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Biochemical Quantitation of PM2 Phage DNA as a Substrate for Endonuclease Assay

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Bacteriophage PM2 has a closed circular form of double stranded DNA as a genome. This DNA from the phage is a useful source for nick-circle endonuclease assay in the fmol range. Due to difficulties in the maintenance of viral infectivity, storage conditions of the phage should be considered for the purification of PM2 DNA. The proper condition for a short-term storage of less than 2 months is to keep the PM2 phage at 4°C; whereas the proper condition for a long-term storage of the PM2 phage for over 2 months is to keep it under liquid nitrogen in 7.5% glycerol. The optimal conditions for a high yield of phage progeny were also considered with the goal to achieve a successful PM2 DNA preparation. A MOI(Multiplicity Of Infection) of 0.03, in which the OD_{600} of the host bacteria was between 0.3 and 0.5, turned out to be optimal for the mass production of PM2 phage with a burst size of about 214. Considerations of PM2 genome size, and the concentrations and radiospecific activities of purified PM2 DNA, are required to measure the endonuclease activity in the fmol range. This study reports the proper quantitation of radioactivity and the yield of purified DNA based on these conditions.

Key words: DNA repair, endonuclease, nick circle assay, PM2 phage

The nick circle assay for endonuclease activity is a very accurate method to measure the nick in a femto (10^{-15}) mole range (Jung *et al.*, 2003). The preparation of the substrate DNA with a high quality is important in order to establish an accurate calculation of the activity. PM2 phage DNA is frequently used as a substrate for the nick circle endonuclease assay based on alkali denaturation because it is unique in that PM2 DNA does not contain RNA primers (Livneh *et al.*, 1979a; Livneh *et al.*, 1979b; Kim and Linn, 1989; Kim *et al.*, 1995; Gille *et al.*, 1996; Jung *et al.*, 2003). The total radioactivity of 1 nmol of PM2 DNA can be measured by either spotting DNA or by irreversible denaturation of DNA with a high alkaline buffer (pH 13.2).

Success in an endonuclease assay depends mainly on the preparation to produce PM2 DNA of high quality which requires the proper maintenance of phage PM2 and the determination of adequate MOI for the best burst size. To date, however, proper conditions have not been established for this purpose. A full understanding of phage PM2 with the interaction of its host cells is, therefore, of utmost importance.

Phage PM2 is an icosahedral bacteriophage and is classified as Corticoviridae. Its genome is composed of double stranded circular DNA that does not have a RNA primer. Phage PM2 was first reported in the late 60's (Espejo and Canelo, 1968). It is the first isolated phage to contain lipids (Hinnen *et al.*, 1976; Brewer, 1979). Two species of the cells, *Pseudoalteromonas espejiana* BAL-31 and *P. sp.* ER72M2, are known to host the phage. The host cell used in this study was first named *Pseudomonas* BAL-31 (Chan *et al.*, 1978) and later reassigned as *Pseudoalteromonas espejiana* BAL-31 (Gauthier *et al.*, 1995).

The general method for the isolation of phage DNA is to use ultracentrifugation for precipitating out phage particles. Because this method is advantageous to obtain highly purified DNA, it was used to purify PM2 DNA (Jung *et al.*, 2003). The whole DNA sequence of the PM2 genome was reported with the size of 10,079 bp (Mannisto *et al.*, 1999).

For the quality preparation of a large quantity of substrate DNA, various storage conditions of phage PM2 were tested at different temperatures with or without the addition of glycerol. In addition, adequate MOI and burst size were determined for this purpose. In this report, biochemical quantitations of the yield of phage and the radiospecific activity of the labeled substrate were introduced for the nick-circle endonuclease assay in the fmol range.

Materials and Methods

Strains and maintenance

PM2 phage and its host strain, *Pseudoalteromonas espe-*

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jiana (BAL 31 and BAL 31-14), were used as described previously (Jung *et al.*, 2003). For labeling PM2 DNA with ^{3}H-thymidine, a thymidine auxotroph (BAL 31-14) strain was used, and the conditions for growth and maintenance of the host strain were considered according to Jung *et al.*, (2003).

