The influence of external factors on bacteriophages—review

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Abstract The ability of bacteriophages to survive under unfavorable conditions is highly diversified. We summarize the influence of different external physical and chemical factors, such as temperature, acidity, and ions, on phage persistence. The relationships between a phage's morphology and its survival abilities suggested by some authors are also discussed. A better understanding of the complex problem of phage sensitivity to external factors may be useful not only for those interested in pharmaceutical and agricultural applications of bacteriophages, but also for others working with phages.

Introduction

Bacteriophages are the most numerous form of life on Earth; ten times more numerous than bacteria (Hendrix 2002; Hanlon 2007). They can be found in all environments where bacteria grow: in the Sahara, hot springs, the North Sea, and polar inland waters (Prigent et al. 2005; Lin et al. 2010; Breitbart et al. 2004; Wichels et al. 1998; Säwström et al. 2008). Phages are detected in ground and surface water, soil, food (e.g., sauerkraut, wine), sewage, and sludge (Lucena et al. 2006; Yoon et al. 2002; Davis et al. 1985; Kumari et al. 2010; Tartera and Jofre 1987). They have also been isolated from humans and animals, for example from feces, urine, saliva, spit, rumen, and serum (Gantzer et al. 2002; Caroli et al. 1980; Bachrach et al. 2003; Nigutová et al. 2008; Keller and Traub 1974). Phages are able to penetrate different organs and tissues, including the central nervous system, and are a part of intestinal flora together with their bacterial hosts (Frenkel and Solomon 2002; Kameyama et al. 2001). They are responsible for 10–80% of total bacterial mortality in aquatic ecosystems and are an important factor limiting bacterial populations (Weinbauer 2004).

Bacteriophages are classified into families with regard to their morphology and size as shown in Table 1. About 96% of them are tailed, but there are filamentous and pleomorphic ones as well (Ackermann 2007; Hendrix 2002). Generally, the phage virion consists of two basic components: nucleic acid (double- or single-stranded RNA or DNA) and a protein envelope. Some have lipids as components of the envelope or of a particular lipid wall (Ackermann 2003).

Various external physical and chemical factors, such as temperature, acidity, salinity, and ions, determine the occurrence, viability, and storage of bacteriophages and can inactivate a phage through damage of its structural elements (head, tail, envelope), lipid loss, and/or DNA structural changes (Ackermann et al. 2004).

Phage morphology and their persistence in extreme environments

Ackermann et al. (2004) showed that tailed phages were the most stable in adverse conditions, but it was not any

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Table 1 Classification of bacteriophages and characteristics of their sensitivity to some external factors such as temperature, acidity, and salinity (phages described in the text are marked in bold)

Family[a]	Members[a]	Nucleic acid and structure[a]	Characteristics of phage sensitivity to some external factors
Myoviridae	**T2** **T4** **ΦgspC** **PFpW-3** **CP-51** **C16** MB08	Linear dsDNA Non-enveloped, contractile tail, consisting of a Sheath and a central tube	Phages from this family may be extremely resistant to a dry environment (desert sands) and may survive large temperature fluctuations (Prigent et al. 2005) Some of them (e.g., T4, C16) proved to be very resistant to long-term (for years) storage (Ackermann et al. 2004) Freeze-drying may be harmful for this family (Clark 1962) T2—stable in pH range 5–9, with maximum at pH 5–6 (Sharp et al. 1946) T4—at 37°C, stable at pH 6–7.4, unstable at pH<5 and 9.2 (Kłak et al. 2010); stable when stored 4 weeks in urine at 6–20°C (Jończyk, unpublished data); its survival after freezing at −196°C was over 65% (Tsutsaeva et al. 1981) ΦgspC—can survive in a very salty environment (Wilson et al. 2004) CP-51—sensitive to low temp. (<0°C); optimum stability at 15°C; for its storage at 0°C, optimal pH is 5.6 (Thorne and Holt 1974)
Siphoviridae	**λ** **T1** **T5** **PFpW-8** **TSP4** P001 P008 MB07	Linear dsDNA Non-enveloped, long non-contractile tail	In the opinion of some authors, the members of this family are generally the most resistant to adverse conditions (Lasobras et al. 1997) Phages from this family may be extremely resistant to a dry environment (desert sands) and may survive large temperature fluctuations (Prigent et al. 2005) Some of them (e.g., T5) proved to be very resistant to long-term (for years) storage (Ackermann et al. 2004) Very stable when stored (for years) in freeze-dried form (Ackermann et al. 2004) λ—good stability at 4°C for over 6 months, stable in a wide range of pH (3–11) for 24 h at 19°C, more stable in distilled water than tap water (Jepson and March 2004) T1—resistant to drying (Faquet et al. 2005); at 37°C, sensitive to pH≤3 (Międzybrodzki et al., unpublished data) TSP4—may survive at high temperatures, optimum is 65°C (Lin et al. 2010)
Podoviridae	**T3** **T7** **PFpW-6** **28B** Kpn5 N22	Linear dsDNA Non-enveloped, short non-contractile tail	Phages from this family may be extremely resistant to a dry environment (desert sands) and may survive large temperature fluctuations (Prigent et al. 2005) T3—at 37°C, stable at pH 5–9.2 (Międzybrodzki et al., unpublished data); its survival after freezing at −196°C was 98% (Tsutsaeva et al. 1981) T7—at 37°C, prefers alkaline conditions; more sensitive to lower pH than T3 (Międzybrodzki et al., unpublished data); at 0.5–2.0°C, optimal long-storage stability at pH 6–8 (Kerby et al. 1949) 28B—very stable (for 6 months) in urine even at pH 9 (Höglund et al. 2002)
Microviridae	**ΦX174**	Circular sDNA Non-enveloped, isometric	ΦX174—its survival after freezing at −196°C was over 80% (Tsutsaeva et al. 1981)
Corticoviridae	**PM2**	Superhelical, circular dsDNA Non-enveloped, isometric, lipid layer in capsid	PM2—stable at pH 6–8, virion, stability strongly dependent on NaCl (10 mmol/L minimum) and $CaCl^2$ (5 mmol/L minimum) (Faquet et al. 2005); completely loses activity after 1 h at pH 5.0 at 37°C (Międzybrodzki et al., unpublished data); freezing and thawing completely removes the protein shell (Kivelä 2004)
Tectiviridae	**PRD1** **AP50**	Linear dsDNA Non-enveloped, isometric, inner lipoprotein vesicle	PRD1—usually stable at pH 5–8 (Faquet et al. 2005); very stable when stored (for years) in broth at 4°C, may also survive at −80°C (Ackermann et al. 2004) AP50—for long storage, should be freeze-dried, because it may lose activity at −80°C or in broth at 4°C (Ackermann et al. 2004)
Leviviridae	**MS2** **PP7** **Qβ** f2	Linear ssRNA Non-enveloped, isometric	MS2 and Qβ—the lowest inactivation was observed at pH 6–8 and temperature of 5–35°C (Feng et al. 2003) Qβ—better survival in an alkaline than acidic environment (Feng et al. 2003) MS2—unstable when suspended in ultrapure water (Governal and Gerba 1997); for long term, should be stored at -80°C rather than at 4°C (Olson et al. 2004); better survival in an acidic than an alkaline environment (Feng et al. 2003) PP7—highly thermostable (Caldeira and Peabody 2007)
Cystoviridae	Phi6	Linear segmented RNA Lipoprotein envelope, spherical	Phi6—stable at pH 6 (ICTVdB Management 2006)

