NOTES

Cold Lability of *Bacillus cereus* Bacteriophage CP-51

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Phage CP-51 was rapidly inactivated when stored at the usual refrigerator temperatures (2 to 4 C) and even more rapidly when exposed to 0 C. The loss in viability resulting from exposure to cold appeared to correlate with the increase in number of phage particles having contracted tails. High concentrations (0.01 M) of Mg^{2+} , Ca^{2+} , or Mn^{2+} stabilized the phage considerably, but even in the presence of these divalent cations, it was much less stable at 0 C than at 15 C.

Bacteriophage CP-51 is a transducing phage for certain strains of *Bacillus cereus* and *Bacillus anthracis* (3-5). It is apparently unusual among phages thus far reported in that it is much more stable at room temperature than at refrigerator temperatures, i.e., 2 to 4 C. Preparations lost up to 90% of their initial numbers of PFU in 24 h when stored at 2 to 4 C (3). This rapid loss of viability made the phage a very unsatisfactory one to use in transduction studies. However, the unexpected finding that the phage was stabilized considerably by storage at room temperature increased its usefulness as a transducing phage. Because cold lability appeared to be unique to CP-51, studies have been carried out to examine the phenomenon of cold-induced inactivation. The results of some of these experiments are reported here.

Phage CP-51 was propagated on *B. cereus* NRRL 569 in nutrient broth-yeast extract medium (NBY) as described previously (3). Procedures used in harvesting the phage, filtering to remove cells, and assaying with spores of 569 as the indicator were the same as those described (3), except that the phage assay plates were incubated at 30 C rather than 37 C.

For electron microscope examination phage samples were used directly in NBY broth. For negative staining samples were either mixed directly with stain or placed on a carbon-stabilized grid, dried to a thin film, and then stained. Samples were stained with 1% phosphotungstic acid or 1% ammonium molybdate, pH 7.0. The stained grids were examined within 24 h of their preparation, and in most cases were examined immediately. Samples were examined in a Philips EM-200 electron microscope at 60 kV as described previously (2).

From results of experiments designed to test the effects of various conditions of storage upon retention of phage viability, it became apparent that the phage was much more stable at room temperature than at 2 to 4 C. Of various temperatures tested, 15 C appeared optimal for stability. The rate of decrease in the number of PFU when phage was held at 0 C was very rapid, and this temperature was chosen for testing the effects of various factors on reaction of the phage to cold. The inactivation of PFU when lysates were held in an ice bath at 0 C is shown in Fig. 1. A freshly prepared lysate retained only 14% of its original PFU after 7 h at 0 C and only 4% after 24 h. Diluting the lysate 1:10 in NBY broth stabilized the phage considerably, 36% being retained after 7 h and 19% after 24 h. Comparable samples that were stored at 15 C lost no detectable activity in the 24-h period represented in the figure. Lysates that were diluted 10-fold in NBY broth (10^{10} or more PFU per ml) retained full activity for 2 weeks or longer at 15 C.

The greater stability at 0 C, as well as at 15 C, afforded by diluting 1:10 in NBY broth could be accounted for by the change in pH. The pH of lysates prepared from growth of phage in NBY soft agar was 7.9 to 8.1. A dilution of 1:10 in NBY broth at pH 6.8 resulted in a final pH near 7.0. In experiments not detailed here the optimal pH for stability of the phage at 15 C was in the range of 6.6 to 7.0 and, as will be shown below, the phage was inactivated rapidly at 0 C at pH values above 7.0. In general, phage suspensions having 10^{10} or more PFU per ml were much more stable under the conditions tested than less concentrated suspensions.

FIG. 1. Inactivation of CP-51 in NBY broth at 0 C. Phage suspensions were held at 0 C and samples were removed at the indicated times and assayed for PFU. The undiluted lysate had an initial titer of 2.8×10^{11} PFU/ml. The 1:10 dilution was made in NBY broth. Control samples held at 15 C lost no detectable activity during the 24-h period.

Table 1 shows the effect of pH on inactivation of phage at 0 C. In those tests in which the phage was suspended in 0.067 M phosphate buffer, the optimal pH of those tested for stability at 0 C was 5.6. At that pH 89% of the initial PFU retained viability after 30 min; however, at pH 7.0 only 1% of the original PFU remained viable, and the loss was even greater at higher pH values. It should be emphasized that the loss in viability in these experiments occurred in response to the low temperature; samples of the same suspensions, with the exception of the one at pH 5.0, retained full activity when held at 15 C. In this and other experiments some loss in viability occurred rapidly at 15 C when the pH of the samples was below 5.6. It seems possible that the phage is unstable at low pH in the buffer systems used as a result of some mechanism other than that involved in cold inactivation.

Table 1 also shows the effect of pH on inactivation of phage with heat. At 45 C there was a rapid loss in viability at the low pH values, i.e., 5.0 and 5.6. At this temperature the

phage was most stable at or near neutral pH values. Thus, the pH optima for stability at 0 C and 45 C were significantly different, 5.6 and near 7.0, respectively.

