Author: K. Porter, B. E. Russ and M. L. Dyall-Smith
Title: Virus - host interactions in salt lakes.
Journal: Current Opinion in Microbiology **ISSN**: 1369-5274 1879-0364
Year: 2007
Volume: 10
Issue: 4
Pages: 418-424
Abstract: Natural hypersaline waters are widely distributed around the globe, as both continental surface waters and sea floor lakes, the latter being maintained by the large density difference between the hypersaline and overlying marine water. Owing to the extreme salt concentrations, close to or at saturation (approximately 35%, w/v), such waters might be expected to be devoid of life but, in fact, maintain dense populations of microbes. The majority of these microorganisms are halophilic prokaryotes belonging to the Domain Archaea, haloarchaea. Viruses infecting haloarchaea are a vital part of hypersaline ecosystems, in many circumstances outnumbering cells by 10-100-fold. However, few of these haloviruses have been isolated and even fewer have been characterised in molecular detail. In this review, we explore the methods used by haloviruses to replicate within their hosts and consider the implications of haloviral-haloarchaeal interactions for salt lake ecology.

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Virus – host interactions in salt lakes

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Abstract

Natural hypersaline waters are widely distributed around the globe, as both continental surface waters and sea floor lakes, the latter being maintained by the large density difference between the hypersaline and overlying marine water. Due to the extreme salt concentrations, close to or at saturation (approx. 35% w/v), such waters might be expected to be devoid of life but, in fact, maintain dense populations of microbes. The majority of these microorganisms are halophilic prokaryotes belonging to the Domain Archaea – "haloarchaea". Viruses infecting haloarchaea are a vital part of hypersaline ecosystems, in many circumstances outnumbering cells by 10 – 100 fold. However, few of these "haloviruses" have been isolated and even fewer have been characterised in molecular detail. In this review, we explore the methods used by haloviruses to replicate within their hosts and consider the implications of haloviral – haloarchaeal interactions for salt lake ecology.

Introduction

Few virus – host systems from the Domain Archaea have been studied (Figure 1), particularly when compared with those of Bacteria and Eucarya. Archaeal viruses were first discovered, by accident, in 1974 [46]. In 2007, there are only approx. 64 archaeal virus and virus-like particle isolates, infecting members of the two major archaeal Kingdoms: Crenarchaeota and Euryarchaeota. The viruses infecting extremely thermophilic crenarchaea are morphologically diverse, and all possess dsDNA genomes, represented by both linear and circular examples. They have been the subject of several thorough reviews in recent years [29 – 33]. Viruses of the methanogenic and extremely halophilic euryarchaea are morphologically less diverse, contain linear dsDNA genomes, and belong to at least three morphological groups: head-and-tail, spherical, and spindle-shaped (Figure 2). Those infecting the extreme halophiles, or "haloarchaea", are perhaps the most thoroughly studied [reviewed in 16]. These "haloviruses" are represented by at least 26 isolates, of which six (ΦCh1, HF1, HF2, His1, His2 and SH1), are currently under examination by groups in Australia, Austria and Finland. Another halovirus, BJ1, has recently been sequenced (GenBank accession number AM419438), but its characteristics have yet to be described. The currently studied halovirus representatives cover the morphological spectrum that has been observed in natural hypersaline environments [18 and 27]. This review focuses on what is currently known about the interactions of haloviruses with their extremely halophilic hosts, the haloarchaea.

Temperate haloviruses

Traditionally, virus – host relationships have been classified as temperate, lytic (or virulent) and chronic [16]. In this review, we will apply these terms, however it must be realised that individual viruses may not conform strictly to these ideal categories.