Table 1 (continued)

Family[a]	Members[a]	Nucleic acid and structure[a]	Characteristics of phage sensitivity to some external factors
Inoviridae	**M13** fd Pf1 Vf33	Circular ssDNA Non-enveloped, filamentous	M13—its optimal pH is 6 and 9 and temperature 37°C (Tey et al. 2009); it can survive at least for 1 h at pH 2 (Międzybrodzki et al., unpublished data)
Lipothrixviridae	**TTV1** SIFV	Linear dsDNA Enveloped, rod-shaped	Members of this family can be found in acidic hot-spring environments (Prangishvili et al. 2001)
			TTV1—it can survive even temperatures >85°C and pH<3 (Goulet et al. 2010)
Rudiviridae	SIRV1 **AFV-1**	Linear dsDNA Non-enveloped, rod-shaped	Members of this family can be found in acidic hot-spring environments (Prangishvili et al. 2001)
Plasmaviridae	L2	Circular superhelical dsDNA Enveloped, pleomorphic	L2—extremely sensitive to heat, relatively cold stable (Faquet et al. 2005)
Fuselloviridae	**SSV1** **SSV2** **SSV3** His1	Circular superhelical dsDNA Non-enveloped, lemon shaped, short spikes at one end	Members of this family can be found in acidic hot-spring environments (Prangishvili et al. 2001)
			SSV1—stable at high temperature (up to 97°C), insensitive to pH 2 but pH<5 reduces its viability, and virions are sensitive to pH>11 (Faquet et al. 2005)
			His1—sensitive to exposure to low salt concentrations; to maintain stability, it should be stored in a high salt solution (18%); stable for a long time at 37°C (Faquet et al. 2005)

[a] Based on Ackermann 2003; Ackermann et al. 2004; Ackermann 2007; Ackermann and Abedon 2000; Ackermann and DuBow 1987; Bettstetter et al. 2003; Buiser et al. 2009; Faquet et al. 2005; Governal and Gerba 1997; Goulet et al. 2010; ICTVdB Management 2006; McAuliffe et al. 2007; Kivelä 2004; Kumari et al. 2010; Kim et al. 2010; Lin et al. 2010; Prangishvili et al. 2001; Pringsulaka et al. 2010; Tey et al. 2009; Wachman and Brown 2010; Wilson et al. 2004

substantial difference in sensitivity between phages with contractile, non-contractile, or short tails, although phages with a large capsid (100 nm in diameter) survive better than phages with a head 60 nm in diameter. The membership in the same family and even close structural similarity may not determine a phage's features and resistance to external factors. The authors observed that the cubic phages PRD1 and AP50, belonging to the *Tectiviridae*, showed different sensitivities to storage conditions. PRD1 survived better when it was stored at −80°C, but AP50 lost its activity after 6 months at the same temperature, surviving better after freeze-drying. PRD1 was detected even after 22 years when it was stored in broth at 4°C, whereas AP50 was not detected after 1 year under the same conditions. Electron microscopic observations showed different changes in phage morphology. For example, in the case of AP50, empty virions were observed, when for PRD1 stored in broth, no phage capsids, but only traces of lipid vesicles, were detected. Lin et al. (2010) isolated from hot springs in China *Thermus* TSP4 phage characterized by high thermostability (the optimum temperature for this phage was 65°C). A relationship between its thermal stability and the presence of an extremely long and flexible tail (785 nm in length and 10 nm in width) was suggested.

Bacteriophages residing in similar environments may have a large variety of morphological forms. For example, phages occurring in hot springs were classified by Ackermann (2007) into five types of archea viruses: SH1, STIV, *Ampullaviridae*, *Bicaudaviridae*, and *Globuloviridae*. SH1 have the same structure as *Tectiviruses*. They are polyhedral and contain a lipid nucleocapsid (a combination of phage nucleic acids and capsid proteins). Phages of this group were found only in a hypersaline lake in Australia. STIV are polyhedral phages infecting hyperthermophilic bacteria and were found in a hot spring in Yellowstone National Park. *Ampullaviridae* have a unique structure in the viral world because they consist of a bottle-shaped mantle, a cone-shaped inner body, a helical nucleocapsid, and polar fibers at the large end. *Bicaudaviridae*, oval or arrow-shaped particles which contain a helical nucleocapsid and grow tail-like appendages at both ends, were found in Italian volcanic springs, as well as in acidic hot springs in China. The virions of *Globuloviridae* consist of helical nucleocapsid lipid containing a spherical envelope, and they are found in Italian hot springs. Members of three families which are dsDNA viruses of the archeon *Sulfolobus* are predisposed to inhibit solfataric fields: *Lipothrixviridae* (e.g., phages TTV1 and SIFV), *Rudiviridae* (e.g., phages SIRV1 and AVF-1), and *Fuselloviridae* (e.g., phages SSSV1, SSSV2, and SSSV3) (Prangishvili et al. 2001; Bettstetter et al. 2003). The existence of bacteriophages in the Sahara was confirmed by Prigent et al. (2005). Twelve morphological phage-like types were detected in sand samples. Six types

