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## Note

## A reliable method for storage of tailed phages

Piotr Golec<sup>a,e</sup>, Kamil Dąbrowski<sup>b,c</sup>, Monika S. Hejnowicz<sup>b</sup>, Agnieszka Gozdek<sup>b</sup>, Joanna M. Łoś<sup>a</sup>, Grzegorz Węgrzyn<sup>a</sup>, Małgorzata B. Łobocka<sup>b,c,\*</sup>, Marcin Łoś<sup>a,d</sup><sup>a</sup> Department of Molecular Biology, University of Gdańsk, Kładki 24, 80-822 Gdańsk, Poland<sup>b</sup> Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Pawińskiego 5A, 02-106 Warsaw, Poland<sup>c</sup> Autonomous Department of Microbial Biology, Faculty of Agriculture and Biology, Warsaw University of Life Sciences, Ul. Nowoursynowska 159, 02-776 Warsaw, Poland<sup>d</sup> Institute of Physical Chemistry of the Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw, Poland<sup>e</sup> Laboratory of Molecular Biology (affiliated with University of Gdańsk), Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Ul. Kładki 24, 80-822 Gdańsk, Poland

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## ABSTRACT

A universal and effective method for long-term storage of bacteriophages has not yet been described. We show that randomly selected tailed phages could be stored inside the infected cells at  $-80\text{ }^{\circ}\text{C}$  without a major loss of phage and host viability. Our results suggest the suitability of this method as a standard for phage preservation.

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The growing interest in tailed phages as the most abundant members of microbial communities and biocontrol agents creates a need for reliable methods of long-term phage preservation. Different preservation methods, such as storage of crude or purified phage lysates at  $4\text{ }^{\circ}\text{C}$ , freezing and storage at  $-80\text{ }^{\circ}\text{C}$  or in liquid nitrogen, and finally storage of dried or lyophilized phages, have been used in different laboratories. Summaries concerning the effectiveness of these methods in preserving various phages have been published (Ackermann et al., 2004; Carlson, 2005; Fortier and Moineau, 2009, and references therein; Zierdt, 1988). Unfortunately, none of these methods appeared universal due to the differences in sensitivity of individual phages to physical conditions and the content of storage media. Only ten out of nineteen phages from nine families tested survived over 10 years without a loss of phage titer, but the most appropriate method of storage was not the same for all these phages (Ackermann et al., 2004). Thus, an individual approach for each phage or usage of several preservation methods in parallel has been

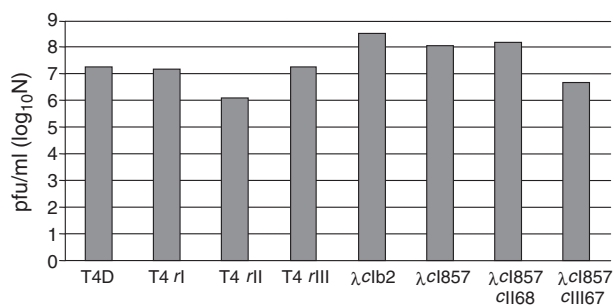
recommended to ensure later phage recovery. Perhaps this is a reason why only 21 of over 500 culture collections registered in the World Data Center for Microorganisms maintain phage deposits (<http://wdcm.nig.ac.jp/hpcc>). Whereas the storage problems may, in the case of temperate phages, be overcome by preserving their lysogens, no universal method for the preservation of obligatory virulent phages or virulent mutants of temperate phages has been described yet.

The encountered problems with the storage of phages differ from phage to phage. In most cases the titers of stored phage lysates decline slowly with time (Ackermann et al., 2004). Sometimes the titer drops are large and thus the lysates have to be refreshed to ensure phage viability, depending on the phage. In such cases, high per-site mutation rates in phage genomes could cause the accumulation of mutations at each refreshment step and lead to differences between the rescued phage and the original isolate (Cuevas et al., 2009; Drake, 1991; Santos and Drake, 1994; Wichman et al., 2005). Some phages are vulnerable to freezing, or even cooling to  $4\text{ }^{\circ}\text{C}$  (Ackermann et al., 2004; Warren and Hatch, 1969). Freeze-drying is particularly harmful to large myoviruses (Clark, 1962; Clark et al., 1962). Another important variable is the titer of stored lysate. On the one hand, at high titers some phages tend to aggregate which attenuates their activity (Carlson, 2005; Serwer et al., 2007a,b, and references therein), and on the other hand, diluting the lysates usually promotes a faster decrease in their titers.