Storage condition

To find out the proper storage condition of PM2 phage, temperatures such as 4°C, -70°C and under liquid nitrogen were tested. In addition to the temperatures, the effect of glycerol (final 7.5%) on storage was also evaluated. Plaque forming units (pfu) of PM2 stored in these conditions were determined by counting plaques after storage for 1 (short-term) and over 2 months (long-term). The starting pfu value for the determination of the storage condition was 5.6×10^9 pfu/ml.

Culture conditions of host cells for interaction with phage

Preparation of the culture medium for the host cells was as follows: 10 ml of 20% glucose, 6 ml of 0.1 M KH_2PO_4 (pH 7.5), 100 ml of 10% casamino acid, 34 ml of 0.1 M $CaCl_2$, and 200 µl of 5% filtered $FeSO_4\cdot7H_2O$ were autoclaved separately, except 5% $FeSO_4\cdot7H_2O$, and mixed with the previously autoclaved and cooled mixture of 0.625 g of KCl, 1 g of NH_4Cl, 10.8 g of $MgSO_4\cdot7H_2O$, 23.2 g of NaCl, 10.8 g of Tris, and 5.4 ml of concentrated HCl in 850 ml of distilled water. Ten mg/ml of thymidine and 4 mg/ml of thiamine were also added to a 1/1000 volume of the complete medium. A single colony of the thymidine auxotroph (BAL 31-14) from the master plate was seeded in 10 ml of the complete culture medium. After an overnight incubation at 30°C, the culture was transferred to 1.5 liter of fresh culture medium and continued to grow.

Titration of phage (plaque forming assay)

Phage extract of the infected culture was centrifugated at 18,000×g for 20 min. The clear supernatant was serially diluted in a phage dilution buffer (2 M of NaCl, 0.01 M of $CaCl_2$, 0.02 M of Tris-Cl, at pH 8.2). Fifty µl of phage in the dilution buffer was added into a 15 ml conical tube containing 450 µl of the culture of host cells. After incubation at room temperature for 10 min, the phage-host mixture was added into 5 ml of top agar [0.7 g of KCl, 1.13 g of $CaCl_2$, 12 g of $MgSO_4\cdot7H_2O$, 26 g of NaCl, 12.1 g of Tris (pH 7.6), 7 g of agar and 1 *l* of distilled water], and maintained at 45°C. Then this agar mixture was vortexed, and the final mixture was overlaid on a growth-agar plate. After the top agar mixture had solidified at room temperature for 2 h, the plate was incubated at 30°C for 24 h, and the plaques were counted.

Determination of optimal MOI and its corresponding burst size

When OD_{600} of BAL 31-14 reached to 0.4~0.5, the original phage solution (5.6×10^9 pfu/ml) at different dilutions was allowed to infect host cells. The pfu concentration from the original phage solution and the number of BAL 31-14 cells (1.06×10^8 cells/ml when its OD_{600} is 0.375) were considered for the determination of MOI. With various MOIs, the mixture of PM2 and BAL 31-14 cells was allowed to interact at 30°C for 16 h. This was then centrifugated at 18,000×g for 20 min, and the pfu from the supernatant was determined by the plaque forming assay. Burst size for the 16 h interaction was calculated as follows. Burst size in this experiment = the pfu concentration of the supernatant after a 16 h interaction/the pfu concentration of the first infecting phage solution.

