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ROLE OF CALCIUM ION IN PROLIFERATION OF PHAGE Xp12 OF *XANTHOMONAS ORYZAE.*[1][2]

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Abstract

Phage Xp12 of *Xanthomonas oryzae* does not multiply in actively growing host cells in a calcium-deficient medium. Calcium can not be replaced by manganese, magnesium or ferric ion. For normal proliferation of the phage, 2.5×10^{-3} M $Ca(NO_3)_2$ is required. Effects of calcium ions on the rate of host bacterial growth, rate of phage adsorption to host cells, penetration of host cells and some stage of the intracellular multiplication process were studied. It was found that calcium ion is needed in bacteriophage invaison, that is, penetration of host cells after adsorption has occurred.

Introduction

When a chemically defined medium which supports normal growth of *Xanthomonas oryzae* was used for the proliferation of Xp12 phage in our laboratory, it was found that this medium did not support the growth of Xp12. It was only after addition of calcium ion to the medium that normal proliferation of Xp12 phage was obtained.

Calcium has been found necessary in other bacteriophage systems and its action seemed to be varied in different systems. This may be due largely to differences in the processes by which new viral particles were formed. Adams (1949) reported that absence of calcium seemed to block bacteriophage dovelopment at a very early stage. Delbrück (1948) demonstrated an adsorption cofactor role for calcium in the case of a mutant type of coliphage T_4. Rountree (1951) found that calcium was required for adsorption of at least some staphylococcal bacteriophages. Luria and Steiner (1954) demonstrated that calcium is required for the penetration of deoxyribonucleic acid of T_5 phage. Kay (1952) concluded that the calcium requirement of a coliphage was for intracellular multiplication.

This paper reports our work on the effect of calcium ion on Xp12

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proliferation.

Materials and Methods

Xanthomonas oryzae strain 507 and its phage Xp12 were used in all experiments. The basal synthetic medium used contained 2g, KH_2PO_4; 3g, $(NH_4)_2HPO_4$; 0.001g, $MnCl_2 \cdot 4H_20$; 0.2g, $MgCl_2 \cdot 6H_20$; 0.001g, $FeCl_3$; 0.05g, cystine; 1g glutamic acid; 15g, sucrose; 1.21g, tris (hydroxy methyl) aminomethane and one liter of deionized, distilled water. For the growth of Xp12, 0.1g, Ca $(NO_3)_2$ per liter was added. All bacteria and phages were grown at 28°C with aeration. The routine methods in phage experiments employed were described by Adams (1950).

For the separation of unlysed infected cells from free phage in the reaction mixture sodium citrate treatment was applied. Previous report (Kuo *et al.* 1971) demonstrated that phage Xp12 is very sensitive to sodium citrate. When phage concentration at 1×10^8 pfu per ml was treated with 0.003 M sodium citrate, almost all phage were killed in one minute. However, sodium citrate affects neither the growth of bacteria itself nor phage that had already penetrated into host cells. This advantage was extensively applied in these experiments to separate unlysed infected cells from free phage in reaction mixture. After host cells were infected with phage, free phage can be removed by diluting reaction mixture with sodium citrate to the final concentration of 0.003 M. After free phages were killed, the reaction mixture was diluted with soft agar and unlysed infected cells assayed by general method.

Results

Dependance of phage proliferation on calcium ion: The effects of calcium on the proliferation of phage and on the growth of host bacteria were determined. Overnight young culture (6×10^8 cells/ml) in basal synthetic medium was equally divided into 4 tubes and they were treated separately in following ways: (1) no addition (2) addition of $Ca(NO_3)_2$, to 2.5×10^{-3} M (3) infection with phage (3×10^9 pfu/ml) (4) addition of $Ca(NO_3)_2$ to 2.5×10^{-3} M and infected with Xp12. They were then incubated at 28°C with aeration. Samples were withdrawn at 0 time and 30-minute intervals thereafter untill 10 hours. The growth and lysis of bacterial cells were measured with spectrophotometer at 450 mμ. At final point the phage titers were also assayed. The results are shown in Figure 1. When bacterial cells grown in calcium-deficient medium were infected with phage Xp12, no lysis was observed. However, when calcium ion was added to the basal synthetic medium a typical lytic pattern was obtained, although calcium was not required for the growth of host bacteria. The 0 time phage titer in calcium-deficient medium was 3×10^9 pfu per ml, and it remained the

