Long-Term Bacteriophage Preservation

Hans-W. Ackermann[1,2]*, Denise Tremblay[1,3], and Sylvain Moineau[1,3,4]

[1]*Félix d'Hérelle Reference Center of Bacterial Viruses,*
[2]*Département de biologie médicale, Faculté de Médecine,*
[3]*Groupe de recherche en écologie buccale, Faculté de médecine dentaire*
[4]*Département de biochimie et microbiologie, Faculté des sciences et de génie,*
Université Laval, Québec, Qc, Canada G1X 4C6
ackermann@mcb.ulaval.ca

*Corresponding author.
Tel.: (418) 656-2131 # 2558, Fax: (418) 656-7666, E-mail: ackermann@mcb.ulaval.ca

---

Introduction

The Félix d'Hérelle Center (WDCM 6) was founded in 1981 with a grant of the Natural Sciences and Engineering Research Council of Canada (NSERC) (3). Its main objective is taxonomical, namely the preservation of type viruses of bacteriophage species. In addition, the Center collects phages with applications in teaching or phage typing, harmful phages from the biotechnological industry, and phages with interesting properties (e.g., capsule- and flagella-specific phages or viruses with exceptionally large DNA size). In addition, the Center provides documentation and welcomes visitors for instruction in phage techniques. Because of budget cuts, the Center lost its NSERC support in 1995 and was self-supporting except for small grants from Laval University in recent times.

The Center (collection.phages@bcm.ulaval.ca) is an instrument of the International Committee on Taxonomy of Viruses (ICTV). Its policy is to select interesting phages from the literature and to request their deposit from the original investigators. Dr. Ackermann was over many years either chairman or vice-chairman of the ICTV Bacterial Virus Subcommittee and therefore in a position to select taxonomically interesting phages. He was the curator of the Center from 1981 until January 2003, when the collection was transferred to a laboratory in the Faculty of Science and Engineering of this university. The present curator, Dr. Moineau, is a specialist of phages for lactic acid bacteria and has a personal collection of 260 phages for these microorganisms. We report phage observations over unusually long periods. Previous investigations of phage survival during storage (5-8) covered relatively short time periods, mostly of 6 weeks to two years, were little or not concerned with phage taxonomy and structure, and sometimes reported unknown phages identified by their collection numbers only.

Phages held

The collection of the Center includes 441 bacteriophages and 376 bacteria representing 59 genera and over 120 species. It is the largest and probably most diversified phage collection in existence. The phages belong to nine families (1, 12) (Table 1). Over 95% of phages are tailed. The collection is rich in phages of enterobacteria, acinetobacters, bacilli, mycobacteria, pseudomonads, rhizobia, and vibrios. Accordingly, the phage hosts include a great number of Class 2 pathogens (e.g., enteropathogenic *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio cholerae).* Many phages were obtained from the ATCC. In turn, about 90 phages were deposited in the ATCC. The identity of newly deposited phages is controlled by electron microscopy. Prior to distribution, phages are controlled for viability and, if necessary, propagated again and examined in the electron microscope. In the last five years (1999-2003), over 700 phages were requested from the collection (average of two phages per week). The requests came from over 100 laboratories located in 21 countries.

Propagation and storage

Phages are propagated at conditions optimal for their bacterial hosts. The most frequently used media are Trypticase Soy agar and Brain Heart Infusion broth (Difco). Some 15 other media are used for particular bacteriophages. Filter-sterilized phage lysates are stored at +4^{o}C and deep-frozen at –80^{o}C and in liquid nitrogen, using plastic vials of 2 ml capacity with 50% glycerol as a cryoprotector (5). Neither chloroform nor other chemicals are added. Propagating and lysogenic bacteria are stored with 15% glycerol at –80^{o}C and in liquid nitrogen. In addition, bacilli are conserved on agar slants at +4^{o}C. Lyophilization was discontinued after initial difficulties, but was recently resumed. The collection thus relies on lysates kept at +4^{o}C for distribution and research and on deep-frozen and freeze-dried specimens for replacement.

Observations

Because of the existence of a considerable literature on phage preservation, it was not planned to investigate phage survival when the collection was started. Although we made numerous observations over the years, they do not represent a systematic investigation. Old lysates and the original phage samples received from depositors were generally not discarded, but were kept at +4^{o}C in a cold room. A large number of old phage lysates accumulated in this way and were now available for reexamination. From many titrations carried out over the years, a general pattern of phage survival emerged (Fig. 1).