Almost predictably, those haloviruses that were discovered inadvertently in laboratory cultures have been found to be temperate, and are carried as prophages. Two well studied examples are ΦH and ΦCh1, which were both isolated after spontaneous lysis of their respective hosts, *Halobacterium salinarum* and *Natrialba magadii* [38 and 49]. They are head-and-tail viruses, resembling the bacteriophages of the family *Myoviridae*, with linear dsDNA genomes of approx. 59 kb (incomplete genome sequence; GenBank accession numbers 405323, 405325, AH004327, S63933, S63992, S63994, X00805, X80161, X80162, X80163, X80164, X52504) and 58.5 kb (GenBank accession number AF440695), respectively. The genome sequences of these viruses share reasonable similarity with several genes of bacteriophages, suggesting that there may be genetic exchange between the Archaea and Bacteria in hypersaline waters (discussed in [43]). ΦH and *Hbt. salinarum* thrive in near neutral pH [38], whilst ΦCh1 and *Nab. magadii* are alkaliphilic, favouring approx. pH 9.5 [49]. Given their distinctly different native environments and isolating hosts, it was somewhat surprising that ΦH and ΦCh1 were found to be closely related, with the nucleotide similarities between the largest sequenced part of ΦH and the central part of ΦCh1 varying between 50 and 97% [21]. This relationship suggests that there may be a wide dispersal of similar viruses throughout the world's hypersaline waters.

Despite their genetic similarities, the behaviours of ΦH and ΦCh1 within their hosts are quite different. Within lysogenised *Hbt. salinarum* cells, the prophage form of ΦH

DNA exists as covalently closed circular molecules, in which one of the terminal repeats present in the linear virus nucleic acid is missing [37]. In contrast, *Nab. magadii* lysogens contain ΦCh1 DNA integrated into the chromosome, with the integration potentially mediated by two putative site-specific recombinases, Int1 and Int2 [35]. Although the *Nab. magadii* chromosomal DNA is not methylated, a proportion of ΦCh1 genomes are modified at adenine residues [6], suggesting that ΦCh1 may use methylation to avoid degradation when infecting other, as yet unidentified host(s).

The behaviour of ΦH during *Hbt. salinarum* infection has been characterised in some detail, with a particular focus on the understanding of the pattern and regulation of ΦH transcription. The presence of the ΦH provirus, or an autonomously replicating plasmid derivative of the virus genome, pΦHL [36], provides immunity to super-infection. Immunity is partially mediated by the *rep*-encoded repressor, which appears to block RNA polymerase from initiating transcription of the major early lytic transcript, T4 [20 and 39]. *rep*-induced immunity is enhanced by transcription of the ΦH gene *per*, however *per* does not provide immunity on its own [41]. As outlined in Figure 3, the divergent promoter arrangement and regulatory network for *rep* are similar to those in bacterial coliphages, such as λ [34]. T4 and *rep* expression are mutually exclusive and T4 repression mediated by Rep may be overcome by transcription from the T4 promoter [42], although the mechanism is as yet unknown.

In addition, an antisense RNA, T_{ant}, complementary to lytic transcript T1, helps to mediate immunity of the host cell to ΦH super-infection [17]. T_{ant} forms a duplex with the first 151 nt of T1, which is subsequently processed by an unidentified ds –

specific RNase. The truncated RNA, lacking a Shine-Dalgarno sequence, is rendered inaccessible to ribosomes [40]. However, immunity induced by T_{ant} does not increase the immunity levels already generated by *rep* [41].

The characterisation of the temperate haloviruses ΦH and ΦCh1 has suggested that these viruses interact with their hosts in much the same manner as "classical" temperate bacteriophages interact with Bacteria. However, many of the basic details remain to be determined, such as the receptors that these viruses use to infect their host cells. The relative importance of this replication strategy in haloarchaea is also unknown, although genes of similar sequence to those of temperate haloviruses and other archaeal viruses have been detected in the genomes of several haloarchaea (*Haloarcula marismortui*, *Hbt. salinarum*, *Haloferax lucentense*, *Haloferax volcanii*, *Haloquadratum walsbyi* and *Natronomonas pharaonis* [2, 5, 9 and 24]), and integrated into chromosomes identified in the metagenome of a hypersaline lake [22], suggesting that temperate haloviruses are significant in natural waters.