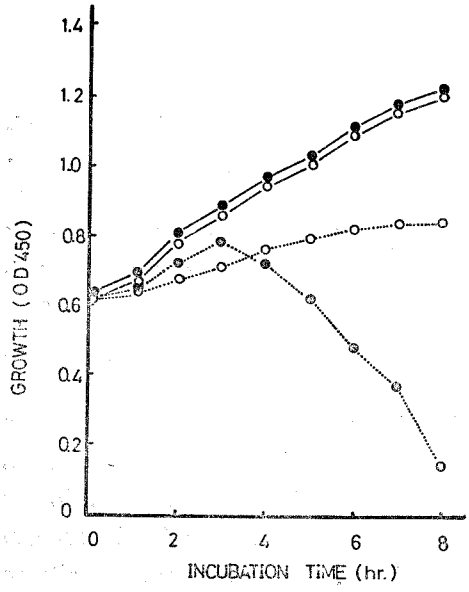

Fig. 1. Effect of calcium on the growth of host cells and lysis of Xp12 infected cells.
○——○ bacterial cells grown in calcium minus medium.
●——● bacterial cells grown in calcium plus medium.
○- - -○ bacterial cells grown in calcium minus medium and infected with Xp12.
●- - -● bacterial tells grown in calcium plus medium and infected with Xp12.

same at the end of the experiment. In the culture supplemented with calcium, the phage titer increased to 2×10^{11} pfu per ml.

There is a possibility that failure to detect increase of phage titer in calcium-deficient medium may be due to inactivation of newly produced phage in a medium of low calcium content, since previous report (Chow *et al.*, 1971) demostrated that Xp12 phage is very unstable in distilled water and addition of calcium ion stabilize the phage particles. It was found that Xp12 was stable in synthetic medium for at least 3 months.

The effect of calcium on proliferation of Xp12 could not be replaced by manganese, magnesium or ferric ion, since these three ions were present in basal synthetic medium

Calcium concentration: The amounts of added $Ca(NO_3)_2$ necessary for optimum proliferation in the calcium-deficient medium were determined. Approximately 2×10^9 pfu per ml phage particles and 4×10^8 cell per ml susceptible bacterial cells were added to a series of tubes containing the calcium-deficient medium plus different amount of $Ca(NO_3)_2$. The tubes were incubated at 28°C for 10 hours and the phage titers of the various tubes determined by plague counting method. The quantitative responses of bacteriophage to various calcium cencentrations are showan in Table 1. There was no phage production, when the medium contained 5×10^{-6} M $Ca(NO_3)_2$. With increasing concentration of $Ca(NO_3)_2$ phage titer gradually increased untill the $Ca(NO_3)_2$ concentration reach 2×10^{-3} M when maximum phage production was obtained. Beyond this $Ca(NO_3)_2$ concentration no further increase in bacteriophage titer was observed.

Table 1. *Effect of calcium concentration on bacteriophage prolifertion*

Concentration of $Ca(NO_3)_2$ (M)	Phage titer (pfu/ml)
2.5×10^{-3}	4.5×10^{11}
2.5×10^{-4}	1×10^{11}
2.5×10^{-5}	5×10^{10}
5×10^{-6}	2×10^{9}
0	2×10^{9}
original titer	3×10^{9}