had icosahedral capsids (without envelope), a large genome, a tail with a contractile sheath, and a base plate with terminal fibers; therefore, they were classified as *Myoviridae*. Two groups with long hexagonal capsids were classified to the *Podoviridae*, and the other four types with long non-contractile tails that were classified to the *Siphoviridae* had the simplest structure and possessed tails, and their genomes were smaller (on average, approximately 100 kb). It was shown that under harsh conditions, such as strong ultraviolet light, desiccation, and large temperature fluctuations, phages belonging to the *Myoviridae* can protect themselves from the extremely dry environment through intercellular location in pseudolysogens or confinement in the biofilm created by bacterial hosts.

Lasobras et al. (1997) suggested there may be some relationship between phage structure and their survivability under adverse environmental conditions. Based on their analysis of phages from sewage and persistently polluted water, which mostly belonged to the *Siphoviridae* family, they suggested that phages with *Siphoviridae* morphology are the most resistant to adverse conditions. However, it seems that there is no fundamental confirmation of this assumption, because it lacks systemic comparative study.

External factors influencing bacteriophages

Temperature

Temperature is a crucial factor for bacteriophage survivability (Olson et al. 2004; Nasser and Oman 1999; Yates et al. 1985; Hurst et al. 1980). It plays a fundamental role in attachment, penetration, multiplication, and the length of the latent period (in the case of lysogenic phages). At lower than optimal temperatures, fewer phage genetic material penetrate into bacterial host cells; therefore, fewer of them can be involved in the multiplication phase. Higher temperatures can prolong the length of the latent stage (Tey et al. 2009). Moreover, temperature determines the occurrence, viability, and storage of bacteriophages.

Atypical environments in which bacteriophages can survive are hot springs (achieving temperatures of 40–90°C). Bacteriophages isolated from such springs in California were tested at low and high temperatures (Breitbart et al. 2004). It was observed that more than 75% of the phage particles remained intact even when incubated on ice (around 0°C). They were more sensitive when boiled at 105°C, as only 18–30% of the phage particles remained intact. The inactivation of phages occurring in dewatered sludge and raw sewage thermal treatment was studied by Mocé-Llivina et al. (2003). They tested thermal resistance of somatic coliphages, phages infecting *Bacteroides fragilis*, and F-specific RNA phages. In both cases, they observed that phages were more resistant to thermal treatment than bacteria. Moreover, F-specific phages were less thermally resistant than other tested phages, incubated both in sludge and sewage. The level of the reduction in titer of somatic coliphages naturally occurring in sludge after thermal treatment at 60°C was 1.0 log after 60 min, and at 80°C it was 2.5 log. Caldeira and Peabody (2007) investigated the role of disulfide cross-links in the protection of phages against thermal denaturation. They tested the highly thermostable *Pseudomonas* RNA phage PP7 and observed that its particles, which only start to denature after heating for 2 min at 90°C, lost their stability after addition of 1,4-dithiothreitol, a reducing agent which destroys disulfide bonds between coat protein dimers by cross-linking a phage icosahedral capsid.

High thermal resistance is characteristic both for environmental and lactococcal bacteriophages. Buzrul et al. (2007) exposed ten bacteriophages of *Lactococcus* spp. suspended in broth at 72°C (15 min) and 90°C (5 min). At lower temperature, only two phages were inactivated, but exposure to 90°C inactivated one half of the investigated phages. Atamer et al. (2008) showed that ≈40% of the *Lactococcus lactis* phages isolated from different German dairies survived heating at 80°C for 5 min when suspended in milk. But almost all phages were completely inactivated when the temperature was raised to 95°C. Two phages, P680 and P1532, proved to be exceptionally resistant to high temperature; P680 phage survived after 5 min of thermal inactivation at 95°C. P1532 phage was detectable even after incubation at 97°C for the same period of time. Milk protected lactococcal phages from thermal inactivation better than other media, such as broth. Therefore, it was suggested to use milk for suspending phages in experiments investigating the influence of different inactivating factors on phage survival. The stability of phages preferring low temperatures that infect *Pseudomonas fragi*, isolated from refrigerated food, was determined at 60°C (Whitman and Marshall 1971). After a 30-min inactivation of the *Pseudomonas* phages ps_1 and wy, the average reductions in phage survival were over 99% and 39%, respectively. There were 15% and 72% decreases in plaque numbers after maintaining the phages in soft agar at 45°C for 2 and 10 min, respectively. It should be recommended that for proper determination of the phage titer (without loss of phage activity), this step should be done as quickly as possible.

The temperature of bacteriophage storage is the most important factor which determines phage activity. Thorne and Holt (1974) observed phages such as the *Bacillus cereus* CP-51 which were sensitive to low temperatures and survived better at room temperature, but the long-term storage of phages at ambient temperature is not generally recommended (Mullan 2001). According to them, an

