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HOST SPECIFICITIES OF FIVE BACTERIOPHAGES ISOLATED FROM *XANTHOMONAS ORYZAE*

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Abstract

Five bacteriophages isolated from diseased leaves were tested for their sensitivity to twelve species of *Xanthomonas*. Xp10, Xp13, Xp20 and Xf attacked only *X. oryzae*, whereas Xp12 also attacked other 5 species of *Xanthomonas*, *X. pruni*, *X. phaseoli*, *X. malvacearum*, *X. monihotis* and *X. citri*. The growth of Xp12 on *X. pruni*, *X. phaseoli* and *X. malvacearum* was normal, but it was rather poor when grown on *X. citri* and *X. monihotis*. The efficiency of adsorption of Xp12 on *X. citri* was also rather low. Possible modification of Xp12 phage by demethylation of its DNA base on *X. citri* was examined. It was found that the methylated base is normal, therefore, the modification of host-phage relation is not caused by the methylation of its DNA base.

Introduction

From general experience, it has been concluded that the *Pseudomonas* phages are less specific than phages for *Xanthomonas* pathogens (Klement and LovreKovich 1960). Species-specific phages have been described for a few pathogens, including *X. pruni* (Eisenstark and Bernstein, 1955) and *X. vesicatoria* (LovreLovich and Klement 1965). For the latter pathogen, there have even been reported that phage strains are specific for tomato and pepper isolates (Dye *et al.* 1964). Later a comprehensive study of phage-host relationships among the *Xanthomonas* have revealed that the situation is largely the same as that in the *Pseudomonas* (Stolp and Starr, 1964). The activity of one particular phage strain is not restricted to attack the isolate of one particular bacterial species, and individual cultures of bacteria react with one or several phages. Furthermore, Vidaver and Schuster (1969) reported that some of the phages isolated from *X. phaseoli* was infectious for ***Pseudomonae phaseolicola.*** The discrepancies between the results obtained by former and later were attributed to the limited number of bacterial strains used by former workers

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compared with the enormous number of bacterial strains used by later workers. The difference in the source from which the phages were isolated might also be responisble for the discrepancy. From the studies on the cross-susceptibility between *Xanthomonas* bacteria and various phages from different sources, Goto and Starr (1972) concluded that the *Xanthomonas* phage, which could be found in soil, showed very broad host ranges. The host range extended not only to the different isolates of a homologous nomenspecies but also to strains of many heterologous nomenspecies. In contrast, the *Xanthomonas* phages isolated from diseased plant tissue usually showed very limited host ranges.

A group of bacteriophages from *X. oryzae* were isolated in this laboratory. They are Xp10, Xp12, Xp13, Xp20 and Xf (Kuo *et al.* 1967). Xp10, Xp13, and Xp20 are normal todeshaped phages. Xp12 is also a todeshaped phage, however, an unusual base, 5-methylcytosine, replaces cytosine in its DNA (Kuo *et al.* 1968). Xf is filamentous, the same type of phage has been reported in *Escherichia coli* and is male specific (Marvin and Hoffmann-Berling, 1963). Since some of these phages possese particular properties, the host specificity was studied. Among these phages, Xp10, Xp13, Xp20 and Xf are highly specific and Xp12 showed broad host ranges. Xp12 attackes 6 species of *Xanthomonas*, therefore, the growth and the efficiency of adsorption of Xp12 on these different hosts and possible change of base composition of Xp12 DNA in the different hosts are studied.

Materials and Methods

Bacterial culture

Xanthomonas oryzae strain 604, 507 and *X. citri* were isolated in this laboratory from diseased leaves. *Agrobacterium rhizogenes* AR7, *A. tumefaciens*, *Pseudomonas solanacearum* PS64, *P. syringae* PSy27, *P. tobasii* B_2, *Erwinia aroideae* EAr 12, *E. ananas* EAr 4, *E. nigrifluens* EN_2, *Corynebacterium faciens* CF 19, *C. michiganense* CM9, *X. campestris* XC4, *X. vesticatoria* XV 21, *X. pruni* Xpr 1, and *X. phaseoli* Xp24 were supplied by Shih-Tien Hsu, Dept. of Plant Pathology, National Chung-Hsing Univ. *X. malvacearum* ID 55, *X. hederae* 4D5, *X. pruni* 8D51, *X. transluscens* 10D5, *X. alfalfa* 12D5 and *X. cyamopsidis* 13D5, were supplied by C. I. Kado, Dept. of Plant Pathology, Univ. of Calif. Davis. *X. monihotis* was supplied by Li-Shieng Leu, Taiwan sugarcane experimental station. During the course of this study the stock cultures were kept on potato sucrose agar slants.