Lytic haloviruses

In more recent years, direct plating has been used to isolate haloviruses. An early example utilised enrichment cultures consisting of a culture of *Hbt. salinarum* inoculated with hypersaline water from a Jamaican lake. A plaque assay from this yielded the lytic head-and-tail virus Ja.1 [45 and 48]. Unfortunately, studies of this and other lytic viruses, such as B10 [45], did not progress beyond one or two publications, and it is probable that these isolates are now lost. It should also be realised that these studies used a very limited host range consisting of various strains of *Hbt. salinarum*. Later studies have also shown that cells belonging to this genus are

usually not dominant in natural hypersaline waters [1, 7, 12 and 23]. To get a better idea of whether lytic haloviruses such as Ja.1 and B10 are examples of halovirus – haloarchaeon interactions that are numerically significant in nature, it will be necessary to integrate studies of virus isolates with metagenomic sequence data showing which are the dominant cells and viruses in these systems.

Other virus-host relationships: the carrier state and persistent infection

Few of the haloviruses obtained by direct plating from natural samples were reported to be strictly lytic. Although there are characterised examples of strictly temperate and lytic haloviruses, the majority of relationships between haloarchaea and their viruses are less rigidly described. They commonly form persistent infections of their host cell populations, and while the nature of these interactions remains to be fully understood, they probably play a significant role in natural waters. For example, persistent infections of *Halorubrum coriense* with HF2 continuously produce low titres of virus, cells are not immune to superinfection, cells plated in overlays (without any virus added) will form plaques, and when plated on solid media, the colonies show a doughnut like morphology because the colony centres tend to lyse (S.D. Nuttall and M.L. Dyall-Smith, unpublished data).

Unlike Bacteria with their rigid peptidoglycan cell walls, many Archaea have only a thin, glycoprotein surface layer (S-layer) protecting the cell membrane, which means that virus release does not necessarily require breakdown of the cell wall and subsequent cell lysis. Viruses of the Crenarchaeota, such as SSV1 and ATV, have been shown to extrude from the cell wall without cell lysis [19, 32]. During halovirus infection, host cells may continually release virus without lysing. Cells do not enter a

lysogenic state, and appear to continue to divide, despite infection. This situation is reminiscent of chronic bacteriophage infections, in which the phage escapes from its host by extruding through the envelope while cells continue to grow [10], or pseudolysogeny, in which the phage nucleic acid remains with the cell until cell death occurs [16].

Persistent infection may benefit haloviruses. In cultures of *Hbt. salinarum* that are persistently infected by the head-tail halovirus S5100, increasing the salinity of the medium decreases the burst size and slows virus maturation. This state is readily reversible as salinity is reduced [13]. Moreover, increased salinity inhibits the adsorption of head-and-tail halovirus Hs1 to *Hbt. salinarum* [47], suggesting that it may be favourable to the virus to be more benign at high salinities but virulent at lower salinities. It has been speculated that when lakes are at high salinity, for example, during a drought, haloarchaea can reach high cell densities. Internal virus numbers and the speed of virus maturation are reduced, allowing cells to continue to divide. In contrast, when the salinity is reduced, for example, after rainfall, viruses are stimulated to reproduce and exit the cells rapidly. Since the cells are likely to be stressed or unable to withstand the change to low osmotic pressure, they are more likely to lyse or die. By increasing their virulence, haloviruses avert death with the host [13]. A direct test of this hypothesis would be to monitor virus populations in a salt lake as salinity rapidly reduces during and after rainfall. Such studies would give a better understanding of the ecological strategies that haloviruses have evolved to allow them to thrive under dramatically changing, and often very hostile, environments.

Of the six haloviruses isolates that are currently under examination, all except ΦCh1 persistently infect their hosts. These five viruses were all obtained by direct plating from Australian hypersaline waters, and include the closely related haloviruses HF1 and HF2, which resemble the head-and-tail bacteriophages of the family *Myoviridae* [26]. HF1 and HF2 have linear, dsDNA genomes of similar size (75.9 kb and 77.7 kb, respectively: Genbank accessions AY190604 and AF222060), and an overall sequence similarity of 94.4%. For the first 48 kb they share identical sequence, except for a single silent nucleotide substitution, while the remaining right-end third of these genomes show extensive differences [44]. This peculiar pattern of similarity can be most simply explained as the result of a recent recombination event occurring between one of these viruses and a third, unidentified relative. These differences are also consistent with the unrelated host ranges of these viruses, as the genes encoding structural proteins (which would include the virus attachment proteins) are located in the regions that differ between them (B. Russ and M.L. Dyall-Smith, unpublished data).