optimum temperature for CP-51 phage stability was 15°C; they showed that fresh propagating lysate retained 14% of the initial plaque-forming unit (PFU) at 0°C after 7 h, and only 4% after 24 h. Interestingly, the nutrient broth–yeast extract medium (NBY) stabilized the phage under cold conditions. After addition of NBY, tenfold diluted phage retained 36% activity after 7 h and 19% after 24 h at 0°C. When samples were stored at 15°C, no loss in activity was observed when they were diluted tenfold in NBY. Lysates retained full activity for 2 weeks. According to electron microscopic examination, CP-51 has a hexagonal head and contractile tail. When phage lysates were stored at 0°C, many of its tails were contracted. When they were stored at 15°C, extended tails rather than contracted were observed, but contracted tails increased during several weeks of storage. As shown by Ackermann et al. (2004), tailed phages were the most resistant to storage and showed the longest survivability; some of them retained viability even after 10–12 years at 4°C. Extremely resistant were T4, T5, and T7. The T4-like *Shigella* phage C16 retained a titer of 10^3 after 32 years of storage under the same conditions. Some phages, such as lipid-containing ones, were not stable after storage at 4°C temperature but could be stored at −80°C or in liquid nitrogen. Jepson and March (2004) tested the influence of storage time at different temperatures on λ phage. They observed that there were no viable phages in liquid (SM buffer) phage stocks at 42°C after 84 days, whereas at 37°C, no phages were detected after 120 days. Good phage stability was observed when they were stored at 4°C for over 6 months. In Mullan's study, *Lactococcus* sp. phage lysates stored at 2–5°C showed insignificant reductions in phage titer by 5–10% after 6 months (Mullan 2001). However, this method was recommended for storage of no longer than 2 months. Although the phages were resistant to freezing and thawing, repeating these procedures too often in short intervals had an adverse influence on the phages' stability. Similarly, Olson et al. (2004) recommend 4°C as the optimum temperature for short (no longer than 40 days) phage storage in wastewater. To protect phages from inactivation during longer periods, they should be maintained at −80°C. They observed that 20% of MS2 phages were inactivated after 8 days of storage at 4°C in comparison to 57% at -80°C. Interestingly, after 290 days, fewer phages were lost at −80°C than at 4°C (75% and 93%, respectively). Bacteriophage storage at −20°C is not recommended because the crystal structure of ice may cause their destruction, as was previously demonstrated by Warren and Hatch (1969). An addition of 5–10% glycerol to the phage suspension may guarantee safe viability and infectivity for 30 days at −20°C or −70°C (Olson et al. 2004).

Previous data indicated that lyophilization is not an efficient method to preserve phage stability because it was the most damaging (Clark 1962). However, the efficiency of lyophilization may depend on its course, and it should be regularly controlled. Puapermpoonsiri et al. (2009) stated that the secondary drying cycle following lyophilization is most important for maintaining phage stability. Freezing in liquid nitrogen is recommended when bacteriophage samples have a low titer (Mullan 2001). According to the observations of Ackermann et al. (2004), different phages lyophilized with the addition of 50% glycerol could survive many years when a vacuum was retained in the ampoules. For example, there was no loss of freeze-dried phages of the *Siphoviridae* family after 21 years. None of the described methods guarantee maintaining the stability for all phages. Nevertheless, recently, Golec et al. (2011) showed that there may be a universal and effective method for storage of tailed phages. They infected cells with phages and froze them. After a few months, they did not observe a significant loss in titers when phages were recovered from melted cells in comparison to freshly infected cells.

Acidity of the environment

Another important factor influencing phage stability is the acidity of the environment. Davis et al. (1985) described the occurrence of phages specific to the lactic acid bacterium *Leuconostoc oenos* in wine. The limiting factor for their activity was pH below 3.5 and SO_2 at a total concentration of 50 mg/L. Phages may persist in an acidic environment, such as sauerkraut, which was described by Lu et al. (2003). They found 24 phage isolates in fermentation tanks with sauerkraut (pH<3.5) after 60 and even 100 days.

Kerby et al. (1949) investigated the stability of T7 phage in buffers (citrate, citrate–phosphate, phosphate, phosphate–borate, and borate buffers) of different pH (3–11) for incubation times of 1–2 weeks at 0.5–2°C. Their observations showed that the optimum for physical stability of this phage was pH from 6 to 8 for long storage. The T7 phage was most stable in phosphate buffer at pH 7, and it lost only 20% of its activity after 2 weeks. It was unstable at pH<4 (after 96 h in citrate or citrate–phosphate buffer, it lost almost all its infectivity), and at pH 3, it completely lost its activity already after 1 h. On the alkaline side, even at pH 9, it retained at least 30% of its infectivity after 2 weeks. In borate buffer of pH>10, almost complete loss of T7 activity was observed after 24 h. Sharp et al. (1946) investigated pH stability of the T2 phage in the pH range of 2 to 11 for 1 h, 1 day, and 1–4 weeks. Generally, the phage was stable in a broad pH range of 5–9, with its maximum at about 5–6. Immediate phage coagulation at pH 2 was observed. At pH 3 and 4, the phages precipitated, but at the higher value, it was reversible, and the phages could be redispersed by shaking. The authors suggested that irreversible coagulation and precipitation might be the factors

limiting phage activity. An insignificant small loss of infectivity near pH 7 was observed. After a 1-h incubation at pH 9, the T2 phage retained full activity. After 24 h at pH 3, about 15% of the virions were infective. After 2 weeks in pH 5–9, 50% of the phages were still active. The λ phage activity was tested by Jepson and March (2004) in the range of pH 2–14. After 24 h of incubation at room temperature (19°C), the phage was highly stable in a wide range of pH; the authors did not observe any significant decrease in its titer at pH 3–11, but at pH 11.8–14 or pH 2, no viable phage particles were observed. Our own studies on the stability of T4 bacteriophage (phages were incubated in buffers of different pH ranging from 1.1 to 9.2 for 1 h at 37°C) showed that the optimum pH was 6.0–7.4 (Kłak et al. 2010). The titer decreased by half at pH 9.2, and no active phages were detected at pH 4.0. Phages T3 and T7 could be detected in the same pH range, although the latter one was more sensitive to lower pH, and it preferred alkaline conditions (unpublished data). The PM2 phage was also sensitive to low pH (it completely lost activity at pH 5.0). T1 phage particles were lost at pH 3.0, and M13 phage survived even at pH 2.

Thorne and Holt (1974) observed that a change in pH of the suspending medium for *B. cereus* CP-51 phage influenced its stability at 0°C. The optimal pH was 5.6; after 30 min of incubation, the phage titer decreased by only 11%, whereas at pH 7.0, it retained only 1% of the initial PFU (10^7). These observations indicate that the change in environmental pH may protect phage activity at a low temperature.