Purification of PM2 DNA by ultracentrifugation

After a 16 h interaction, the phage-host mixture was chilled on ice for 10 min. Then it was centrifugated at 9,100×g, at 4°C for 15 min. A bacterial orange pellet was discarded and the supernatant was taken for the use. The phage was pelleted by ultracentrifugation of this supernatant at 50,000×g using a Beckman 45 Ti rotor, at 4°C for 3 h. Each pellet was resuspended in 5 ml of the phage suspension buffer (1.0 M of NaCl, 0.02 M of Tris-Cl, at pH 8.1). Because this pellet was very difficult to resuspend, care needed to be taken to make sure that all of the materials were in the suspension. The phage suspension was centrifugated at 12,000×g, at 4°C for 20 min to remove the remaining host cell debris. This step was repeated until the orange color in the phage pellet disappeared in order to exclude the bacterial cell debris from the phage solution. This was also done in order to obtain a clear phage solution. After the clear phage pellet was taken, it was resuspended in 4 ml of the phage suspension buffer. Cesium chloride (CsCl) was added to the phage solution in accordance with the formula described below. [weight of adding CsCl = 0.3588×weight of phage solution]. After adding CsCl, the CsCl-phage solution was ultracentrifugated at 140,000×g by a Beckman NVT100 rotor, at 25°C for 24 h. The white band was drawn off from the gradient by a syringe and dialyzed in 1 liter of BE buffer (0.1 M of NaCl, 20 mM of Tris, pH 7.5, 1 mM EDTA) at 4°C for 3 h. After dialysis, 10% of the sarkosyl solution was added to the dialyzed phage suspension to 0.5% as the final concentration and incubated at room temperature for 5 min. The solution became clear immediately when the sarkosyl was mixed in because the lipids wrapping the phage particles were broken in this step. To remove the remaining protein and clear the phage solution, the phage solution was subjected to a phenol extraction. The aqueous phase was collected, and 5 M of NaCl was added to 0.5 M of the final concentration. The final solution was sequentially dialyzed at 4°C in dialysis buffer #1 (1 M of NaCl, 0.01 M of Tris, 1 mM of EDTA, pH 7.5), #2 (0.01 M Tris, 0.2 mM of EDTA, pH 7.5), #3 (0.01 M of Tris, pH 7.5) and #4 (0.01 M of Tris, 2% glycerol, pH 7.5) for 12 h each. The final solution containing PM2 phage DNA was stored in 50 µl aliquots at -70°C.

Results and Discussion

Storage conditions of PM2 phage (Table 1)

To obtain a large quantity of double stranded DNA of the PM2 phage in a limited time, it is very important that the viruses are maintained in a highly active state as well as the host cells. It is necessary to find an optimal condition for the storage of a virus, in which the storage temperature and glycerol concentration of the stock solution have an effect on the infectivity of the virus stock. In this study, we investigated the storage condition of PM2 phage by changing the glycerol concentration and storage temperature using the plaque forming assay as mentioned previously. Table 1 shows the results of the plaque forming assay under various storage conditions. The most effective storage condition is to keep the phages in 4°C. In this case, the pfu concentration of the phage stock did not change throughout the period of 1 month (Table 1). Other concentrations decreased significantly when compared to the concentration at 4°C. The addition of a 7.5% glycerol solution to 4°C storage had a more adverse effect in both the short- and long-term storage, whereas the addition of glycerol to -70°C storage had a better effect on the storage than in the storage without glycerol. This effect of glycerol was most evident when the storage was frozen with liquid nitrogen. This shows that the pfu concentration was reduced dramatically when the solution was in the process of freezing. However, once the pfu concentration of the storage was frozen under -70°C or with liquid nitrogen, it appeared to be stable. The optimal short-term storage condition (for 1 month) is to keep the phage stock in 4°C; whereas the long-term optimal storage condition (over 2 months) is to keep it with 7.5% glycerol under liquid nitrogen.

Determination of optimal MOI for PM2 phage

After finding the best storage conditions of a virus, we tried to determine optimal MOI for the mass production of the phage. Empirically, the burst size of the PM2 phage was known to be around 100. Therefore, we performed the assay by diluting the phage supernatant up to 10^{-11}. When the infection was performed at 0.03 of MOI, the burst size in the 16 h interaction was the highest (Fig. 1

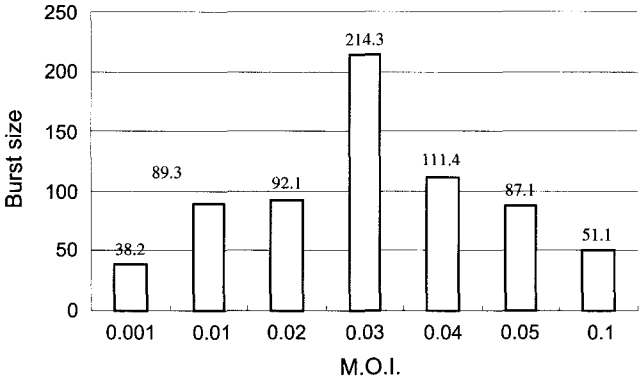

Fig. 1. A variation of burst size by changing MOI in the case of PM2 phage infection. For the determination of optimal MOI, we used the phage solution with 5.6×10^9 pfu/ml. When OD_{600} of the BAL 31-14 culture reached 0.375, the number of observed cells was 1.06×10^8 cells/ml. When OD_{600} of the BAL 31-14 culture reached 0.4~0.5, host cells were infected with the phage at indicated MOI. Sixteen hours later, the phage extract from the culture was centrifugated at 18,000×g for 20 min, and the clear supernatant containing the phage particles was used for the plaque forming assay. This assay was performed in triplicate.