1. Lysates stored at +4^{o}C

Lysates are stored in screw-cap tubes of 40 ml. Occasionally, a dematious mycelium developed in a lysate, but it never affected phage titers. Tailed phages were mostly stable regardless of their host, taxonomic position, or morphology. Contrary to previous observations (7), phages with contractile tails were as stable as phages with long, noncontractile or with short tails. Phages with large capsids of 100 nm in diameter were as stable as phages with heads of 60 nm. In particular, valuable experimental models such as phages of the T series, the λ group, and ϕ29 and its relatives remained viable over 10-12 years. Phages of the T4 and T7 phage groups appeared to be extremely resistant. For example, the T4-like *Shigella* phage C16, a historical phage described in 1933 by Burnet (4), had a titer of 10^3 after 32 years. The general behavior of tailed phages, as indicated by frequent titrations, is that phage titers drop by 1 log in the first year and then remain stable or decline slowly by approximately one log per year.

The principal exceptions were viruses of the type of coliphages P2 and Mu. These viruses are characterized by fragile tails, a particular that is visible in the electron microscope. Upon contraction, the tail sheath of these phages becomes loose, and slides along the tail tube and finally off the tail. Phages of this type, which occur in many Gram-negative bacteria (e.g., enterics, *Haemophilus, Pasteurella, Pseudomonas, Rhizobium, Vibrio*) (12) must be watched. Coliphage Mu is particularly sensitive; it is inactivated after only a few months of storage (7) and may lose 8 logs in a year. However, the titer of its close relative, coliphage D108, decreases by only one log over 5 years. There are thus individual variations. One exceptional virus, *Bacillus* phage CP-54Ber, is cold-sensitive (loss of 7 logs over 3 months) and is best kept at room temperature or included into bacterial spores (12).

The icosahedral ("cubic") phages in the collection fell into two groups: phages with lipids and those without. The filamentous phages had no lipid components. In a general way, lipid-containing phages proved unstable and had to be watched closely and to be propagated every two years. Again, there were exceptions and individual variations. For example, cystovirus ϕ6 and tectivirus PRD1 were as stable as most tailed phages, but tectiviruses Bam35 and AP50 lost 8 logs in one year and had to be retrieved from samples in liquid nitrogen. By contrast, phages without lipids were relatively stable. These observations parallel those of Greiff (1969) on the cryosensitivity of animal viruses (9).

2. Storage at -80^{o}C and in liquid nitrogen

Tailed phages were relatively stable. In many cases, phages were more stable in liquid nitrogen than at –80^{o}C and +4^{o}C. The behavior of cubic and filamentous phages was more erratic. On several occasions, lipid-containing and other phages which had died in lysates stored at +4^{o}C, were recovered from frozen samples kept at -80^{o}C or in liquid nitrogen. Others, for example corticovirus PM2 and

tectivirus AP50, were extremely unstable in all conditions. Deep-freezing was often disappointing (Table 2) and did not prove superior to simple storage of lysates at +4^{o}C. Some phages even seemed to be inactivated faster during freezing than during storage at +4^{o}C. It is unclear whether this is due to rapid freezing and thawing or if it can be prevented by addition of DSMO, milk, or peptones. Bacteria of the genera *Aeromonas, Bacteroides, Brucella,* and *Vibrio* seem to be relatively sensitive to storage at -80^{o}C and should be propagated every 5 years.

3. Lyophilization

Phages and bacteria were lyophilized using a VirTis model 10-47 freeze-drying apparatus (Gardiner, New York). Additives were 50% glycerol for phages and 10% bovine serum albumin for bacteria. Glass beads were sometimes added to the sample. Ampoules were checked with a vacuum tester. One month after lyophilization, most phage titers had decreased by approximately 1 log. One year later, the vacuum had deteriorated in most ampoules and the phages therein had lost their viability. After this disastrous experience, lyophilization of phages and bacteria was halted, but the ampoules were stored in a cold room. In 2003, over 20 years later, all ampoules with intact vacuum were found to contain viable phages. Conversely, bacteria and ampoules with deteriorated vacuum contained no viable material. We attribute this relative failure to the development of tiny cracks in the ampoules during the sealing process.

