FACTORS INFLUENCING THE ASSAY OF THE BACTERIOPHAGE OF *XANTHOMONAS ORYZAE* BY PLAQUE COUNT[1]

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(Recieved July 14, 1966)

Introduction

Phages specific for *Xanthomonas oryzae* are demonstrable in the water of rice field, irrigation cannals, and rivers. The concentration of the phage is correlated with the number of its host bacterium, and is indicative of the quantity of the pathogen reproducing in nature. It has been demonstrated that when the phage can be detected abundantly in a rice paddy field in advance of the time of disease development, the disease appears soon after the phage can be detected (Tagami 1959, Yoshimura 1963). If we can count the phage titer in a fixed place in a given rice field or irrigation cannals, we can estimate the number of actively multiplying bacteria in the field. This would enable us to forecast the ensuring disease. There are, however, many problems to be taken into consideration in the practice of the phage lysis technique for the forecasting of disease.

In our laboratory the phages of *X. oryzae* were collected from different parts of this island and classified into several strains based on their host specificity. During this study it was found that the number and the morphology of the plaque differed among different culture conditions. Since the principle assumption of the test is that phage number is representitive of the number of host bacteria multipling in the water of rice field, it is necessary to develop a best method for the quantitative determination of this phage. In the present study factors influencing the number and size of plaques were examined and finally a better method is thereby suggested.

(1) The authors wish to express their gratitute to Dr. H. W. Li for his encouragement and final revision of the manuscript. Paper No. 56 of the Scientific Journal Series, Institute of Botany, Academia Sinica. This research was supported partly by Joint Commission on Rural Reconstruction and National Council on Science Development.

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Materials and Methods

Organism: The phage was isolated from the water of rice field with bacterial strain X_{501}. Single plaque isolate was suspended in distilled water and stored in the refrigerator. Bacterium, *X. oryzae*, was isolated in this laboratory from diseased plants. Single colony isolate was preserved in sterile distilled water in a test tube and stored at room temperature (DeVay 1963). Fresh culture was prepared for each experiment by transferring a loop of water suspension to nutrient slants. The strain X_{501} was used throughout this study.

Madia: For the growth of bacterium, the liquid medium, contained peptone, 5 g; yeast extract, 5 g; glucose, 1 g; K_2HPO_4, 5 g; and water 1000 ml., was used. Solid medium was prepared by adding 15 g agar, per liter. For the phage plaque assay, unless otherwise stated, potato-peptone agar contained potato, 200 g; peptone, 5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $Na_2HPO_4 \cdot 12H_2O$, 2 g; sucrose, 15 g; agar, 15 g and water 1000 ml., was used.

Propagation of phage: Growth medium inoculated with *X. oryzae* was incubated at room temperature on a shaker for 10 hr. A drop of phage suspension was added, and the culture was continuously cultivated until there was a reduction in turbidity, indicating that there was substantial lysis take-place. This last step took about 5–10 hours. All crude lysates, filtered through the bacterium-proof filter to remove unlysed bacteria and debris, were stored at 4°C prior to assaying.

Preparation of plates: Potato peptone agar plates (15 ml per petri dish) were dried overnight in an incubator at 37°C. The overlayer media (3 ml) previously prepared in test tubes were melted and held at 45°C in an water bath prior to being used. These samples of overlayer media were incubated with 0.5 ml of bacterium suspension which had been cultivated in liquid culture for 10–14 hours. Tenfold serial dilutions of filtered lysate were made with sterile distilled water, 0.5 ml of the appropriate dilution of phage was added to an inoculated overlayer tube. The overlayer tube was shaken and the mixture was poured onto a plate. The plate was rocked gently to insure a uniform overlayer, and the overlayer was allowed to harden before placing the plate in a 28°C incubator. All assays were plated in triplicate.

Plaque counting: Plaque counts were made on a Erma colony counter after 10 hours of incubation at 28°C.

Results

Plate media: The various media, employed in plate preparation for plaque count were: (1) potato, 200 g; peptone, 5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $Na_2HPO_4 \cdot 12H_2O$, 2 g; sucrose, 15 g; (2) peptone, 5 g; potato, 200 g; sucrose, 15 g;

(3) potato, 200 g; sucrose, 15 g; (4) Na-glutamate, 2 g; $MgCl_2 \cdot 6H_2O$, 1 g; K_2HPO_4, 0.1 g; Fe (EDTA-Fe), 1 mg; sucrose, 5 g. All above media were prepared in 1 liter water with 15 g agar. As indicated in Table 1, the number and size of plaques were not significantly affected by the different plate media used. All of above media could be used for assaying, however, the plaque formation was clearer and shaper in medium 1 and 2.

