Bot. Bull. Academia Sinica XII: 57-65 (1971)

STABILITY OF PHAGE Xp12[1]

TEH-YUAN CHOW, YING-TZU LIN
and TSONG-TEH KUO[2]

Abstract

In distilled water at pH below 6.0, 25 mM phosphate buffer at pH 7.0, or 50 mM tris buffer at pH 7.0 inactivated the phage Xp12, high hydogen-ion or the higher molarity of buffer solution caused more inactivation. The Xp12 was also unstable in monovalent cations and organic acids. When Xp12 was suspended in 15 mM of these ions, the phage lost its titer in 15 minutes at room temperature. Whereas this phage was stable in divalent cations. It was well known that divalent cations have protective effect to the bacteriophages. In the case of Xp12, only Ca ion was demonstrated. Inactivation could be prevented by $CaCl_2$, Mg or Mn ions failured to demonstrate such kind of effect. The inactivation was not reversible, since after inactivation the phage solution was diluted with nutrient medium or added Ca ion, no active phage particles were recovered.

The structure of inactivative products was analyzed with CsCl density gradient centrifugation. It was found that the intact phage particles were destroyed by these treatments.

Introduction

Phage Xp12, isolated from *Xanthomonas oryzae*, has an elliptical hexagonal head of 76×55 mμ and a tail of 6×133 mμ. It possesses an unusual base, 5-methylcytosine, which completely replaces cytosine in its deoxyribonucleic acid (Kuo *et al.* 1968a, Kuo *et al.* 1968b). During the studies on the purification of Xp12 it was found that Xp12 was very unstable in various ionic environments. When partially purified phage particles were diluted with water or phosphate buffer, or when phage particles were further purified with CsCl density gradient centrifugation, the phage titer was observed to decrease rapidly. In order to study the structure of Xp12 phage particles with electron-microscope, the phage has been stained with 1% phosphotungstic acid, although this stain

(1) This work was supported by Biology Research Center, Academia Sinica and JCRR. Paper No. 112 of the Scintific Journal Series Inst. Bot., Academia Sinica.

(2) Assistant Research Fellow, Research Assistant and Research Fellow, respectively.

method was generally used in other bacteriophages, it caused the destruction of Xp12 phage structure. These unusual properties made the difficulty of the study of this phage. However, these difficuties could be improved by addition of a protective agent. In this investigation the conditions cause the inactivation of phage particles and a protective agent stabilizing Xp12 phage particles are reported.

Materials and Methods

Xanthomonas oryzae strain 507 and bacteriophage Xp12 were used in all the experiments. For the comparison, phage Xf and Xp10 were also used. The medium used contained, 2 g KH_2PO_4, 3 g $(NH_4)_2HPO_4$, 1 mg $MnCl_2$. 4 H_2O, 1 mg $FeCl_3$, 0.2 g $MgSO_4$, 1.21 g tris-HCl, 1 g glutamic acid, 50 mg cysteine and 15 g sucrose. The medium was finally adjusted to pH 6.8 with HCl.

The phages were multiplied in bacteria which were grown in early log phase at 28°C. Bacteria were inoculated at a multiplicity of 2 and aerated continuously for 16 hours. When lysis became evident, unlysed cells were removed by centrifugation at 8,000×G for 20 minutes. The phage particles were precipitated by centrifugation at 50,000×G for 60 minutes. The resulting pellets were resuspended in 10 mM tris buffer at pH 8.0.

For the experiments of inactivation test, a fresh dilution was prepared form single lysate containing 1×10^{12} pfu per ml, which was stored in the refrigerator. The activity of the stock solution was checked frequently and found to be constant throughout the period of study.

The CsCl density gradient columns were prepared from 4 ml each of buffer solution containing 3.2 g of CsCl in a 5 ml plastic centrifuge tube, 0.2 ml of the phage preparation from differential centrifugation was added and mixed. The tubes were then centrifuged for 24 hours at 40,000 rpm in the SW 50 L rotor of the Beckman model L ultracentrifuge. After centrifugation, 2-drop fractions were collected, and each fraction was diluted with 1 ml of 10 mM tris buffer and measured their absorbancy at 260 mμ and also assayed for phage titers. All plaque counts were derived from platings in PS agar (Kuo *et al.* 1968b) made by the standard technique (Adams 1959).