Bacteriophage

Bacteriophages Xp20, Xp10, Xp12 and Xf were isolated from infected rice leaves and were preserved in this laboratory. Xp20 Xp10 and Xp12 are all

todeshaped and Xf is filamentous.

Media

For the propagation of bacteriophages and their hosts, and phage plating, the medium (PS medium) containing potato extract (from 200 g fresh potato); peptone, 5.0 g; sucrose, 15.0 g; $Na_2HPO_4 \cdot 12H_2O$, 2.0 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; and one liter distilled water was used. For study of growth and adsorption of phages the SM medium containing sucrose, 15.0 g; L-glutamic acid, 1.0 g; cystine, 0.05 g; $(NH_4)_2HPO_4$, 3.0 g; KH_2PO_4, 2.0 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $Ca(NO_3)_2$, 0.1 g; $MnCl_2 \cdot 4H_2O$, 0.001 g; $FeCl_3$, 0.001 g; tris (hydroxy methyl) aminomethane 1.21 g and one liter distilled water was used.

Propagation of bacteriophages

Phage lysates were prepared by adding the phage to log-phase bacterial cells at 4×10^8 cells per ml at a ratio of phages to bacteria of 0.1. After incubation at 28°C for 16 hrs. the lysates were harvested and stored at 4°C. The final titers for Xp10, Xp20 and Xp13 were around $5 \times 10^{10} - 1 \times 10^{11}$ pfu per ml and that for Xp12 and Xf could reach 2×10^{12} pfu per ml.

Results

Host range of bacteriophage isolated from X. oryzae

Both spot tests with concentrated phage suspensions and quantitative tests were used to examine the susceptibility of 12 species of Xanthomonas to 5 phages isolated from *X. oryzae*. The results are showed in Table 1. Xf, Xp10, Xp13 and Xp20 attacked only *X. oryzae*, and Xp12 attacked 5 other species of *Xanthomonas*.

Since Xp12 could attack 5 species of *Xanthomonas*, it was considered important to learn whether these phage strains are specifically active against *Xanthomonas*. For this purpose, their activity were checked against a variety of bacteria from different systematic categories. The following cultures were tested for susceptibility to the 5 phages. They are *Agrobacterium rhizogenes* AR7, *A. tumefaciens*, *Pseudomonas solancearum* PS 64, *P. syringae* Psy27, *P. tobasii* B_2, *Erwinia aroideae* EAr12, *E. ananas* EAn4, *E. nigrifluens* EN_2, *Corynebacterium faciens* CF19, *C. michiganense* CM9, *Bacillus subtilis* and *Escherichia coli*. All these cultures proved to be non-susceptible to any of 5 phages.

Plaque morphology of Xp12 on different species of hosts

In the usual incubation period and growth condition (16 hrs 28°C) Xp12 produced clear plaque with sharp irregular border. The plaque diameter on

Table 1. *Host specificity of Xanthomonas oryzae phages on 15 plant pathogenic Xanthomonas spp.*

Bacterial strains \ Phages	Xf	Xp10	Xp12	Xp13	Xp20
Xanthomonas oryzae					
507	+	+	+	+	+
507-R12	+	+	—	+	+
X. pruni					
Xpr1	—	—	+	—	—
8D51	—	—	—	—	—
X. phaseoli					
Xp24	—	—	+	—	—
X. malvacearum					
1D55	—	—	+	—	—
X. monihotis	—	—	+	—	—
X. citri	—	—	+	—	—
X. vesicatoria	—	—	—	—	—
X. campestris	—	—	—	—	—
X. hederae (4D5)	—	—	—	—	—
X. transluscens (10D5)	—	—	—	—	—
X. alfalfa	—	—	—	—	—
X. cyamopsidis	—	—	—	—	—

X. oryzae, and *X. phaseoli* was 5 to 7 mm, that on *X. malvacearum*, *X. pruni* and *X. monihotis* was 2 mm, and that on *X. citri* was 1 mm.