The method we described here is simple. It overcomes the above mentioned difficulties and may represent a step ahead in the

\* Corresponding author at: Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Ul. Pawińskiego 5A, 02-106 Warsaw, Poland. Tel.: +48 22 5921300; fax: +48 22 6584636.

E-mail addresses: [pgolec@biotech.ug.gda.pl](mailto:pgolec@biotech.ug.gda.pl) (P. Golec), [kamild@ibb.waw.pl](mailto:kamild@ibb.waw.pl) (K. Dąbrowski), [hejmonika@ibb.waw.pl](mailto:hejmonika@ibb.waw.pl) (M.S. Hejnowicz), [agnes@ibb.waw.pl](mailto:agnes@ibb.waw.pl) (A. Gozdek), [juno1@interia.pl](mailto:juno1@interia.pl) (J.M. Łoś), [wegrzyn@biotech.ug.gda.pl](mailto:wegrzyn@biotech.ug.gda.pl) (G. Węgrzyn), [lobocka@ibb.waw.pl](mailto:lobocka@ibb.waw.pl), [malgorzata\\_lobocka@sggw.pl](mailto:malgorzata_lobocka@sggw.pl) (M. Łobocka), [Marcinlos@rocketmail.com](mailto:Marcinlos@rocketmail.com) (M. Łoś).



**Fig. 1.** Ability of phage infected *E. coli* MG1655 cells to release mature phages after 45 months of freeze-storage. *E. coli* MG1655 cells of overnight cultures were infected with different T4D or  $\lambda$  phage mutants (M.O.I. = 0.1–0.5), left for 5 min at 37 °C, supplemented with glycerol to the final concentration of 15%, frozen and stored at –80 °C. To check the ability of infected and frozen cells to release mature phages upon melting, portions of cell suspensions were scratched from the surface of frozen stocks, thawed, and 1  $\mu$ l of each portion was resuspended in 50 ml of ice-cold LB medium. Cells were harvested by centrifugation in a cooled microcentrifuge for 2 min, at 4 °C, resuspended in fresh, cold LB, washed again three times in the same way to remove free phages, and resuspended in 50  $\mu$ l of fresh LB. Cell suspensions depleted of free phages were immediately used to prepare serial dilutions and to measure the number of infective centers, as described in the text.

standardization of phage preservation procedures. In this method we utilize bacterial cells infected with a phage as a reservoir of phage DNA and a source of mature phages.

In our routine work, we observed that *E. coli* cells freshly infected with mutants of bacteriophage T4 or lytic mutants of  $\lambda$  phage and frozen to –80 °C can form infective centers when melted and mixed

with the appropriate phage sensitive cells (Fig. 1). The infective centers could be recovered after over three years of freeze-storage, with their titer remaining high. They did not represent unadsorbed phages, as the latter were removed by centrifugation of cell suspensions and washing of harvested cells in fresh medium before the assays. Encouraged by these results, we used nine randomly selected tailed phages of various genome lengths that infect Gram-negative or Gram-positive bacteria and belong to different phage families (myoviruses: Muclts62, P1vir, T4D and A5W; siphoviruses:  $\lambda$  papa and: bIBB29; and podoviruses: phiKDA1, phiAGO1.3 and 933W; Table 1), to test whether freshly infected bacterial cells can serve as a shelter for phage preservation in frozen liquid media. Five of these phages were obligatory virulent (T4, A5W, phiKDA1, phiAGO1.3 and bIBB29), two were temperate ( $\lambda$  papa and 933W) and two were virulent mutants of temperate phages (P1vir and Muclts62). The *stx*-converting temperate phage 933W was selected for this experiment as it is so unstable in unfrozen lysates that its titers fell down over 20-fold, even after such a short time as overnight storage at 4 °C (Plunkett et al., 1999; our unpublished results).

Cells from overnight cultures grown at 37 °C (*L. lactis* at 30 °C) in LB medium (Sambrook et al., 1989) were diluted in fresh LB supplemented with MgSO<sub>4</sub> (10 mM) and CaCl<sub>2</sub> (10 mM) to OD<sub>600</sub> of approximately 0.2 and grown with shaking (*L. lactis* without shaking) until the late exponential or early stationary phase. Next, the cells were cooled to room temperature, infected with a phage at the multiplicity of infection (M.O.I.) between 0.1 and 0.5, and kept without shaking for 5 or 15 min. The 15 min incubation time was used in nearly all experiments as it appeared sufficient to adsorb from 72 to 99% of the tested phages (Table 2). A sample of each mixture, taken