Results

Stability of Xp12 in water and buffer solutions: Phage Xp12 stocks at 1×10^{12} pfu per ml were diluted to 1×10^{7} pfu per ml with distilled water in different hydrogen-ion concentrations which were adjusted with HCl, or sodium phosphate buffer in different molarities. These suspensions were incubated at 28°C for 30 min, then phage titers were assayed with standard technique. The results are shown in Table 1, phage Xp12 particles were very sensitive

Table 1. *Inactivation of Xp12 by distilled water and phosphate buffer.*

Medium used to dilute and equilibrate phage at 28°C		Time			
		0 min.	30 min.	24 hr	48 hr
H_2O	pH 5.0	1×10^7	4×10^2	—	—
	pH 6.0	1×10^7	1.5×10^6	3.1×10^3	1×10^1
	pH 8.0	1×10^7	1×10^7	1×10^7	1×10^7
phosphate buffer pH 7.0					
	0.005 M	1×10^7	1×10^7	1×10^7	1×10^7
	0.01 M	1×10^7	3×10^6	1×10^6	6×10^5
	0.025 M	1×10^7	1×10^2	1×10^2	1×10^2
	0.05 M	1×10^7	4×10^1	—	—
phosphate buffer 0.05 M+Mg SO_4 1 mM		1×10^7	1×10^7	4×10^5	1×10^4
phosphate buffer 0.05 M+$CaCl_2$ 0.3 mM		1×10^7	1×10^7	1×10^7	1×10^7

to various ionic environments, even in change of hydrogen-ion concentration in distilled water caused the change of phage titer. In distilled water phage Xp12 particles were stable at pH 8.0 and unstable at pH below 5.0. In phosphate buffer at pH 7.0 Xp12 particles were stable at concentration of 5 mM, but inactivated when the buffer solution was incresed to 10 mM.

In order to clarified the relationship between inactivation of phage and buffer at different molarities and hydrogen-ion concentrations, the above experiments were repeated with tris buffer. Tris buffer in different molarities of 10 mM, 50 mM and 100 mM were adjusted to pH 7.0, 7.5, 8.0, 8.5 and 9.0 respectively. Then phage stock was diluted to 1×10^9 with these solutions.

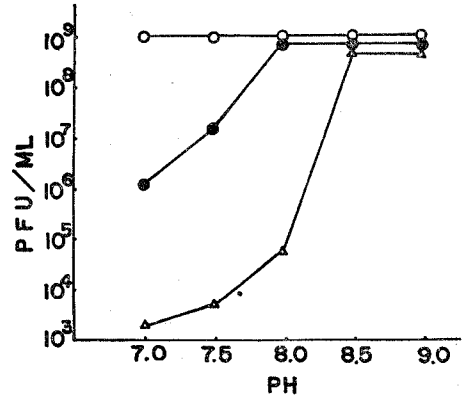

Fig. 1. Effect of pH and molarity of tris buffer on the inactivation of Xp12. Xp12 was diluted in the solutions with different pH and molarity of tris-HCl buffer. ○—○—○ 0.01 M tris-HCl, ●—●—● 0.05 M tris-HCl, and △—△—△ 0.1 M tris-HCl.

The results are shown in Figure 1, the phage particles were more stable at higher pH than at lower pH's. Superimposed on the pH effect is an ionic strength, the phage particles are always stable in the lower concentrated buffers. The phage Xp12 particles can be kept in 10 mM tris buffer at pH 8.0 at room temperature for at least 41 days.

Inactivation of Xp12 in cations and organic acid solutious.