Table 2. Determination of optimal MOI for PM2 phage infection by a plaque forming assay

Dilution factor MOI	10^{-3}	10^{-5}	10^{-7}	10^{-9}	10^{-11}
0.001	clear	uc	107	–	–
0.01	clear	uc	258	–	–
0.02	clear	uc	250	6	3
0.03	clear	uc	600	32	1
0.04	clear	uc	312	10	0
0.05	clear	uc	244	–	–
0.1	clear	uc	143	–	–

(uc: Too many to count, uncountable)

Table 1. Pfu concentrations from various storage conditions of PM2 phage

storage condition \ storage time		Fresh	1 month (short-term)	2 months (long-term)
4°C	pfu/ml	5.6×10^9	5.8×10^9	3.0×10^7
	relative fold	1	1.04	5.3×10^{-3}
4°C, 7.5% glycerol	pfu/ml	4.2×10^9	2.6×10^8	4.8×10^4
	relative fold	1	6.2×10^{-2}	1.1×10^{-5}
–70°C	pfu/ml	5.6×10^9	1.6×10^4	1.1×10^4
	relative fold	1	2.8×10^{-6}	2.0×10^{-6}
–70°C, 7.5% glycerol	pfu/ml	4.2×10^9	5.9×10^4	6.6×10^3
	relative fold	1	1.4×10^{-5}	1.6×10^{-6}
liquid nitrogen, 7.5% glycerol	pfu/ml	4.2×10^9	6.0×10^5	5.8×10^5
	relative fold	1	1.4×10^{-4}	1.4×10^{-4}

(decrease fold = phage concentration of 1 month or 2 months/phage concentration of fresh storage)

and Table 2). From this result, we concluded that, when the PM2 phage infects BAL 31-14, the optimal MOI for the mass production of phage DNA is 0.03.

Calculation of radiospecific activity and yield for the endonuclease assay

After the preparation of ^{3}H-labeled DNA of a PM2 phage, it is necessary to know the yield and the radiospecific activity of DNA, because the DNA concentration, the yield and the radiospecific activity are important criteria for the assay. The current study introduced the calculation methods. To calculate the concentration of purified DNA, we used formula (1).

$$A_{260} = \varepsilon \times c \times l \quad \cdots\cdots (1)$$

[l: length of sample (cm), ε: constant number ($l \cdot cm^{-1}$ mmol), c: concentration of sample (mM)]

Using formula (1), the concentration of our sample (purified DNA) was 0.283 mM because its absorbance (260 nm) was 1.84. To know how much tritium was incorporated into DNA, we checked the radioactivity of the sample by a scintillation counter. For this, equation (2) is needed.

$$\text{Specific activity} = R/V \times c = R \times \varepsilon \times l/V \times A_{260} \quad \cdots\cdots (2)$$

(V: Volume of sample, R: a measured value of radioactivity)

Concerning formula (2), we calculated the specific activity by checking A_{260} of the sample. The specific activity of purified DNA was 4.99×10^{3} cpm/nmol.

Finally to know the efficiency of the DNA preparation, we calculated the yield using the fact that the genome of the phage PM2 has 20,158 nucleotides. It was calculated by using formula (3)(4)(5)

$$P = c \times V \text{ (V: volume of purified DNA)} \quad \cdots\cdots (3)$$

$$T = \text{final pfu (pfu/ml)} \times \text{volume of total culture growth (ml)} \times 20158 \text{ (nucleotide/pfu)}/6.0 \times 10^{23} \quad \cdots\cdots (4)$$

$$\text{Yield} = \text{amount of prepared phage DNA (P)} / \text{amount of phage DNA in the culture mixture (T)} \quad \cdots\cdots (5)$$

In our result, the pfu level of the culture infected with the phage (final pfu) was 1.01×10^{11} pfu/ml. Therefore, our preparation yield was 19.5%. Normally, it has been known that previous preparation yields were calculated to be between 15% and 30%.

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