**Table 1**  
Effectiveness of phage storage in frozen infected cells and in frozen lysates.

Phage host (strain) <sup>a</sup>	Phage (genome size in kb) <sup>b</sup>	Phage family <sup>c</sup>	Phage source	Initial pfu/ml <sup>d</sup>	Decrease in pfu/ml after freezing/melting (in logs) <sup>e,g</sup>	Storage time (months)	Further decrease in pfu/ml after storage (in logs) <sup>f</sup>
<i>Escherichia coli</i> (MG1655)	T4D (169)	M	Infected cells	1.8 × 10 <sup>4</sup>	0	8	0
			Lysate	1.2 × 10 <sup>12</sup>	n.d.	10	4
<i>Staphylococcus aureus</i> (PS80)	A5W (137)	M	Infected cells	3.0 × 10 <sup>7</sup>	0	11	0
			Lysate	2.6 × 10 <sup>9</sup>	0	11	1
<i>Escherichia coli</i> (N99)	P1vir (94)	M	Infected cells	2.0 × 10 <sup>7</sup>	0	10	0
			Lysate	1.8 × 10 <sup>8</sup>	4	10	2
<i>Escherichia coli</i> (MG1655)	933W (62)	P	Infected cells	1.7 × 10 <sup>7</sup>	1	3	0
			Lysate	5.2 × 10 <sup>10</sup>	1	3	2
<i>Escherichia coli</i> (MG1655)	$\lambda$ papa (48)	S	Infected cells	8.4 × 10 <sup>4</sup>	0	8	0
			Lysate	2.8 × 10 <sup>9</sup>	n.d.	10	2
<i>Enterobacter</i> sp. (Ecl1)	phiKDA1 (45)	P	Infected cells	5.1 × 10 <sup>8</sup>	0	5	0
			Lysate	5.8 × 10 <sup>9</sup>	1	7	1
<i>Escherichia coli</i> (N99)	Muclts62 (37)	M	Infected cells	6.6 × 10 <sup>7</sup>	1	4	0
<i>Escherichia coli</i> (C600)	Muclts62	M	Infected cells	1.2 × 10 <sup>8</sup>	3	6	0
			Lysate	1.0 × 10 <sup>8</sup>	5	6	0
<i>Lactococcus lactis</i> (IL1403)	bIBB29 (29)	S	Infected cells	4.0 × 10 <sup>8</sup>	0	6	0
			Lysate	1.0 × 10 <sup>8</sup>	0	6	0
<i>Staphylococcus aureus</i> (PS80)	phiAGO1.3 (17)	P	Infected cells	3.3 × 10 <sup>7</sup>	0	9	0
			Lysate	6.7 × 10 <sup>6</sup>	0	9	0

<sup>a</sup> The Ecl1 strain is an environmental isolate which was typed to *Enterobacter* sp. based on the sequence analysis of its 16S rDNA and the *gyrB* gene (our unpublished results). Strains used as hosts of other phages were described previously (C600: Sambrook et al., 1989; N99: Shimada et al., 1972; MG1655: Blattner et al., 1997; PS80: Asheshov, 1969; IL1403: Hejnowicz et al., 2009).

<sup>b</sup> Phages were from the collections of the Biology Department of Gdańsk University: T4D (Miller et al., 2003), 933W (Plunkett et al., 1999),  $\lambda$  papa (Hendrix and Duda, 1992), the Institute of Biochemistry and Biophysics PAS: P1vir (Sternberg and Maurer, 1991; Łobocka et al., 2004), Muclts62 (Howe, 1973), bIBB29 (Hejnowicz et al., 2009), and from the collection of the Ludwik Hirsfeld Institute of Immunology and Experimental Therapy, PAS: A5W. Phages phiKDA1 and phiAGO1.3 are our new environmental isolates that are currently under study (data to be published elsewhere). Phage genome sizes are given according to the following GenBank entries: NC 000866 (T4), AF503408 (A5W), AF234172 (P1vir), AF125520 (933W), NC 001416 ( $\lambda$ ), AF083977 (Muclts62), NC\_011046 (bIBB29), and according to our unpublished sequencing results (phiKDA1, phiAGO1.3). The taxonomic classification of phiKDA1 and phiAGO1.3 to the *Podoviridae* family is based on electron micrographs (data to be published elsewhere). Muclts62 and 933W, which are exceptionally unstable in lysates were obtained for the purpose of initiating these studies by induction of their lysogens.

<sup>c</sup> Abbreviations mean respectively: M – Myoviridae, S – Siphoviridae, P – Podoviridae.