Previous report (Kuo *et al.*, 1971) demonstrated that Xp12 were unstable in sodium citrate and monovalent cations, therefore, the effect of other organic acids and cations were studied. The phage stock at 1×10^{12} pfu per ml were diluted to 1.6×10^{8} pfu per ml in 150 mM of various monovalent and divalent cation solutions and 15 mM organic acid solutions. The organic acid solutions were adjusted with NaOH to pH 7.0 before use. These suspensions were incubated at room temperature for 15 minutes then phage titers were assayed. The results are shown in Table 2, Xp12 particles were rapidly killed in organic acids and monovalent ion solutions, but stable in the divalent cations even when the concentration of divalent cations were increased to 1.0 M. In order to check the stability of other bacteriophages Xp10 and Xf of *X. oryzae* were

Table *2. Inactivation of Xp12 by cations and organic acids.*

Cations and organic acids	0 min. (pfu/ml)	15 min. (pfu/ml)
NaCl 0.15 M	1.6×10^{8}	2×10^{5}
$NaNO_3$ 0.15 M	1.6×10^{8}	2×10^{5}
KCl 0.15 M	1.6×10^{8}	1.2×10^{8}
KNO_3 0.15 M	1.6×10^{8}	1.6×10^{8}
$CaCl_2$ 0.15 M	1.6×10^{8}	1.5×10^{8}
$MgCl_2$ 0.15 M	1.6×10^{8}	1.6×10^{8}
$MgSO_4$ 0.15 M	1.6×10^{8}	1.4×10^{8}
Ca $(NO_3)_2$ 0.15 M	1.6×10^{8}	1.5×10^{8}
$Ca(NO_3)_2$ 1 M	1.6×10^{8}	1.6×10^{8}
$MgCl_2$ 1 M	1.6×10^{8}	1.6×10^{8}
$CaCl_2$ 1 M	1.6×10^{8}	1.6×10^{8}
$MgSO_4$ 1 M	1.6×10^{8}	1.6×10^{8}
Na-acetate 0.15 M	1.6×10^{8}	2.2×10^{3}
Na-acetate 0.015 M	1.6×10^{8}	1.2×10^{8}
Na_3-citrate 0.015 M	1.6×10^{8}	2×10^{4}
Succinic acid 0.015 M	1.6×10^{8}	6.2×10^{4}
Malonic acid 0.015 M	1.6×10^{8}	3.4×10^{2}
Maleic acid 0.015 M	1.6×10^{8}	7.1×10^{2}
Citric acid 0.015 M	1.6×10^{8}	6.7×10^{2}
Na_3-citrate 0.015 M+$CaCl_2$ 1 mM	1.6×10^{8}	1.4×10^{8}

tested as well, they were just stable through the treatment.

Protective action of Ca and Mg ions.

Since Xp12 particles were stable in Ca and Mg ion solutions, the pretect effect of both ions were studied. Mg or Ca ions in concentration of 1 mM were added respectively to the solutions unstabilizing Xp12 particles. The results are shown in Table 1 and Table 2. In the present of Ca ion, Xp12 particles were protected, however, Mg ion did not have the same kind of effect. The inactivation of Xp12 particles in various ionic environments apparently is a permanent nature, since after the inactivation occurred, no active phage particles were recovered either by the addition of Ca ion to the inacivated phage suspension or by the dilution of inactivated phage with distilled water at PH 8.0.

In general, divalent cations were believed to have protective effect on the phage particles, however, in this case only Ca ion was effective. This conclusion was further supported by the protection of Xp12 particles from the inactivation by heating. Xp12 particles at 2×10^{12} pfu per ml were diluted to 2×10^{7} pfu per ml with 10 mM tris buffer at pH 8.0, 5 mM or 1 mM of Ca or Mg ion was added respectively and incubated at 60°C. The samples were taken at 5 minutes intervals and assayed for phage titers. As indicated in Figure 2, Xp12 particles in 10 mM tris buffer sulution at pH 8.0 were stable at 20°C, when temperature was increased to 60°C the phage particles were rapidly destroyed in 5 minutes. Addition of Ca ion stabilized the phage par-