The lysis and multiplication of Xp12 in different host bacteria

Since Xp12 attacked 6 different species of *Xanthomonas*, the growth of Xp12 in these six different hosts was studied. Overnight young culture (8×10^8 cell/ml) in SM medium was transfer to new medium and grew for another 6 hours. The phage at multiplicity of 1 were added, samples were withdrawn at zero time and one hour intervals up to 12 hours. The lysis of host bacteria was measured directly with spectrophotometer at 450 mμ and multiplication of phage was also assayed by plaque count. The lysis of host are shown in Fig. 1, when Xp12 infected *X. oryzae*, host cells lysed at three hour after phage infection, however, when other 5 species of *Xanthomonas* was infected, cell lysis was detected at four hour after phage infection.

The multiplication of Xp12 in different host bacteria are shown in Fig. 2, two type of multiplication patterns could be observed. When Xp12 was grown on *X. oryzae, X. malvacearum, X. phaseoli, X. pruni* the titer could reach 5×10^{12}

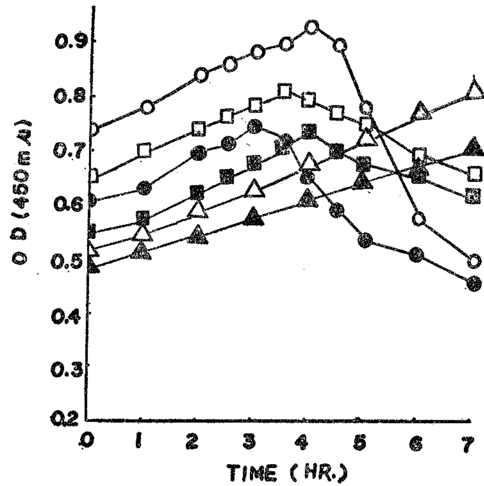

Fig. 1. Lysis of different bacterial hosts by Xp12 phage. ○——○: *X. malvacearum*; □——□: *X. citri*; ●——●: *X. oryzae*; ■——■: *X. pruni*; △——△: *X. campestris*; ▲——▲: *X. vescatoria.*

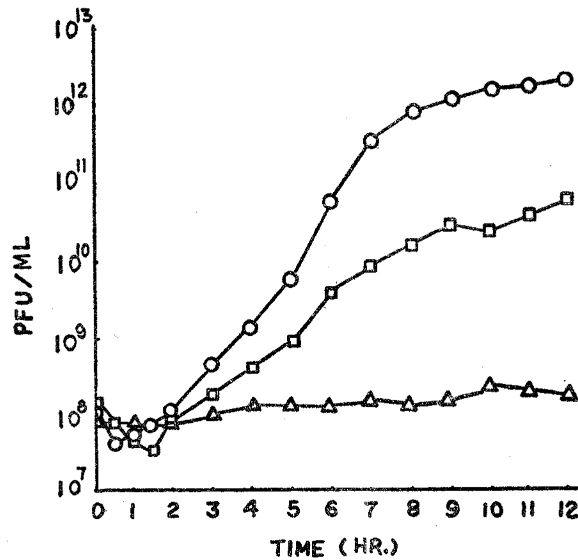

Fig. 2. Production Xp12 phage on different hosts. ○——○: *X. oryzae, X. malvacearum, X. phaseoli* and *X. pruni*; □——□ :*X. citri* and *X. monihotis*; △——△: *X. vesicatoria.*

pfu per ml. When it was grown in *X. citri* and *X. monihotis* only 2×10^{11} pfu per ml was obtained after 12 hours incubation.

Adsorption of Xp12 phage on different host

The experiments were conducted to see whether Xp12 can adsorb to those *Xanthomonas sp.* which are not infected by Xp12, or any difference in the

efficiency of adsorption on different susceptible hosts. To overnight culture at 1×10^9 cells per ml, phage Xp12 at multiplicity of 0.1 were added. The mixture was incubated at 28°C with aeration. At 0 time and 10 minutes intervals, 1 ml of samples were withdrawn and immediatly cooled down in centrifuge tubes. The tubes were centrifuged for 10 minutes at 5000 g to sediment the cells. Following centrifugation, supernatant was removed and phage particle were titered by plaque counting method. The results are shown in Fig. 3, the adsorption of Xp12 on *X. oryzae* was faster and more completed than that on *X. citri.* No adsorption was observed on the hosts which were not infected by Xp12.