Table 1. *Effect of different media on the plaque formation*

Media	1	2	3	4
Number of plaques	178	188	173	174
Size of plaques (dia. mm)	3	3	3	3

(1) Potato, 200 g; peptone, 5 g; sucrose, 15 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $Na_2HPO_4 \cdot 12H_2O$, 2 g; (2) Potato, 200 g; peptone, 5 g; sucrose, 15 g; (3) Potato, 200 g; sucrose, 15 g; (4) Na-glutamate, 2 g; $MgCl_2 \cdot 6H_2O$, 1 g; K_2HPO_4, 0.1 g; sucrose, 5 g; Fe (EDTA-Fe), 1 mg.

For testing the effect of salts or sugar on plaque formation, the medium 2 was used for assaying. The addition of inorganic salts e. g. sodium chloride and calcium chloride to the plating media had no effect upon plaque formation. When sucrose in medium 2 was replaced with glucose, the number of plaque was ineffective but the size of plaques was smaller.

Age and concentration of bacterial cells: The age of the bacterial cells to be added to the overlayer medium was varied from 12 to 96 hours. At the end of incubation the concentration of bacterial cells was adjusted to about 5×10^9 cells/ml with colorimetric method. The results indicated in Table 2, the maximum number of plaque was obtained when the young bacterial cells (10 hr.) were used. The number of plaques was remarkably decreased when aged bacterial cells were used, however, the size and the time of plaque formation were not different among the different age of bacterial cells used. It was recommended that about 10 hr cultures would be the best age for the assay and it was routinely used in the preparation of bacterial cell suspension.

Table 2. *Effect of the age of bacterial cells on the plaque formation*

Age of bacterial cells (hr)	10	24	36	48	60	72	84	96
Number of plaques	105	102	91	71	36	36	45	32
Size of plaques (mm)	3	3	3	3	3	3	3	3

The concentration of bacterial cells to be added to the overlayer medium was varied from 2.5×10^{10} to 2.5×10^8 cells/ml. As indicated in Table 3, the maximum number of plaques was obtained when the concentration of bacterial cells in overlayer was in the range from 2.5×10^{10} to 2.5×10^9 cells/ml. When

the concentration of bacterial cells 5×10^8 cells/ml was used, the growth of bacteria was slow, the number of plaques decreased sharply and the size of plaques became very big and unclear (Fig. 1). If more than 30 plaques were present on a plate, they blended together and it was difficult to determine the exact number. When few plaques were present, counts from duplicate did not check well.

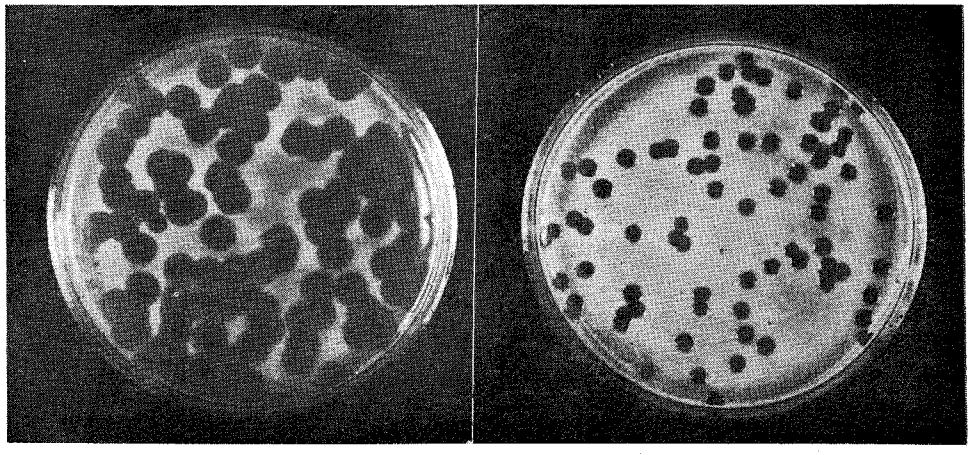

Fig. 1. Effect of the concentration of bacterial cells on the plaque formation right; 5×10^9 cells/ml, left; 2.5×10^8 cells/ml.

Table 3. *Effect of the concentration of bacterial cells on plaque formation*

Concentration of bacterial cells	Time of examination (hr)							
	6		8		10		20	
	Size (mm)	Number	Size (mm)	Number	Size (mm)	Number	Size (mm)	Number
2.5×10^{10}	1	81	2	88	3	88	4	88
5.0×10^9	1	65	2	92	3	92	5	92
2.5×10^9	—	0	2	84	3	91	6	91
5.0×10^8	—	0	—	0	3	38	8	38
2.5×10^8	—	0	—	0	—	0	8	34

The time of plaque formation was also different when different concentrations of bacterial cells were used. The plaque formation could be detected within 5 hr when the concentrations of bacterial cells 2.5×10^{10} to 5.0×10^9 cells/ml were used, whereas the plaque formation was delayed to 18 hours when the bacterial concentration at 2.5×10^8 cells/ml was used.