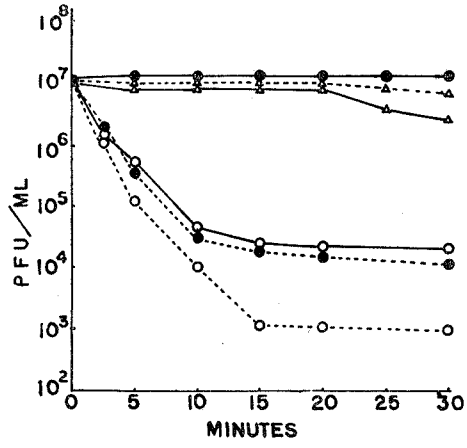

Fig. 2. Effect of divalent cations on the inactivation of Xp12 by heating. Xp 12 was suspended in 0.01 M tris-HCl pH 8.0, and incubated at the following conditions. ●—●—● at 20°C, ●----●----●; at 60°C; △----△----△ added 5 mM $CaCl_2$, incubated at 60°C; △—△—△ added 1 mM $CaCl_2$, incubated at 60°C; ○----○----○ added 5 mM $MgCl_2$, incubated at 60°C: and ○—○—○ added 1 mM $MgCl_2$, incubated at 60°C.

ticles, moreover, increasing the Ca ion concentration increased the protective effect. On the contrary, addition of Mg^{++} did not have any protect effect, but caused phage inactivation. Furthermore increasing the concentration of Mg ion increased the degree of phage inactivation.

Application of the protective agent: Since $CaCl_2$ possesses protective effect to phage particles, the $CaCl_2$ was applied to stabilize the phage particles during the purification of Xp12 or other preparation which inactivated phage particles. Addition of 50 mM $CaCl_2$ to the solutions during purification could prevent the loss of any phage titers.

When Xp12 particles were further purified with CsCl density gradient centrifugation almost all phage particles were destroyed. A comparison was made in CsCl density gradient centrifugation with and without Ca ion. The results are shown in Figure 3, when Ca ion was present a sharp UV absorption peak was obtained at the density $\rho=1.5210$. Phage assay indicated this peak was Xp12. When Ca ion was omitted UV absorption was greatly decreased and phage titers was almost negligible, and the UV absorption and phage assay peaks did not coincide to each other, maximum UV absorption peak shift to

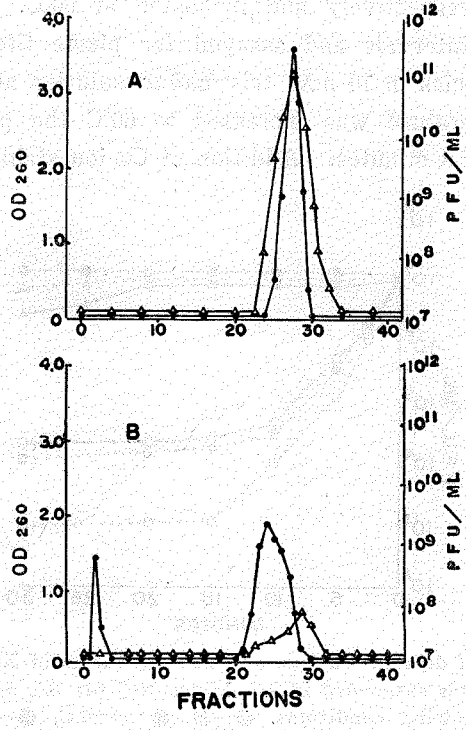

Fig. 3. Comparison of the CsCl density gradient centrifugation pattern of Xp12 in the solution with and without $CaCl_2$. ●—●—● optical density, △—△—△ phage titer. (A) with $CaCl_2$. (B) without $CaCl_2$.

the position at density $\rho=1.5370$, and litter shoulders were observed at both sides of maximum peak. The one which located at the position of density $\rho=1.5210$ should be intact phage particles since maximum phage assay peak was detected at this position. Meanwhile, a new UV absorption peak was observed at bottom, which is supposed to be a DNA peak. Both decrease of phage titer and change of UV absorption spectrum strongly indicated that phage structure was destroyed, and DNA was extruded out of phage particles.