Feng et al. (2003) investigated the survivability of coliphages (MS2 and Qβ) in water and wastewater with regard to the effects of different temperatures and pH on the phages. Both phages presented the lowest inactivation rate in the pH range of 6–8 and temperature range of 5–35°C. MS2 survived better in an acidic than in an alkaline environment, but the opposite was true for Qβ. Wick et al. (2006) showed that MS2 could survive for 66 h in a 0.1-mol/L HNO_3 without any decrease in the number of phage particles.

Höglund et al. (2002) studied the survival of bacterial viruses in source-separated urine (pH 9). They observed only an insignificant inactivation rate of 28B phage at 5°C during 6 months; however, at 20°C, it was 20 times greater. In PBS (pH 7.4), which was used as a control, the inactivation was only approximately twice as much at 20°C than at 5°C. The decrease in the phage titer at 20°C might have resulted from the conversion of urea to ammonia, which is a factor inactivating viruses. These results were confirmed by Vinnerås et al. (2008), who showed that the stability of phage 28B, MS2, and phiX174 increased with urine dilution and lowering incubation temperature. Our own unpublished data on T4 phage stability in human urine showed no substantial change in phage titer even after 4 weeks of incubation in urine both at 6°C and at room temperature, presenting good phage stability. Chandran et al. (2009) observed that the survival of MS2 is better in diluted or fresh urine in comparison with stored urine. Moreover, the influence of temperature and pH on the phage inactivation was higher at 30°C than 15°C.

It was shown that hydrogen ion concentration influences phage aggregation. For example, MS2 phages showed significant ability to aggregate when the pH was less than or equal to the phage isoelectric point (pI=3.9) (Langlet et al. 2007). Their aggregates could be up to 6 μm in diameter. This may cause a decline in phage count and an easier elimination of aggregates through their adsorption on membranes than single virions.

Salinity and ions

Osmotic shock has been shown to inactivate bacteriophages. Whitman and Marshall (1971) observed that psychrophilic *Pseudomonas* phages (wy and ps₁) had reduced persistence in highly concentrated solutions of NaCl or sucrose. The phage ps₁ diluted in 4 mol/L NaCl showed a 99% decrease in viability, while the viability of the phage wy was reduced by only 26%. However, a 2-mol/L sucrose solution caused a decrease in viability of ps₁ by 50% and of wy by 48%. The same investigators observed that in 0.1% citrate in soft agar medium, the viability of both phages was reduced by 30%.

Several bacteriophages were isolated from marine water of different salinities. Wichels et al. (1998) studied 22 phages which they found in water near Helgoland in the North Sea. All of them had tails and icosahedral heads of 50.2 to 99.3 nm, and they were classified into three different families: 11 phages to *Myoviridae*, 7 to *Siphoviridae*, and 4 to *Podoviridae*. No similarity in DNA structure was shown among phages belonging to different families present in this area. Also, Hidaka (1971) tested the stability of five marine bacteriophages in media with the addition of different inorganic salts (distilled water, 0. 5% NaCl solution, 3% NaCl solution, artificial seawater diluted sixfold, artificial seawater, and seawater broth). They observed that all phages were most inactivated in a medium containing 0.5% NaCl than in the other media. It suggests that the phages had the highest activity in salt concentrations roughly equivalent to seawater. Seaman and Day (2007) successfully isolated bacteriophages from a soil sample of salt plains in OK (USA). The salinity of the groundwater in this area varies between 4% and 37%, and soil salinity, between 0.3% and 27% (Wilson et al. 2004). One of those phages, ΦgspC, a member of the *Myoviridae* family, has an unusually large genome (340 kb). The authors suggested that this large genome may encode

environmentally relevant genes that probably increase the phage adaptation to some environments. Interesting observations made by Leibo and Mazur (1969) revealed that when the T4B phage was rapidly transferred from a concentrated to a dilute solution, the phage activity depended on the initial salt concentration of the solution in which the phage was suspended. The phage inactivation occurred by rapid dilution, but it did not decrease when the phage was slowly diluted. A rapid change in osmotic pressure may cause phage DNA to extrude from the tail or their heads to break. This occurred when phages were diluted from high salt concentration to low concentration solutions (Lark and Adams 1953). Yamamoto et al. (1968) who investigated the inactivation of the T5 phage similarly observed streaking decrease in phage activity achieved immediately after rapid dilution. Furthermore, the sensitivity of the T5 phage to chelating agent shock (sodium citrate or ethylenediaminetetraacetic acid) increased when the concentration of the chelating agent increased. Interestingly, higher inactivation was observed with low concentrations of chelating agents. The same authors observed that the inactivation of the phage by chelating agents was reduced by ionic solution (such as 0.85% NaCl).