<sup>d</sup> The measurements of the number of pfu/ml were performed as described in the text. In the case of experiments with infected cells, phages were adsorbed to cells for 15 min before freezing the mixtures. Unadsorbed phages were not removed from the mixtures.

<sup>e</sup> In each case the first measurement of the number of pfu/ml was performed one to two weeks after freezing of the infected cells or lysates.

<sup>f</sup> The consecutive measurements were performed every week or two for the first two months of storage and every 4–12 weeks for the next months, using different aliquots of the same stock of infected cells or lysates. Only the results of the last measurements are shown for each phage. Each assay of pfu/ml was performed in triplicate. In the case of Muclts62 and Muclts62 infected cells, plates for the assays of the number of pfu/ml were incubated at 42 °C.

<sup>g</sup> n.d. – not determined.

after the incubation of bacteria with the phage, was depleted of bacterial cells and assayed for phage titer to verify the efficiency of phage adsorption. The remaining portion of each mixture was either washed out from unadsorbed phages and supplemented with cold glycerol to the final concentration of 15%, or supplemented with glycerol without prior washing (Fig. 2, Table 1). Aliquots of mixtures were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further studies. Aliquots of phage lysates supplemented with glycerol were frozen as controls at the same time. Portions of infected cells and phage lysates taken either before freezing or at different times after freezing served to measure the number of pfu/ml. The measurements were performed using the double agar overlay plaque assay of Adams as described previously (Łoś et al., 2008), after mixing appropriately diluted, infected cells or phage lysates with cells of exponential cultures of sensitive bacteria, grown in LB medium and supplemented with 10 mM  $\text{MgSO}_4$  and 10 mM  $\text{CaCl}_2$ . The melting of frozen suspensions of infected cells or of phage lysates was performed for 15–60 min in an ice water bath just before the measurement.

The influence of freezing–melting and freeze-storage on the titer of phage lysates varied (Table 1, Fig. 2). Only the titers of lysates of *Staphylococcus aureus* phage phiAGO1.3 and *Lactococcus lactis* phage bIBB29 did not drop either shortly after freezing or later. The titers of other phage lysates dropped more or less dramatically as a result of freezing–melting or further freeze-storage.

The storage of phages in frozen, infected cells appeared superior to their storage in frozen lysates (Table 1, Fig. 2). Either there was no decrease in the number of infective centers (pfu/ml) with frozen and melted infected cells, as compared to freshly infected cells, or the observed decrease did not exceed one log, unless the strain which was used as a host for the infecting phage was not hypersensitive to freeze–thawing (Table 1; C600 infected with Mucts62, see below). In no case the decrease in the number of infective centers occurred as a result of prolonged freeze-storage. Clearly, the freezing of freshly infected cells does not influence the integrity of these cells neither does it influence the ability of infecting phages to continue their interrupted development to the extent that would prevent efficient phage recovery.

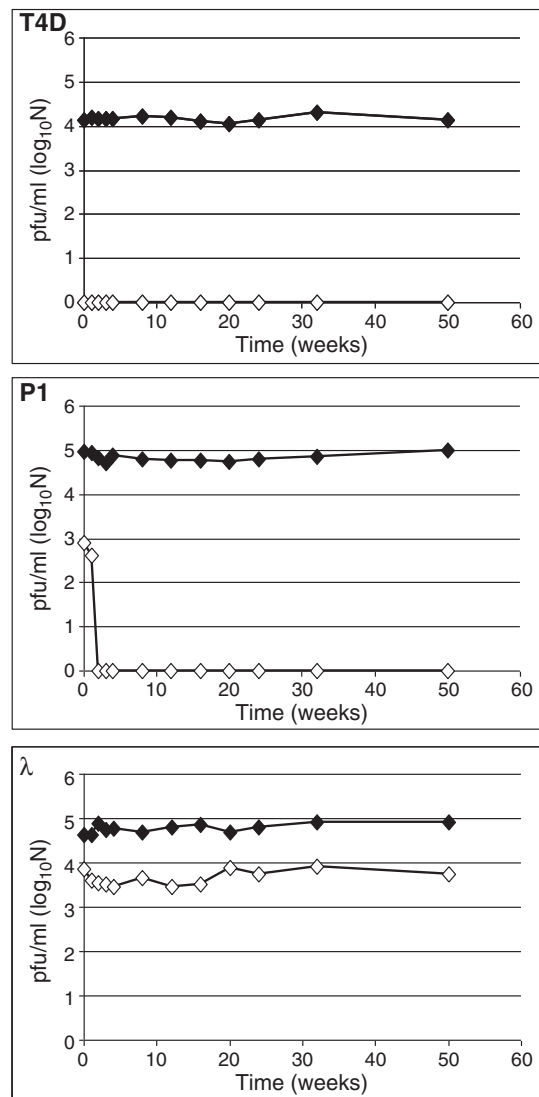
The common *E. coli* laboratory strain C600 appeared to be the only poor host for phage storage. For example, the number of infective centers that could be recovered after freezing and melting of C600 infected with Mucts62 decreased by three logs. One of the numerous mutations in C600, *rfbC*, increases the lethality of cells in response to stress (Berlyn, 1998; Han et al., 2010; Liu and Reeves, 1994; Stevenson et al., 1994). Indeed, we found that the number of colony forming units in cultures of C600 cells alone decreased by one log as a result of freezing and melting with the same method that was used for