4. Electron microscopy

Various "old" lysates were examined in a Philips EM 300 electron microscope. Tailed phages in showed the same types of damages that were regularly present, albeit much less frequently, in fresh preparations: namely "ghosting" or loss of DNA, head disruption, tail contraction, loss or disruption of tails or tail sheaths, or loss of base plates and tail fibers. The type of damage depended on the phage; e.g., some phages tended to loose DNA and others showed contracted tails. Similar alterations were described in phages subjected to lyophilization or freezing and thawing (10, 13). In some "old" preparations, only rare empty heads were observed and tails had simply vanished. On the other hand, biologically active lysates always contained intact particles. Again, T4-type phages proved to be particularly resistant. "Cubic" and filamentous phages showed few spectacular alterations. In several cases, the phages had simply disappeared and were no longer detectable. In other cases, empty capsids or inner vesicles persisted (Table 3). These observations are difficult to quantify because, due to the vagaries of ultracentrifugation and grid preparation, intact and damaged phages tend to collect on different fields of grids.

Problems

At one time, phages appeared to die at an accelerated rate. The problem was traced to a detergent used to detach animal cells from the glassware of a nearby virological laboratory. Since all glassware, including the tubes for phage storage, was cleaned in the same batches, it is possible that traces of the detergent persisting in the tubes inactivated phages by long-term contact. Another problem was caused by unstable plasmids, which led to the loss of some 10 plasmid-dependent RNA and filamentous enterobacterial phages. A final and novel problem, entirely man-made, is the present system of regulations against shipment of minor pathogens of containment level 2. It is certain that this collection could not have been assembled with the present restrictions in use.

Conclusions

Lysates of most tailed phages and "cubic" or filamentous phages without lipids are easily stored over 5-10 years, but there are many individual variations. Phage titers should be expected to decline by 1 log per year. Lipid-containing phages tend to be more fastidious and unstable, and should be controlled yearly. Chloroform should not be used for preservation because it inactivates one third of tailed and all lipid-containing and filamentous phages (2); however, chloroform may be added to individual phages if they are known to be resistant. The general watchword is "Know your phage."

Storage of lysates at +4^{o}C alone is dangerous and must be backed by deep-freezing or lyophilization; however, deep-freezing at -80^{o}C or in liquid nitrogen does not guarantee phage survival. Our phages appear to be more sensitive to freezing and lyophilization than expected from earlier reports (5-8), but this may be explained by our exceptionally long storage periods. Cryovials should be prepared in duplicate or triplicate and stored in several locations.

Replacement lysates should be prepared from cryovials to minimize genetic drift. Lyophilization, if done properly, seems to be equivalent to preservation in liquid nitrogen. Ampoules should be checked regularly for proper vacuum.

References

1. **Ackermann, H.-W**. 2003. Bacteriophage observations and evolution. Res. Microbiol., **154,** 245-251.
2. **Ackermann, H.-W., and DuBow, M.S**. 1987. Viruses of Prokaryotes. II. Natural Groups of Bacteriophages. CRC Press, Boca Raton, FL, p. 27.
3. **Ackermann, H.-W., Martin, M., Vieu, J.-F., and Nicolle, P.** 1982. Félix d'Hérelle: his life and work and the foundation of a bacteriophage reference center. ASM News, **48,** 346-348.
4. **Burnet, F.M.** 1933. The classification of dysentery-coli baceriophages. III. A correlation of the serological classification with certain biochemical tests. J. Pathol. Bacteriol., **37,** 179-184.
5. **Clark, W.A.** 1962. Comparison of several methods for preserving bacteriophages. Appl. Microbiol., **10,** 466-471.
6. **Clark, W.A., and Geary, D.** 1969. The collection of bacteriophages at the American Type Culture Collection. *In* Freezing and Drying of Microorganisms, ed. Tokio Nei, University Park Press, Baltimore, pp. 179-187.
7. **Clark, W.A., and Geary, D.** 1973. Preservation of bacteriophages by freezing and freeze-drying. Cryobiology, **10,** 351-360.
8. **Clark, W.A., and Klein, A.** 1966. The stability of bacteriophages in long term storage at liquid nitrogen temperatures. Cryobiology, **3,** 68-75.
9. **Greiff, D.** 1969. Comparative studies of the cryobiology of viruses classified according to their physico-chemical characteristics. *In* Freezing and Drying of Microorganisms, ed. Tokyo Nei, University Park Press, Baltimore, pp. 69-80.
10. **Steele, P.R.M.** 1976. Morphological manifestations of freezing and thawing injury in bacteriophage T4Bo. J. Hyg. (Camb.), **77:**119-127.
11. **Van Regenmortel, M.H.V., C.M. Fauquet, D.H.L. Bishop, E. Carstens, M.K. Estes, S. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle, and R. Wickner,** eds. Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 63-120, 267-284, 645-650.
12. **Van Tassell, R.L., and Yousten, A.A.** 1976. Response of *Bacillus thuringiensis* to bacteriophage CP-51. Can. J. Microbiol., **22,** 583-586.
13. **Vieu, J.F., and Croissant, O.** 1966. Lyophilisation du bactériophage Vi II de *Salmonella typhi*. Arch. Roum. Pathol. Exp. Microbiol., **25,** 305-318