Adams (1949) checked the stability of bacteriophage T5 incubated at 37°C in salt solutions (phosphate buffer, buffer plus citrate, and buffer plus calcium). He observed that the phage was stable in the calcium ion solution but lost its activity in phosphate buffer, whereas it was rapidly inactivated in citrate solution. No phage particles were detected after 2 h of incubation in 10 mmol/L phosphate buffer with 2 mmol/L citrate (pH 7). He also showed that divalent metals at millimolar concentrations might prevent phage inactivation. He supposed that the increase in T5 stability in the presence of different anionic solutions resulted from complex formation between the phage particle and ion. Mylon et al. (2009) studied MS2 phage stability in different solutions of LiCl, NaCl, KCl, and $CaCl_2$ in a range of 0.01–1.0 mol/L. Their observations revealed that monovalent salts did not influence phage aggregation. In contrast, the growth rate of the phage aggregates increased with an increasing calcium salt concentration. It was suggested that this resulted from neutralization of the negatively charged moieties on the phage surface by cation binding. The chemical composition of water may also influence phage stability. The stability of five *Flavobacterium* phages (PFpW-3, PFpC-Y, PFpW-6, PFpW-7, PFpW-8) isolated from pond water collected from Japanese ayu farms was tested for 21 days at 18°C (Kim et al. 2010). There were no significant changes in the phage titer in pond water, autoclaved filtered water, or broth during the first 3 days of incubation, but their stability decreased below the detection limit in pond water after 10 days. The persistence of MS2 and PRD1 phages was compared in tap water and ultrapure water system samples at room temperature and pH increasing from 7.6 to 8.9 and at stable pH 7 (Governal and Gerba 1997). There was no significant decrease in concentration of PRD1 during the experiments, but MS2 showed a different decrease in survivability in different types of water. The highest inactivation was in post reverse osmosis water. These observations were explained as resulting from phage structure. The genetic material of PRD1 is DNA, which is generally a more stable acid than RNA of MS2. After removing contaminants, water becomes a "more powerful solvent," and the possibility to degrade the phage genetic material increases. Moreover, phage PRD1 has internal lipids which increase its resistance to degradation in ultrapure water. It was suggested that ultrapure water, being an aggressive solvent, attacks the virus surface through a mechanism of direct oxidation. It causes head degradation, dispersion of capsids, tail fragmentation, and release of viral genetic material into the water environment. Jepson and March (2004) observed that phage was more stable at ambient temperature when stored in distilled water than in tap water in which its titer decreased by 2–3.5 log after 2 weeks. It was suggested that halogenating agents in tap water may inactivate phages. According to Thorne and Holt (1974), the addition of 10 mmol/L Mg^{2+} to the NBY may protect CP-51 phage against inactivation under unprofitable temperature. A 1-h incubation of phage lysates at 0°C at pH 6.8 with magnesium ions caused no detectable loss in phage activity. In comparison, 60% decrease in initial phage titer was observed when there was no Mg^{2+}. Similarly, other authors suggest that some metal ions may protect phages against inactivation. Interestingly, Ca^{2+} (1 or 5 mmol/L) could protect Xp12 phage particles suspended in 10 mmol/L Tris buffer solution at pH 8.0 against inactivation by heating at 60°C (Chow et al. 1971). On the other hand, Mg^{2+} in a concentration of 5 mmol/L increased thermal inactivation of the phage. Therefore, it was suggested that addition of 5 mmol/L $CaCl_2$ to solutions may prevent a loss of phage titer during the purification process. Kuo et al. (1971) observed phage Xp12 dissociation by sodium citrate in Tris buffer at pH 7.5 at room temperature. Phage particles exposed to 3 mmol/L sodium citrate presented decomposition in DNA and empty heads and tails. Gupta and Yin (1995) showed that bacteriophage T7 lost its activity with half life after 30 s when was exposed to 6 mol/L urea used as denaturing component. Whang et al. (1996) presented that 1 mmol/L metal ions may slow or accelerate T7 phage inactivation by urea. As they observed, divalent metal ions (Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}) stabilized activity in the presence of urea, in contrast to trivalents (Al^{3+} and Au^{3+}) which destabilized phages. The presence of either of the ions caused loss of phage titer more than 50-fold even at concentrations of 0.25 mmol/L.

Conclusions

Bacteriophages can be resistant to unfavorable physical and chemical factors, such as low and high temperatures, pH, salinity, and ions. Thus, they can settle in extreme environments. Based on the literature, it seems that phage features in that field are highly diversified and may differ not only among families, but also within them. So far, anyone who is going to preserve phages should "know his phage," as suggested by Ackermann et al. (2004). High bacteriophage resistance for external factors is important for stability of phage preparations. However, this feature is disadvantageous for industry when maintenance of the activity of bacterial strains is important. Inactivation of phages in different environments is multifactorial. It is known that the variation of one factor that influences phages may change phage sensitivity to the others (Thorne and Holt 1974; Müller-Merbach et al. 2004). One can expect that the growing interest of the pharmaceutical and agricultural industries in phages will result in new data on phage survivability and methods of their preservation.

References

Ackermann HW (2003) Bacteriophage observations and evolution. Res Microbiol 154:245–251

Ackermann HW (2007) 5500 phages examined in the electron microscope. Arch Virol 152:227–243

Ackermann HW, Abedon ST (2000) Bacteriophage names 2000. A compilation of known bacteriophages. http://mansfield.osu.edu/~sabedon/names.htm. Accessed 2 Jan 2011

Ackermann HW, DuBow MS (1987) Viruses of prokaryotes, volume I: general properties of bacteriophages. CRC Press, Boca Raton (USA)

Ackermann HW, Tremblay D, Moineau S (2004) Long-term bacteriophage preservation. WFCC Newslett 38:35–40

Adams MH (1949) The stability of bacterial viruses in solutions of salts. J Gen Physiol 32:579–594

Atamer Z, Dietich J, Müller-Merbach M, Neve H, Heller KJ, Hinrichs J (2008) Screening for and characterization of *Lactococcus lactis* bacteriophages with high thermal resistance. Int Diary J 19:228–235

Bachrach G, Leizerovici-Zigmond M, Zlotkin A, Naor R, Steinberg D (2003) Bacteriophage isolation from human saliva. Lett Appl Microbiol 36:50–53

Bettstetter M, Peng X, Garrett RA, Prangishvili D (2003) AFV1, a novel virus infecting hyperthermophilic archaea of the genus Acidianus. Virology 315:68–79

Breitbart M, Wegley L, Leeds S, Schoenfeld T, Rohwer F (2004) Phage community dynamics in hot springs. Appl Environ Microbiol 70:1633–1640

Buiser PB, Baliling JG, Esguerra RR, Galut AG, Oliveros SD, Dela Cruz-Papa DMA (2009) Two new tailed pseudomonas bacteriophages from the Philippines. Philippine Journal of Systematic Biology 3:29–39

Buzrul S, Öztürk P, Alpas H, Akcelik M (2007) Thermal and chemical inactivation of lactococcal bacteriophages. LWT 40:1671–1677

Caldeira JC, Peabody DS (2007) Stability and assembly *in vitro* of bacteriophage PP7 virus-like particles. J Nanobiotechnol 5:1–10

Caroli G, Armani G, Levre E, Jefferson TO (1980) Finding of *E. coli* phage in urinary tract infections. Ann Sclavo 22:857–860