Effect of calcium on the adsorption of Xp12: Experiments were performed to determine if the effect of calcium could be attributed to the increased rate of adsorption of bacteriophage particles to host bacteria in the presence of calcium. For these studies, bacterial cells grown in the calcium-deficient medium was transfered to two tubes; one was used as calcium miuns treatment and the other contained 2.5×10^{-3} M $Ca(NO_3)_2$. The phage at 1×10^{8} pfu per ml was added to above tubes contained 2×10^{9} cells per ml host bacteria. The content of the tubes were mixed and the tubes incubated at 28°C with aeration. At 0 time and 10 minutes intervals, 2 ml of samples were withdrawn, and immediately cooled down in centrifuge tubes. The tubes were centrifuged for 10 minutes at 5,000×G to sediment the cells and any bacteriophage particles adsorbed to them. Following centrifugation supernatant was removed and phage perticales were titered by the plaque counting method. The results are given in Figure 2. The numbers of phage particles remaining in the supernatants decreased with time but there were no difference in both tubes. The adsorption of Xp12 phage in basal synthetic medium was rather slow, it takes 60 minutes to reach 70% adsorption.

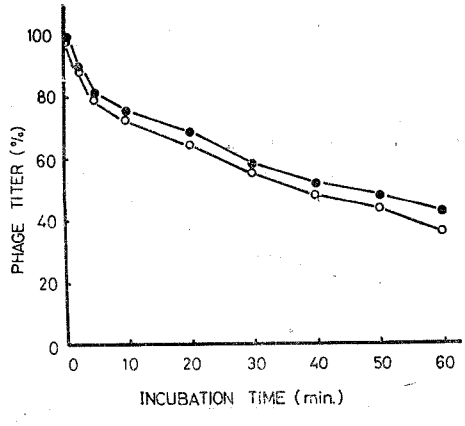

Fig. 2. Effect of calcium on the phage adsorption.
●——● without calcium
○——○ added $Ca(NO_3)_2$ to 2.5×10^{-3} M

Effect of calcium on penetration of phage Xp12: If calcium assists the phage penetration, addition of calcium to cells on which particles of bacteriophage have been adsorbed should permit the particles to invade and multiply unless such adsorption results in inacivation of attacking virus by killing of host cells. The *X. oryzae* cells were infected with Xp12 in synthetic medium and incubated at 28°C for 60 minutes. After adsorption the reaction mixture was divided to two part, one part served as control and the other part was added $Ca(NO_3)_2$ to the final concentration of 2.5×10^{3} M. At 5 minutes intervals, the samples were taken and diluted with sodium citrate. By this treatment free phage can be titered by plaque counting method. The results are given in Fig. 3, the infected cell could be detected in the synthetic medium supplied with calcium and no detectable infected cells was observed in calcium-deficient medium. Compared to the time required for phage adsorption the time for penetration is very short, it takes only 5 minutes.

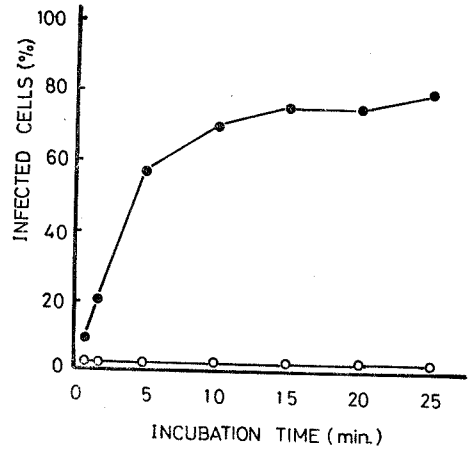

Fig. 3. Effect of calcium on the penetration of phage Xp12.
○—○ without calcium
●—● added $Ca(NO_3)_2$ to 2.5×10^{-3} M