Agar concentration and amount of overlayer medium: The concentration of agar in overlayer was varied from 0.6 to 1.4 percent. As shown in Table 4, there was no different in the number of plaques among different concentrations

of overlayer agar used. The decrease in agar concentration enlarged the size of plaques, but soft agar below 0.5% had a tendency to break. Increase agar concentration above 1.4% had difficulty to plate evenly before condensation. Accordingly 0.8 or 1.0% was the best concentration which could be handled conveniently and these concentrations were recommended.

Table 4. *Effect of the concentration of overlayer agar on plaque formation*

Concentration of overlayer	0.6%	0.8%	1.0%	1.2%	1.4%
Number of plaques	98	107	101	103	96
Size of plaques (mm)	3	3	2.5	2.5	2

The amount of overlayer medium was varied from 3 to 6 ml. As indicated in Table 5, the number of plaques was much higher when 3 ml of overlayer was used. The size of plaques was not different from one another when different amount of agar was used.

Table 5. *Effect of the amount of overlayer agar on the plaque formation*

Amount of overlayer	3 ml	6 ml
Number of plaques	123	85
Size of plaques (mm)	3	3

pH of the plating medium: The pH of media was adjusted directly with HCl and NaOH. The pH of both basal layer medium and the overlayer medium was varied from pH 5 to 8. Very poor bacterial growth was observed when pH of the medium was below pH 5.0 or above pH 8.0. As indicated in Table 6, the maximum number of plaques was obtained when the pH of medium was at 6 or 7. Since the growth of bacteria was very good, the plaques were very clear. When pH of plating media was at 5 or 8 the time of plaque formation was slow and the number of plaques was also decreased.

Table 6. *Effect of pH of plating medium on the plaque formation*

pH	5	6	7	8
Number of plaques	108	177	170	144

Temperature and period of incubation: Incubation temperature of the plates was varied from 15° to 35°C. As indicated in Table 7, there was no significant difference of the count of phage plaques observed in the temperature range 25° to 30°C. At low temperature (15-20°C) the growth of bacterial

cells was slow, the count of phage plaques decreased, the time of plaque formation became slow and the plaques became very undistinguishable.

Table 7. *Effect of incubation temperature on plaque formation*

Incubation temperature (°C)	Time of examination (hr)					
	6		12		18	
	Number	Size (mm)	Number	Size (mm)	Number	Size (mm)
15	0	—	90	0.6	90	1.5
20	75	0.6	88	3.0	88	4.0
25	105	1.0	105	3.0	105	4.0
30	102	1.0	102	3.0	102	4.0
35	103	1.2	103	2.5	103	3.0

Influence of the period of incubation was also investigated. A thin mate of host cell growth was observed with clearly discernible plaques after about 6 hr incubation. The size of plaques increased during incubation of inoculated plates, usually reaching a maximum at 20 hr. While, the number of plaques reached to the maximum after 8 hr. The plates were ordinarily observed after 10 hours.

Conclusion

Based on the experimental results obtained, the following conclusion could be made. The important factors affecting the number of plaques were the number and age of bacterial cells, and the amount of overlayer media. While the factors affecting the size of plaques were the number of bacterial cells and the concentration of agar in overlayer. About the effect of pH and temperature for incubation of the plates it was indicated that the growth of the phages was best under the condition which were optimum for the growth of the host bacterium. Other four strains of bacteriophage, classified by their plaque morphology and specificity to host bacteria were also examined, the plaque formation of three phage strains fell in same kind of results, and one strain had little difference in plaque formation from others which did not form plaques in medium 4.

Summary

The factors influencing the quantitative measurement of bacterial cells of *X. oryzae* by plaque count of bacteriophage was studied. The number of plaque formation was affected by the age and amount of bacterial cells used for assaying, and different culture conditions. Medium 1 was the best medium for assaying but general potato-sucrose agar could be used also. The best

overlayer medium was 0.8% agar. For the age and number of bacterial cells, ten-hour cultures and 5.0×10^9 cells/ml were recommended. The best pH of plate media was 6 to 7 and the best incubation temperature was 25-35°C.

影響測定白葉枯病病原菌之噬菌體形成溶菌斑之因子

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使用 Adams 氏之洋菜雙層法（agar layer method）來定量白葉枯病病原菌（Xanthomonas oryzae）之噬菌體（Bacteriophage）時，發現溶菌斑（plaque）之數目及形態受到宿主菌（host cell）的年齡，濃度以及不同的培養條件所影響。根據實驗結果；一般的馬鈴薯—蔗糖培養基均適於測定此噬菌體之溶菌斑，在幾種不同之培養基中，馬鈴薯—蔗糖—燐酸鈉鹽（詳細成分請看 medium 1）培養基上溶菌斑最清楚。上層培養基（overlayer）之洋菜濃度以 0.8% 爲宜。測定噬菌體所採用之細菌懸濁液以震盪培養 10小時之細菌其濃度在每毫升含 5.0×10^9 個爲宜。形成溶菌斑最適合之酸鹼度是 pH 6 至 pH 7。最適合的培養溫度是 25°～35°。

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