Xp12 phage particles were also unstable in 1% phosphotungstic acid (pH 7.2). When Xp12 phage particles were stained with phosphotungstic acid, usually only ghosts were observed under electron-microscope (Figure 4). Evidently DNA was extruded out from the phage particles. The stain method could be improved by using 2% uranyl acetate (Figure 4) or by addition of 0.5 mM Ca ion to the phosphotungstic acid.

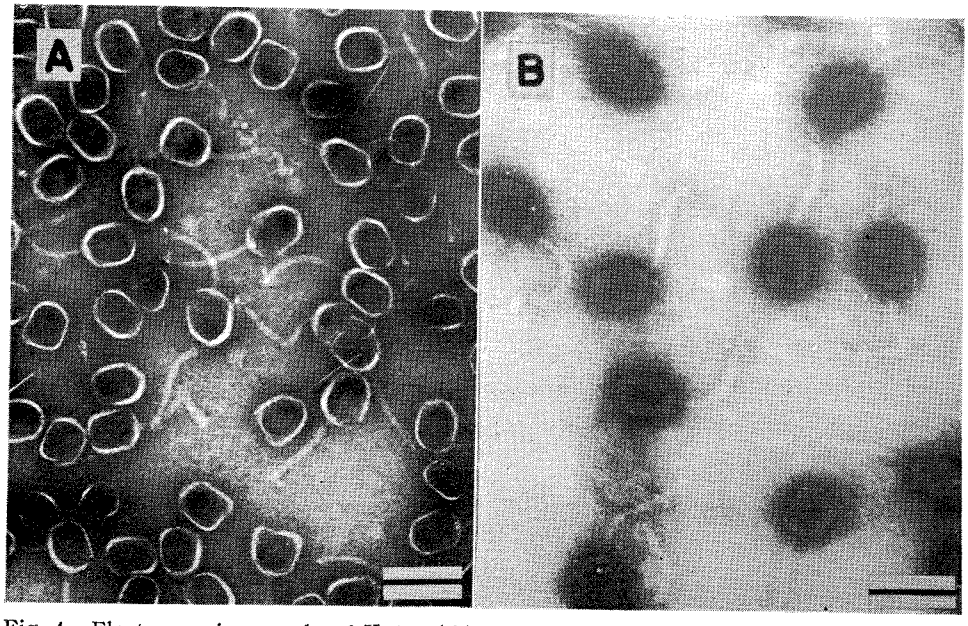

Fig. 4. Electron micrograph of Xp12. (A) stained with 1% phosphotungstic acid, left only empty head. (B) stained with 1% uranyl acetate, intact phage particles. Bars represent 100 mμ.

Discussion

It is surprising that Xp12 tends to decompose at room temperature in distilled water, diluted buffers of virous kinds of ionic enviroment, but is stable in nutrient medium. Same phenomenum was reported with T_1 phage (Puck 1949), it was shown that in distilled water or dilute phosphate buffer, T_1 phage undergoes a transformation to a sensitized form that loses its ability

to form plaques, if it is immediately added to a suspension of host cells in broth. This transformation can be reversed by allowing such sensitized phage to recover in plain broth before the addition of the cells, and the senitization can be prevented by $CaCl_2$. It was concluded that sensitization produces a block in some steps of the virus metabolic cycle occuring after adsorption. Although Xp12 was inactivated in same way but may not draw out same conclusion, since inactivation of Xp12 was not reversible and the destruction of phage particles was observed in inactivated phage preparations.