Chandran A, Pradhan SK, Heinonen-Tanski H (2009) Survival of enteric bacteria and coliphage MS2 in pure human urine. J Appl Microbiol 107:1651–1657

Chow TY, Lin YT, Kuo TT (1971) Stability of phage Xp12. Bot Bull Academia Sinica 12:57–65

Clark WA (1962) Comparison of several methods for preserving bacteriophages. Appl Microbiol 10:466–471

Davis C, Silveira NFA, Fleet GH (1985) Occurrence and properties of bacteriophages of *Leuconostoc oenos* in Australian wines. Appl Environ Microbiol 50:872–876

Faquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (2005) Virus taxonomy: eighth report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, London

Feng YY, Ong SL, Hu JY, Tan XL, Ng WJ (2003) Effects of pH and temperature on the survival of coliphages MS2 and Qbeta. J Ind Microbiol Biotechnol 30:549–552

Frenkel D, Solomon B (2002) Filamentous phage as vector-mediated antibody delivery to the brain. Proc Natl Acad Sci USA 99:5675–5679

Gantzer Ch, Henny J, Schwartzbrod L (2002) *Bacteroides fragilis* and *Escherichia coli* bacteriophages in human feces. Int J Hyg Environ Health 205:325–328

Golec P, Dabrowski K, Hejnowicz M, Gozdek A, Łoś JM, Węgrzyn G, Łobocka M, Łoś M (2011) A reliable method for storage of tailed phages. J Microbiol Meth. doi:10.1016/j.mimet.2011.01.007

Goulet A, Pina M, Redder P, Prangishvili D, Vera L, Lichière J, Leulliot N, van Tilbeurgh H, Ortiz-Lombardia M, Campanacci V, Cambillau C (2010) ORF157 from the archaeal virus *Acidianus* filamentous virus 1 defines a new class of nuclease. J Virol 84:5025–5031

Governal RA, Gerba CP (1997) Persistence of MS-2 and PRD-1 bacteriophages in ultrapure water system. J Ind Microbiol Biotechnol 18:297–301

Gupta K, Yin J (1995) Metal recognition by in-vitro selection. Biotechnol Bioeng 45:458

Hanlon GW (2007) Bacteriophages: an appraisal of their role in the treatment of bacterial infections. Int J Antimicrob Agents 30:118–128

Hendrix RW (2002) Bacteriophages: evolution of the majority. Theor Popul Biol 61:471–480

Hidaka T (1971) Isolation of marine bacteriophages from seawater. Bull Jpn Soc Sci Fish 37:1199–1206

Höglund C, Ashbolt N, Stenström TA, Svensson L (2002) Viral persistence in source-separated human urine. Adv Environ Res 6:265–275

Hurst CJ, Gerba CP, Cech I (1980) Effects of environmental variables and soil characteristics on virus survival in soil. Appl Environ Microbiol 40:1067–1079

ICTVdB Management (2006) 00.021.0.01.001. Pseudomonas phage φ6. In: Büchen-Osmond C (ed) ICTVdB – The Universal Virus Database, version 4. Columbia University, New York

Jepson CD, March JB (2004) Bacteriophage lambda is highly stable DNA vaccine delivery vehicle. Vaccine 22:3413–1419

Kameyama L, Fernandez L, Bermudez RM, Garcia-Mena J, Ishida C, Guarueros G (2001) Properties of a new coliphage group from human intestinal flora. Rec Res Develop Virol 3:297–303

Keller R, Traub N (1974) The characterization of Bacteroides fragilis bacteriophage recovered from animal sera: observations on the nature of bactericides phage carrier cultures. J Gen Virol 24:179–189

Kerby GP, Godwy RA, Dillon ES, Dillon ML, Csáky TZ, Sharp DG, Beard JW (1949) Purification, pH stability and sedimentation properties of the T7 bacteriophage of Escherichia coli. J Immunol 63:93–107

Kim JH, Gomez DK, Nakai T, Park SC (2010) Isolation and identification of bacteriophages infecting ayu Plecoglossus altivelitis altivelitis specific Flavobacterium psychrophilium. Vet Microbiol 140:109–115

Kivelä H (2004) Marine icosahedral membrane-containing dsDNA bacteriophage PM2: virion structure and host cell penetration. PhD Thesis, University of Helsinki

Kłak M, Międzybrodzki R, Bubak B, Jończyk E, Weber-Dąbrowska B, Górski A (2010) Studies on the gastrointestinal transit and blood penetration of a therapeutic staphylococcal bacteriophage. Abstract no. 209, First International Congr. Viruses of Microbes, Paris

Kumari S, Harjai K, Chibber S (2010) Isolation and characterization of Klebsiella pneumonia specific bacteriophages from sewage samples. Folia Microbiol 55:221–227

Kuo TT, Chow TY, Lin YT, Yang CM (1971) Specific dissociation of phage Xp12 by sodium citrate. J Gen Virol 10:199–202

Langlet J, Gaboriaud F, Gantzer C (2007) Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage. J Appl Microbiol 103:1632–1638

Lark KG, Adams MH (1953) The stability of phages as a function of the ionic environment. Cold Spring Harb Symp Quant Biol 18:171–183

Lasobras J, Muniesa M, Lucena F, Jofre J (1997) Relationship between the morphology of bacteriophages and their persistence in the environment. Water Sci Tech 35:129–132

Leibo SP, Mazur P (1969) Effect of osmotic shock and low salt concentration on survival and density of bacteriophages T4B and T4o$_1$. Biophys J 6:747–772

Lin L, Honh W, Ji X, Han J, Huang L, Wei Y (2010) Isolation and characterization of an extremely long tail Thermus bacteriophage from Tegchong hot springs in China. J Basic Microbiol 50:452–456

Lu Z, Breidt F, Plengvidhya V, Fleming HP (2003) Bacteriophage ecology in commercial sauerkraut fermentations. Appl Environ Microbiol 69:3192–3202

Lucena F, Ribas F, Duran AE, Skraber S, Gantzer C, Campos C, Morón A, Calderón E, Jofre J (2006) Occurrence of bacterial indicators and bacteriophages infecting enteric bacteria in ground water in different geographical areas. J Appl Microbiol 101:96–102