The possibility that intracellular multiplication of Xp12 rather than penetration was affected can not be eliminated. Therefore, the following experiment was conducted. Bacteria were grown in calcium-deficient medium overnight, then transfered to new synthetic medium and incubated at 28°C for one hour. The concentration of bacterial cell was 5×10^{8} cell per ml. $Ca(NO_3)_2$ was added to the final concentration of 2.5×10^{-3} M and heavily infected with phage Xp12 (1.6×10^{10} pfu/ml). The reaction mixture was incubated at 28°C with aeration for 20 minutes. After adsorption and penetration were accomplished the reaction mixture was cooled down. Free phage and calcium ions were removed by washing with synthetic medium by centrifugation twice. Finally the infected bacterial cells were suspended in new medium and then divided into two part, one was supplied with calcium and incubated at 28°C with aeration while the

other one was not. At different time intervals samples were withdrawn and total phage titer were measured after dilution with tris buffer, and unlysed infected cells of the samples were also followed with the help of sodium citrate. The results are shown in Figure 4. The latent period for this treatment required 3 hours, it was slightly longer than that of usual treatment. During latent period total phage titer was similar to the count of unlysed infected cells. No free phage was released. After three hours, host cells started to lyse and free phages released. The total increase of phage title in medium with or without calcium ion were almost identical. Apparently calcium had no effect on intracellular multiplication.

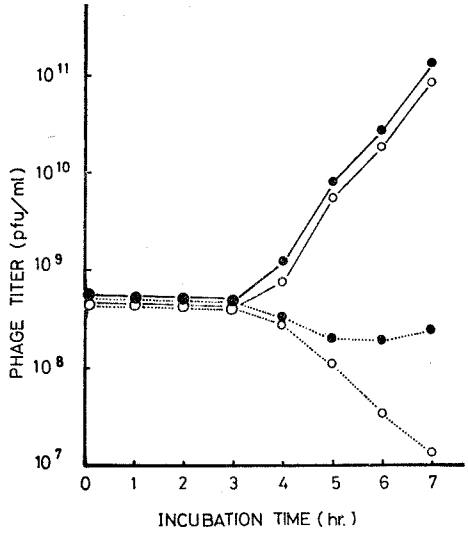

Fig. 4. Effect of calcium on intracellular multiplication and phage liberation of Xp12.
●——● Total phage titer in calcium plus medium.
○——○ Total phage titer in calcium minus medium.
●- - -● Unlysed infected cells in calcium plus medium.
○- - -○ Unlysed infected cells in calcium minus meium.

After 3 hours of incubation the infected cells gradually decreased and leveled off at 5 hours in calcium-supplimented medium. On the contrary the number of unlysed infected cell were sharply decreased in the calcium-deficient medium. This was taken to mean that the washing has been sufficient to removed calcium that second penetration was not possible.

Conclusion

With the adventage of sodium citrate treatment developed in this laboratory, unlysed infected cells can be separated from free phage in reaction mixture. Using this technique it could be clearly demonstrated that the failure of Xp12 phage proliferating in calcium deficient culture was caused by the requirement of calcium in the step of phage penetration into host cells.

Effect of calcium on the phage penetration has been reported with lactic streptococcus bacteriophage and T_5 phage of *Escherichia* coli. Potter and Nelson

(1953) demostrated that calcium is required for the lactic streptococcus phage invasion, and manganese can replace calcium. Luria and Steiner (1954) also dermonstrated that calcium is required for penetration of T_5 phage of *Escherichia coli*. Magnesium can replace calium, and calcium appears to be still needed after the penetration stage, for a full yield of phage in synthetic medium. In the case of Xp12 phage the calcium affects phage penetration only and can not be replaced by magnesium or manganese.

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鈣離子在水稻白葉枯病菌噬菌體 Xp12 繁殖過程中的任務

郭宗德 周德源 林英子

水稻白葉枯病病原菌噬菌體 Xp12 在寄主能生長的合成培養基（缺鈣）中不能繁殖。加鈣則能營正常的生長。本研究曾就鈣之影響在寄主本身及噬菌體感染和繁殖每一步驟做一通盤的檢討。結果發現鈣離子對寄主本身的生長，噬菌體附着到寄主細胞壁，噬菌體在細菌體內的繁殖，和噬菌體溶化寄主細胞都無關。而發現鈣離子的缺乏阻止噬菌體 DNA 的侵入寄主體內，由於此步驟受抑上而影響整個噬菌體之繁殖。最合適之硝酸鈣濃度爲 2.5×10^{-3} M。鎂、錳、鐵等離子不能代替鈣。