Electron microscope examination of CP-51 revealed that the phage particle had a head presenting a hexagonal profile and a contractile tail (Fig. 2a). Examination of phage preparations that had been chilled revealed that many of the tails were contracted (Fig. 3a and b). Preparations that were stored at 15 C showed a large majority of extended rather than contracted tails (Fig. 2b) although the proportion of contracted tails increased with time through several weeks of storage. Tests with the *B. subtilis* transducing phage, SP-10, which also has a contractile tail, showed that exposure to 0 C did not result in contraction of tails or a loss in viability.

Biswal et al. (1) reported that the *B. subtilis* phage, SP-50, lost viability when stored in the absence of Mg^{2+} and that the loss in viability correlated with contraction of tails. Although those authors did not report any effect of temperature on tail contraction, their results suggested to us that Mg^{2+} might affect the stability of CP-51. Table 2 shows the results of testing various concentrations of $MgSO_4$ in a Tris-maleate buffer at pH 6.7. Concentrations between 0.005 M and 0.1 M were effective in increasing the stability of the phage at 0 C. The optimal concentration tested was 0.05 M; in other tests the optimal concentration ranged from 0.01 M to 0.05 M, depending on the pH and constitution of the suspending medium. Electron micrographs revealed that 0.01 M Mg^{2+} was very effective in maintaining tails in the extended condition when the phage was chilled.

TABLE 1. Inactivation of phage CP-51 at 0, 15, and 45 C in phosphate buffer at various pH values^a

pH	Viable phage (% of initial PFU) after		
	30 min at 0 C	30 min at 15 C	15 min at 45 C
5.0	72	67	0.5
5.6	89	95	9
6.0	61	100	53
6.6	7	105	77
7.0	1	100	77
7.6	<0.05	105	56
8.0	<0.05	105	13

^a A 0.1-ml sample of phage suspension containing 1.8×10^{10} PFU per ml was added to 10 ml of 0.067 M phosphate buffer at each of the pH values given, and 2-ml portions were held at the indicated temperatures.

FIG. 2. Electron micrographs of CP-51. (a) Fresh lysate and (b) phage after 144 h at 15 C. Bar = 0.5 μ m.

FIG. 3. *Electron micrographs of CP-51 after chilling at 0°C for (a) 25 h and (b) 144 h. Bar = 0.5 μ m.*

TABLE 2. Effect of Mg^{2+} on stability of CP-51^a

Suspending medium	pH	MgSO ₄ added (molarity)	Viable phage after one h at 0 C (% of initial PFU)
Tris-maleate buffer	6.7	0	7
Tris-maleate buffer	6.7	0.005	18
Tris-maleate buffer	6.7	0.01	20
Tris-maleate buffer	6.7	0.05	44
Tris-maleate buffer	6.7	0.10	36
NBY broth	5.4	0	71
NBY broth	6.0	0	57
NBY broth	6.8	0	40
NBY broth	5.4	0.01	71
NBY broth	6.0	0.01	86
NBY broth	6.8	0.01	100

^a The Tris-maleate buffer contained 0.05 M Tris and 0.05 M maleic acid adjusted to pH 6.7 with 10 N NaOH. NBY broth with or without MgSO₄ was adjusted to the given pH values by the addition of 6 N HCl. A 0.1-ml sample of phage suspension was added to 10 ml of broth or buffer to give approximately 10⁸ PFU per ml, and 2-ml portions were held in an ice-water bath for 1 h. No loss in viability was detected in control samples held at 15 C for 3 h.

An interesting relationship between the presence or absence of Mg^{2+} and the optimal pH for phage stability at 0 C is also shown in Table 2. For those tests the phage was suspended in NBY broth adjusted to the desired pH with HCl. When Mg^{2+} was omitted from the NBY broth, stability at 0 C increased with decreasing pH. Inclusion of 0.01 M Mg^{2+} in the NBY broth resulted in increased stability with increasing pH. The time period of exposure to 0 C in this experiment was 1 h. At pH 6.8 and with 0.01 M Mg^{2+} there was no detectable loss in viability; however, at pH 6.8 in the absence of Mg^{2+} there

was a loss of approximately 60% of the original number of PFU.

Although data are not shown here, 0.01 M Ca^{2+} or Mn^{2+} was also effective in increasing stability to cold. Negative results with 0.01 M NaCl and $(NH_4)_2SO_4$ indicate that the effect was not merely one of salt concentration. Mixtures of the three cations, Ca^{2+} , Mg^{2+} , and Mn^{2+} , were not tested for additive effects.

The experiments reported above on lability of CP-51 at low temperatures were done with unpurified phage preparations. Routinely phage yields ranging from 10¹¹ to 3 × 10¹¹ PFU per ml were obtained and for most of the stability tests lysates were diluted about 1,000-fold to give approximately 10⁸ PFU per ml. Thus any extraneous materials in the lysates were also diluted 1,000-fold and it seemed unlikely that they might be affecting the results. To test this, however, some phage was purified by two successive bandings in sucrose gradients. The purified phage could not be distinguished from unpurified preparations with respect to instability at low temperatures.

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