**Table 2**  
Adsorption efficiency of selected phages to cells of their hosts<sup>a</sup>.

Phage adsorption time (min)	% adsorbed phages <sup>b</sup>							
	T4D	A5W	P1vir	$\lambda$ papa	phiKDA1	phiAGO1.3	bIBB29	Mucts62
5	92	51	87	91	85	30	72	96
10	95	67	98	95	95	51	90	97
15	95	75	99	96	98	72	94	98

<sup>a</sup> The adsorption of Mucts62 was measured with *E. coli* C600 as a host. Hosts of other phages were as featured in Table 1.

<sup>b</sup> Cells of late exponential or early stationary phase, grown in LB medium at  $37^{\circ}\text{C}$  (*L. lactis* at  $30^{\circ}\text{C}$ ) were infected with a phage at M.O.I. between 0.1 and 0.5 and incubated at room temperature without shaking. At the time indicated samples were withdrawn from each suspension of cells with a phage, and centrifuged for 20 s in a microcentrifuge. Supernatants were quickly filtered through the Filtrapur Syringe Filter, 0.20  $\mu\text{m}$  pore size (Sarstedt) or chloroformed, and used to prepare dilutions in LB medium. The number of p.f.u. in each diluted phage suspension was assayed as described in the text. The fraction of adsorbed phages was calculated by the subtraction of the fraction of unadsorbed phages from the total number of phages that were used for the infection (100%). Each result is the average of at least three independent assays.



**Fig. 2.** The influence of freeze-storage on the survival of phage infected *E. coli* cells and their ability to release mature phages. *E. coli* MG1655 cells of overnight cultures were infected with bacteriophage T4D, P1vir or  $\lambda$ , as indicated, at the multiplicity of infection (M.O.I) between 0.1 and 0.5, left for 5 min at  $37^{\circ}\text{C}$ , harvested by centrifugation, washed three times with the initial volume of cold LB to remove unadsorbed phages, resuspended in 1 ml cold LB containing glycerol (15%), and used to make 50  $\mu\text{l}$  aliquots. The aliquots were immediately frozen at  $-80^{\circ}\text{C}$  and stored frozen. At times indicated, single aliquots were melted in an ice-water bath, and centrifuged in a microcentrifuge for 2 min at  $4^{\circ}\text{C}$ . Supernatants were removed, supplemented with 10  $\mu\text{l}$  of chloroform, vortexed vigorously to remove any viable bacteria, and used to prepare serial dilutions to measure the titer of free phages (white diamonds). The harvested cells were resuspended in the initial volume of fresh, cold LB medium, and used to prepare serial dilutions to measure the number of infective centers (black diamonds), as described in the text.

phage infected cells (data not shown). No similar problem was observed with other phage hosts that were used in this work.

Taken together, our results indicate that the preservation of tailed phages in the form of DNA in freshly infected cells is a more reliable method for phage storage than any method of preservation of mature phages in the form of crude or purified lysate. This method is especially useful in the case of new phage isolates of unknown structure whose laboratory propagation has not been optimized yet, or in the case of phages of fragile virion structure. Results of our preliminary, short term studies with MS2 and Q $\beta$  show that ssRNA phages can also be safely stored in this way. Routinely, we recover the infected bacteria by scraping the top of their frozen stock with a sterile

toothpick or a pipette tip, and mix them with phage-sensitive bacteria to propagate the phage.

Cryopreservation of bacteria in appropriate growth media supplemented with glycerol or other cryoprotectant is a standard storage method in the vast majority of world strain collections (De Paoli, 2005; Smith and Ryan, 2008). All these collections could be easily enriched with phage deposits simply by the addition of frozen stocks of phage-infected cells and the corresponding non-infected cells.

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