McAuliffe O, Ross RP, Fitzgerald GF (2007) The news phage biology from genomics to applications. In: Mc Grath S, van Sinderen D (eds) Bacteriophage genetics and molecular biology. Caister Academic Press, Norfolk, pp 125–159

Mocé-Llivina L, Muniesa M, Pimenta-Vale H, Lucena F, Jofre J (2003) Survival of bacterial indicator species and bacteriophages after thermal treatment of sludge and sewage. Appl Environ Microbiol 69:1452–1456

Mullan WMA (2001) Bacteriophage isolation and purification. [On-line] UK. Available at http://www.dairyscience.info/isolation-and-purification-of-bacteriophages.html. Accessed 2 Jan 2011

Müller-Merbach M, Rauscher T, Hinrichs J (2004) Inactivation of bacteriophages by thermal and high-pressure treatment. Int Dairy J 15:777–784

Mylon SE, Rinciog CI, Schmidt N, Gutierrez L (2009) Influence of salt and natural organic matter on the stability of bacteriophage MS2. Langmuir. doi:10.1021/la902290t

Nasser A, Oman S (1999) Quantitative assessment of the inactivation of pathogenic and indicator viruses in natural water sources. Water Res 33:1748–1752

Nigutová K, Štyriak I, Javorský P, Pristaš P (2008) Partial characterization of Enterococcus faecalis Bacteriophage F4. Folia Microbiol 53:234–236

Olson MR, Axler RP, Hicks RE (2004) Effects of freezing and storage temperature on MS2 viability. J Virol Meth 122:147–152

Prangishvili D, Stedman K, Zillig W (2001) Viruses of the extremely thermophilic archaeon Sulfolobus. Trends Microbiol 9:39–43

Prigent M, Leroy M, Confalonieri F, Dutertre M, DuBow MS (2005) A diversity of bacteriophages forms and genomes can be isolated from the surface sands of Sahara Desert. Extremophiles 9:289–296

Pringsulaka O, Patarasinpaiboon N, Suwannasai N, Atthakor W, Rangsiruji A (2010) Isolation and characterisation of a novel Podoviridae-phage infecting Weissella cibaria N 22 from Nham, a Thai fermented pork sausage. Food Microbiol. doi:10.1016/j.fm.2010.10.011

Puapermpoonsiri U, Ford SJ, van der Walle CF (2009) Stabilization of bacteriophage during freeze drying. Int J Pharm 389:168–175

Säwström CH, Lisle J, Anesio AM, Priscu JC, Laybourn-Parry J (2008) Bacteriophage in polar inland waters. Extremophiles 12:167–175

Seaman PF, Day MJ (2007) Isolation and characterization of a bacteriophage with an unusually large genome from the Great Salt Plains National Wildlife Refuge, Oklahoma, USA. FEMS Microbiol Ecol 60:1–13

Sharp DG, Hock A, Taylor AE, Beard D, Beard JW (1946) Sedimentation characters and pH stability of the T2 bacteriophage of Escherichia coli. J Biol Chem 165:259–270

Tartera C, Jofre J (1987) Bacteriophages active against Bacteroides fragilis in sewage-polluted waters. Appl Environ Microbiol 53:1632–1637

Tey BT, Ooi ST, Yong KC, Tan Ng MY, Ling TC, Tan WS (2009) Production of fusion m13 phage bearing the disulphide constrained peptide sequence (C-WSFFSNI-C) that interacts with hepatitis B core antigen. J African Biotechnol 8:268–273

Thorne CB, Holt SC (1974) Cold liability of Bacillus cereus bacteriophage CP-51. J Virol 14:1006–1012

Tsutsaeva AA, Visekantsev IP, Mikulinskiy YE, Butenko AE (1981) Effect of low temperatures of the survival and intracellular multiplication of Escherichia coli bacteriophages (in Russian). Mikrobiologiia 50:292–244

Vinnerås B, Nordin A, Niwagaba Ch, Nyberg K (2008) Inactivation of bacteria and viruses in human urine depending on temperature and dilution rate. Water Res 42:4067–4074

Wachman HA, Brown CJ (2010) Experimental evolution of viruses: Microviridae as a model system. Phi. Trans. R. Soc. B. 365:2495–2501

Warren JC, Hatch MT (1969) Survival of T3 coliphage in varied extracellular environments. Appl Microbiol 17:256–261

Weinbauer M (2004) Ecology of procaryotic viruses. FEMS Microbiol Rev 28:127–181

Whang T, Daly B, Yin J (1996) Metal-ion discrimination by phage T7. J Inorg Biochem 63:1–7

Whitman PA, Marshall RT (1971) Characterization of two psychrophilic *Pseudomonas* bacteriophages isolated from ground beef. Appl Microbiol 22:463–468

Wichels A, Biel SS, Gelderblom HR, Brinkhoff T, Muyzer G, Schutt CH (1998) Bacteriophage diversity in the North Sea. Appl Environ Microbiol 64:4128–4133

Wick CH, Elashvili I, McCubbin PE, Birenzvige A (2006) Determination of MS2 bacteriophage stability at low pH using the integrated virus detection system (IVDS). [On-line] Available at http://www.stormingmedia.us/92/9237/A923754.html. Accessed 2 Jan 2011

Wilson C, Caton TM, Buchheim JA, Buchheim MA, Schneegurt MA, Miller RV (2004) DNA-repair potential of *Halomonas* ssp. from the Salt Plains Microbial Observatory of Oklahoma. Microb Ecol 48:541–549

Yamamoto N, Fraser D, Mahler HR (1968) Chelating agent shock of bacteriophage T5. J Virol 2:944–950

Yates MV, Gerba CP, Kelley LM (1985) Virus persistence in ground water. Appl Environ Microbiol 31:778–781

Yoon SS, Barrangou-Poueys R, Breidt F Jr, Klaenhammer TR, Fleming HP (2002) Isolation and characterization of bacteriophages from fermenting sauerkraut. Appl Environ Microbiol 68:973–976