibility that 34H was susceptible to infection only during a particular stage of its growth cycle but did not succeed in synchronizing growth. Were the hypothesis true, one would expect to observe a relationship between EOP and the frequency or number of dividing cells, which we did not (Fig. 2).

With the exception of 3 non-marine PHS examined at 3.5 to 4°C (Olsen et al. 1968, Kulpa & Olsen 1971, Sillankorva et al. 2004a), the 9A–34H system at -1°C has a shorter latent period (4 to 5 h) and larger burst size (55) than the other known cold-active PHS at similarly low temperatures. As reported for several cold-active PHS (Olsen et al. 1968, Kulpa & Olsen 1971, Whitman & Marshall 1971b, Sillankorva et al. 2004a), the latent period was shorter (2.5 to 3 h) at a warmer temperature (8°C), although Sillankorva et al. (2004a) found that this effect depended on the temperature of cell growth before infection. In contrast to these studies, however, an increased burst size was not observed at 8°C , instead being 1 order of magnitude smaller than that measured at -1°C (Fig. 5). Likewise, the phage production rate, defined as the burst size divided by the latent

period (following Zachary 1976), was also an order of magnitude higher at -1°C (and a factor of 5 higher if the rise times were included with the latent period). If considered in the context of genome replication, the release at -1°C of 55 viruses per infected cell, each with an 80 to 90 kb genome, is equivalent to the replication of a 4.4 to 5.0 Mb genome, comparable in size to that of 34H (5.4 Mb; Methé et al. 2005) but requiring only 0.1 to 0.2 of the generation time of 34H at -1°C to be produced. In contrast, at 8°C the infection cycle lasted 0.2 to 0.3 of the 34H generation time, but only ~ 0.5 Mb of phage genomes were made. These results may imply an extremely cold-active, phage-encoded enzyme, such as a DNA polymerase. They also emphasize that optimum conditions for phage production do not necessarily correspond to those for bacterial growth.

Besides the well-resolved one-step growth curves at -1 and 8°C , the consistency of normalized phage concentrations in unfrozen samples after 0 and 5 d, and again after 10 and 15 d, allows an estimation of the latent period and burst size at -12 to -10°C as 5 to 10 d and 5 viruses, respectively (Fig. 3A). At the slightly colder (-13 to -12°C) temperatures of the second experiment, the microscopically-observed stepwise increase in viral concentrations approximately 2 wk apart (Fig. 3B) is reminiscent of a synchronized infection (although no effort was made to synchronize infection in this experiment) with a latent period of <14 d. This apparent synchronization may again reflect phenotypic plasticity. The lengthening of the latent period and reduction of the burst size relative to -1°C are more in line with the observations of prior investigators and perhaps better understood as a response to temperature stress rather than temperature per se. We note also that measurements below -10°C may be biased by prior growth of the cells at -1°C (rather than -10°C) for the reasons already discussed, although the stepwise increase in viruses shown in Fig. 3B implies that at least some infections occurred once cells had acclimated to the experimental temperature.

The one-step growth curves at -1 and 8°C have in common long rise times comparable in length to the latent period. Asynchronous infection is unlikely to account for this observation, since the adsorption period was only 10 min, after which the phages were removed and cells washed. Since rise times are frequently not reported, the prevalence of this feature among marine PHS is not clear, although rise times comparable to latent periods are apparent in some PHS studied by Proctor et al. (1993) and Suttle & Chan (1993). In terms of TEM estimates of virus-induced mortality, long rise times may be important in estimating the fraction of the latent period when virus-like particles are visible; in particular, some inconsistency

appears in how or whether rise times and latent periods are distinguished (Proctor et al. 1993).

Finally, at both -1 and 8°C , the latent period of the 9A–34H system was considerably shorter than the corresponding doubling time of the bacterium under the relevant conditions (>48 h at -1°C and >17 h at 8°C ; Huston 2003 and present Fig. 2). Contrary to the common assumption of equality, the ratio of the latent period to the generation time (γ) was thus only 0.1 to 0.2 (at -1°C) and 0.2 to 0.3 (at 8°C), depending on whether or not the rise time was added to the latent period. If this feature ($\gamma < 1$) is common to other marine or cold-active phages and their hosts, models must account for it or risk a substantial underestimation of the importance of viral processes in the ocean. In particular, Binder (1999) showed that TEM estimates of virus-induced mortality are very sensitive to the assumed value of γ : for example, relative to the common assumption that $\gamma = 1$, if the true value of γ is 0.5 and the average frequency of visibly infected cells is 2%, then mortality due to viruses will be underestimated by approximately half (see Fig. 6 in Binder 1999).

CONCLUSIONS

Besides its unprecedented cold activity, Phage 9A exhibits different traits on its 2 identified hosts, which can reflect host history (for example, prior starvation) as well as immediate circumstances. Much of the work with cold-active phages, and marine PHS more generally, has not been attentive to these possibilities. In addition to making such work difficult to interpret, oversights of this nature may blind us to important nuances of phage ecology. For example, the different temperature and salinity ranges of plaque formation by 9A on *Colwellia psychrerythraea* 34H and *C. demingiae* ACAM 459^T (as well as the effect of prior starvation on the temperature range on 34H) hint at ‘niche partitioning’ of hosts in the ocean; i.e. at the vulnerability of different hosts to a given phage varying with environmental conditions, possibly relevant to genetic exchange and viral traffic between biomes (Sano et al. 2004). These and other documented characteristics of the 9A–34H system, including the change in EOP with culture age and the undetectable adsorption rate, imply phenotypic plasticity rather than mutational changes. The results of this study thus direct attention specifically to the impact of viruses on microbial mortality and diversity in the cold ocean but, more generally, to viral interactions with and influences on cellular phenotypes, such as surface-associated receptors, polymeric substances and released enzymes, affecting both the ability of the virus to infect its host(s) and the biogeochemical role of the host.

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