Another dominant morphotype is represented by the distantly related spindle-shaped haloviruses His1 and His2 of the *Salterprovirus* group, both infecting *Haloarcula hispanica* [4 and 5]. Morphologically, these viruses resemble archaeal viruses of the family *Fuselloviridae*, but genetically these groups are unrelated. In addition, the halovirus genomes are linear dsDNA of 14.5 kb (accession AF191796) and 16.1 kb (accession AF191797), respectively, while fusellovirus genomes are circular dsDNA. The %GC of the His1 and His2 genomes is approx. 20% lower than that of their host, *Har. hispanica*, suggesting that they may use an alternative host that more closely matches in GC content and codon usage [5]. The third, dominant virus morphology

seen in salt lake waters are spherical VLPs. This group is represented by halovirus SH1, which also infects *Har. hispanica,* as well as an uncharacterised *Halorubrum* isolate [28]. Despite morphological similarities to some known virus groups (e.g. tectiviruses), the 30.9 kb genome of this virus (GenBank accession number AY950802) appears unrelated to all other characterised virus groups [3].

The lytic virus – host relationship in these cases are not classical. Single-step growth curves for His1, His2 and SH1 all show that virus particles are continually liberated from host cells without cell lysis. Cells continues to divide for at least 15 hr but eventually die and lyse [5 and 28] (Figure 4). None of these haloviruses appear to encode a lysis gene that is related to characterised holin or lysin genes [3, 5, 43 and 44]. It may be that the prolonged infection, and continual exit of virus particles damages the cells and restricts their ability to repair, eventually leading to cell death and breakdown of the cell wall. All five haloviruses form clear plaques on host cells, but also readily enter unstable carrier states in liquid cultures, with host cells continually liberating virus [4; C.R. Bath, S.D. Nuttall, K. Porter and M.L. Dyall-Smith, unpublished data].

Laboratory isolates of haloviruses are unlikely to exactly represent the dominant types found in natural environments. The inital isolation is highly selective and continual passaging of virus isolates is likely to select for mutants with improved growth characteristics. For example, repeated passaging of the head-and-tail haloviruses S41 and S50.2 dramatically increased their virulence; apparently due to a more rapid adsorption [14]. There are many possible explanations for this behaviour. The characteristics may be a reflection of the necessity for the viruses to adapt to

laboratory conditions before efficient adsorption and replication can occur. Alternatively, in natural environments, slowly adsorbing viruses may be favoured. This selection may be due to viruses benefiting if they do not instantly release their DNA every time that they encounter a potential host. Instead, the behaviour may be due to viruses adsorbing well under a range of salinity conditions, instead of optimally under ideal conditions.

Although difficult and potentially impractical, to get a true understanding of the interactions between haloarchaea and the haloviruses persistently infecting them, it may be more prudent to mimic environmental conditions with low nutrient media or investigate strains of both viruses and hosts that have not undergone extensive passaging. One approach could be to adapt the SYBR Green I staining method used for studying marine viruses and cells [16 and 25] to study the binding of virus particles to their host cells in natural hypersaline waters (Figure 5).

The continuing story

Elucidating the interactions between haloviruses and their hosts is in its infancy, and it is perhaps worth pointing out some of the major areas of ignorance, particularly concerning the initial stages of virus infection. Only a few studies have determined rates of halovirus particles binding to cells, and no cell surface receptors have yet been identified. The rather bare external surface of most haloarchaea would suggest that S-layer proteins and flagella are likely candidates. No haloviral attachment proteins have been identified, but it might be expected that the tail fibres and base plate proteins of head-tail haloviruses are likely to perform this role, by analogy to their bacteriophage counterparts. Once bound to cells, the mechanisms of DNA entry

have not been examined, and will be particularly interesting in the cases of spindle and spherical haloviruses. No crystal structure has been determined for any halovirus. Clearly, there is considerable scope for improving our understanding of halovirus-host interactions and it cannot be assumed they will be similar to well-studied bacteriophage examples.