The destruction of phage particles were observed in T_4 phage of *E. coli* (Kark and Adams 1953). When phage particles in solution with high salt concentration was diluted to low salt concentration, the DNA extruds out from the tail or breakage of phage head occurred. It was caused by the rapid change of osmotic pressure. Because of this reason phage particles are generally preserved in concentrated salt solutions. However, the explaination could not satisfy the case of Xp12, since Xp12 was extremely unstable in solutions with high salt concentration. And increasing salt concentration proportionally increased the degree of phage inactivation. Furthermore Ca ion stabilized the phage particles in concentration as low as 10^{-4} M. These evidences indicated that these agents act in some way rather than osmotic pressure.

The importance of divalent cation in the stabilization of bacteriophages has long been recognized (Lark and Adams 1953). Adams (1949) reported that 10^{-3} M concentrations of the divalent cations Ca, Ba, Sr, Mg, Mn, Co, Ni, Zn, Cd and Cu have a marked stabilizing effect and that sodium ion above 0.1 M also stabilizes the viruses. It is apparent that the ability to stabilize phage T_5 against heat inactivation is a rather general property of metal cations. In the present paper, the protective effect of divalent cations was limitted to only Ca ion. Mg, and Mn did not have any pretective effect on Xp12.

When phage Xp12 was inactivated by various ionic environments, certain physiological and morphological changes occurred either simultaneously or in association with the loss of infectivity. From CsCl density gradient centrifugation pattern (Figure 3) and electron microscopic study (Figure 4) of Xp12 phage indicate phage particles were destroyed by those treatments. Kuo *et al.* (1971) demonstrated that when Xp12 phage particles were exposed to 3 mM sodium citrate, phage was decomposed into DNA, empty heads and tails. These results indicated that the effect of the cations or organic acids on the stability of phage Xp12 may be most simply explained on the assumption that some metal cation is firmly bound to the labile site, which contribute to the structure stability of the site. The breaking of some bonds, linking the metal cation to the site, would decrease the stability of the site.

噬菌體 Xp12 之穩定性

周德源 林英子 郭宗德

噬菌體 Xp12 在水溶液中，其穩定性與溶液中之酸度及溶解質之濃度有密切之關係，在蒸餾水中酸度在 5.0 以下保持 30 分鐘，其活性就完全喪失。在酸度 7.0 之磷酸鹽緩衝液中，當緩衝液濃度高於 25 mM 時，其活性也在 30 分鐘內完全喪失，在同酸度之 tris-HCl 緩衝液，當濃度高於 50 mM時亦能使其不活化。Xp12 在一價金屬離子或有機酸溶液中也不穩定。這些離子在 15 mM 之濃度下，5 分鐘內就使此等噬菌體喪失活性。Xp12 在兩價金屬離子中很穩定。雖然兩價金屬離子對一般噬菌體都有保護之作用，但對 Xp12 祇有鈣離子具有此等作用，而鎂離子則缺如。上述這些 Xp12 之不活化之現象均爲不可逆的。依氯化銫異濃度離心法及電子顯微鏡之觀察得悉此等不活化均由噬菌體之結構破損所致。

Literature Cited

ADAMS, M. H. Bacteriophages, Interscience publishers, Ins. New York, 1959.

ADAMS, M. H., The stability of bacterial viruses in solutions of salts. J. Gen. Physiol. **32**: 579-582, 1945.

KUO, T. T., T. C. HUANG, and M. H. TENG, 5-Methylcytosine replacing cytosine in deoxyribonucleic acid of a phage of *X. oryzae*. J. Mol. Biol. **34**: 373-375, 1968a.

KUO, T. T., T. C. HUANG, R. Y. WU and C. P. CHEN, Phage Xp12 of *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson. Cana. J. Microbiol. **14**: 1139-1142, 1968b.

KUO, T. T., T. Y. CHOW, Y. T. LIN and C. M. YANG, Specific dissociation of phage Xp12 by sodium citrate. J. Gen. Virol. **10**: 199-202, 1971.

LARK, K. G., and M. H. ADAMS, The stability of phages as a function of the ionic environment. Cold Spring Harber Symposia Quant. Biol. **18**: 171-183, 1953.

PUCK, T. T. A reversible transformation of T_1 bacteriophage J. Bacteriol. **57**: 647-655, 1949.