Table 1. Bacteriophages in collection

Family	Shape	Nucleic acid	Particulars	Member	Number
Myoviridae	Tailed	dsDNA	Tail contractile	T4	157
Siphoviridae		"	Tail long, noncontractile	T1	174
Podoviridae		"	Tail short	T7	88
Corticoviridae	Cubic	"	Lipid layer in capsid	PM2	2*
Tectiviridae	"	"	Inner lipoprotein vesicle	PRD1	4
Cystoviridae	"	dsRNA	Lipoprotein envelope	ϕ6	3
Leviviridae	"	ssRNA		PP7	7
Microviridae	"	ssDNA		ϕX174	1
Inoviridae	Filamentous	"		Pf1	5

*Including a phage of uncertain affiliation.

Table 2. Lyophilization vs. cold storage

Family	Phage	Lyophilization		+4°C		-80°C		N_2
		Years	Loss	Years	Loss	Years	Loss	Loss
Myoviridae	T2	21	3	10	3	12	4	2
	T4	21	4	7	-	12	4	3
	T6	21	2	6	-	12	4	2
	P1	21	6	3	2	12	8*	3
	P2	16	2	10	5	12	7*	7*
Siphoviridae	T1	21	-	10	1	12	1	1
	T5	21	-	12	-	12	7	1
	λvir	21	-	7	1	12	2	3
Podoviridae	T3	21	5	7	3	12	5	6
	T7	21	1	3	-	12	3	6
	N4	21	-	8	1	12	-	-
	ϕ29	21	4	5	1	12	-	-
Corticoviridae	PM2			1	5	3	8*	6
Tectiviridae	PRD1	21	5	3	1	12	3	4
	AP50	21	3	1	8	0.5	8*	8*
Cystoviridae	ϕ6	21	5	6	1	12	2	6
Leviviridae	C-1	19	4	6	-	13	1	3
Microviridae	ϕX174	21	4	4	1	12	-	4
Inoviridae	Vf33	16	7*	4	8	11	4	2

Loss, decrease in logs; N_2, liquid nitrogen; *, complete inactivation; -, no loss.

Table 3. Morphological changes after storage in broth (+4°C)

Family	Phage	Years	Phages present	Phages intact	Main observations
Myoviridae	BW-1*	17	+++	+/-	Empty heads, contracted tails
	C16*	32	+++	+/-	Empty heads, contracted tails
	K19Q	7	+	+/-	Disrupted heads without tails
	Twort	12	-	-	Empty heads (traces)
Corticoviridae	PM2	4	-	-	Disappearance
Tectiviridae	PRD1	22	+	+/-	Mostly empty phages
	AP50	1	-	-	No capsids, traces of lipid vesicles
Cystoviridae	ϕ6	4	-	-	Loss of envelope, persistence of dodecahedral polymerase complex
Leviviridae	C-1, C-2, Iα	5-10	Traces	+/-	Partial disappearance
Microviridae	ϕX174	22	Traces	-	Partial disappearance
Inoviridae	Ike, I_2 -2, Pf1	11-10	-	-	Disappearance

Years, storage time in years; *, T4-type phages; +++, large quantities; +, present; -, absent.

Fig. 1 Hypothetical behavior of tailed "phage X" under various storage conditions