It is also important to know whether the halovirus – haloarchaeon combinations at our disposal actually represent significant players in natural ecosystems. The dominant haloarchaeal members of hypersaline waters are now available [8, 11 and 12] but it is not clear whether these dominant species are infected by the dominant haloviruses, or if it is because relatively few viruses infect them that they have flourished. In this laboratory, we have found that direct plating of water samples onto lawns of haloarchaea belonging to the more environmentally dominant species gives much higher virus isolation rates compared to using lawns of commonly used laboratory strains, such as *Hfx. volcanii* DS2. While preliminary, these results suggest that the dominant species probably remain susceptible to the heavy lytic/persistent viral load present in hypersaline waters [K Porter and ML Dyall-Smith, unpublished data]. The upcoming metagenomic studies of salt lakes will provide an unprecedented view of the genetic diversity present in these systems, and will provide many questions, but since they examine only part of the biological information (DNA sequences), they should be seen as complementing studies of the whole organisms. In this context, concerted efforts to study the roles and impacts of viruses that infect the dominant microbial groups will provide significant insights into the microbial ecology of these extreme environments, including virus diversity, the selection pressures driving cell

evolution, lateral gene transfer, nutrient cycling and the regulation of host cell densities.

Acknowledgements

We gratefully acknowledge the support of the Department of Microbiology and Immunology. BER was supported by a Melbourne Research Scholarship. K.P. was supported by an Australian Postgraduate Award. M.D-S. has had support from the Australian Research Council.

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Figures

Figure 1. The three Domains of Life. Rooted phylogenetic tree showing the three Domains of life, Archaea, Bacteria and Eucarya. The numbers of virus families and type species recognised by the International Committee on the Taxonomy of Viruses, as of 2006, are given.

Figure 2. Examples of the three haloviral morphological groups. These uncharacterised halovirus isolates represent the three morphological groups: head-and-tail (**A**), spherical (**B**), and spindle-shaped (**C**). Scale bar is 200 nm in **A** and **C**, and 100 nm in **B**.

Figure 3. Schematic map of the transcription of the 11.5 kb "L-region" of temperate halovirus ΦH. Transcripts T1, T2, T3, T4, T4', T6 (*rep*) T7, T8, T9 (*per*), T10, TLX1, TLX3, TISH1.8 and T_{ant} are depicted. Rep acts to repress T4 transcription, by binding to the promoter of T4. Expression of *per* enhances this repression, whilst transcription from T4 prevents the repression. *per* and T4 act by an unknown mechanism. T_{ant} acts to repress T1 translation by forming a duplex with T1. The duplex is subsequently processed by a RNase, thus preventing ribosome binding and translation. Map not to scale.

Figure 4. Single-step growth curve of SH1 infected *Har. hispanica*. Cells were infected at high multiplicity (30 pfu/cell), washed of unadsorbed virus, and the cell density and production of virus followed over time. Between 5-6 hr post-infection, during the major rise period, there was a dramatic increase in virus titre but the culture did not lyse. The cells continue to divide until about 15 hr post-infection before

extensive lysis is observed. Cell growth was followed by measuring absorbance at 550 nm (Red line). Infectious virus, including both free and intracellular virus, was followed by plaque assay (virus infective centres) (Blue line). Dashed black line, the mid-point of the rise period, when virus release occurs.

Figure 5. SYBR Green I stained preparation of cells and virus like particles (arrows) from a saltern crystallizer pond (Cheetham Salt Works, Victoria, Australia). Square cells, almost certainly members of the genus *Haloquadratum*, dominate the prokaryotic population. Photographed using a fluorescence microscope with an oil-immersion, 100 × objective lens. Scale bar is 5 μm.