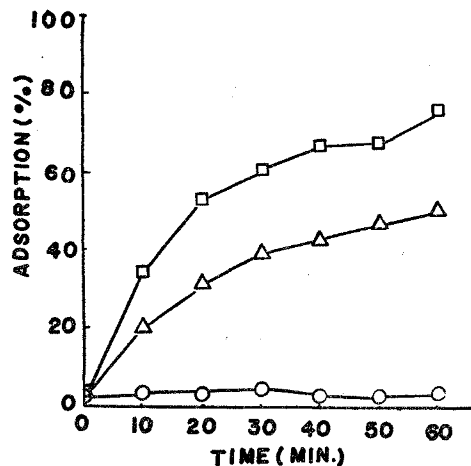

Fig. 3. Adsorption of Xp12 phage on different hosts. □——□: *X. oryzae*, *X. pruni* and *X. malvacearum*; △——△: *X. citri*; ○——○: *X. vescatoria*, *X. campestris* and *X. hederae*.

Identification of DNA bases of Xp12 grown in X. citri

The Xp12 DNA has a unusaul base, 5-methylcytosine replacing cytosine, in its DNA. Since the growth of Xp12 in *X. citri* was rather poor, it was suspected that whether the base composition was changed after Xp12 were grown on *X. citri,* therefore, the Xp12 phages grown on *X. citri* were purified and the base composition of Xp12 DNA was analyzed.

Phages were propagated in *X. citri* which grew exponentially in PS medium. The phages were purified by differential centrifugation and sucrose gradient centrifugation. Contamination by host DNA and RNA was removed by incubating the phages with DNase and RNase before phage DNA extraction. DNA was extracted with phenol and isolated by the procedure of Marmure (1961) from the last 95% ethanol precipitation step on. The purified phage DNA was hydrolysed in 12 N perchloric acid for 60 minutes at 100°C and liberated bases

were separated by paper chromatography. Three solvent systems were used for identification of bases. (1) To 65 ml of isopropanol is added 16.7 ml of concentrated HCl (12N). After mixing, water is added to 100 ml; (2) 86% (v/v) aqueous n-butanol; (3) 86% (v/v) aqueous n-butanol, with 5% by volume of concentrated ammonia added to the solvent in the bottom of tank. Authentic bases were used for comparison. In all these solvents 4 bases coincided exactly with authentic guanine, 5-methlycytosine, adenine and thymine. Although the growth of Xp12 was poor in *X. citri*, it seemed that there was no change in base composition of Xp12 DNA.

Discussion

Based on host specificity the bacteriophages isolated from *X. oryzae* could be divided into two distinquished groups. One group attacked only *X. oryzae* and the other group attacked many different species of *Xanthomonas*. Goto and Starr (1972) have compared the behavior of the twelve Stolp-Starr phages which were mainly isolated from soil with that of other *Xanthomonas* phages which were isolated from diseased materials. They concluded that *Xanthomonas* phages isolated from soil showed broad host range and that from diseased plant tissue showed limited host ranges. In this investigation all of our *X. oryzae* phages were isolated from diseased plant materials. Therefore the conclusion of Goto and Starr is true in the case of Xp10, Xp13, Xp20 and Xf, however, Xp12 is an exception. It is also possible that Xp12 was from soil by contamination.

Viral properties have frequently been reported to undergo non-heritable change upon passage through certain host strains, but very little was known about the molecular mechanism, for such host controlled modifications. A hypothesis postulated that enzymic methylation of DNA bases is responsible for host-specific modification (Gold and Hurwitz 1963). Since the Xp12 possesses 5-methylcytosine in its DNA, and its growth was poor in *X. citri*. It was thought that the poor growth of Xp12 in *X. citri* might cause by the modification of the methylation of DNA base. However, the result showed that it was not the case.

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Xanthomonas oryzae 的幾種噬菌體的寄主特異性

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自水稻白葉枯病病原菌分離到的幾種噬菌體，依其對寄主的特異性可分爲二類：一類其寄主特異性很窄，如 Xp10，Xp13，Xp20 和 Xf. 祇感染 *X. oryzae* 一種。一類其寄主特異性很寬，如 Xp12 除能感染 *X. oryzae* 以外，還感染 *X. pruni*，*X. citri*，*X. phaseoli*，*X. malvacearum* 和 *X. monihotis*。Xp12 在 *X. pruni*，*X. phaseoli* 和 *X. malvacearum* 上生長情形與其在 *X. oryzae* 上相同，但在 *X. citri* 及 *X. monihotis* 上卽較差。其對 *X. citri* 的吸附率也較低，Xp12 的 DNA 中有一不正常的氮鹽基 (methylated cytosine)。Xp12 在 *X. citri* 上繁殖不良可能與此不正常的氮鹽基有關，但分析結果顯示生長不良並不影響此不正常的氮鹽基的改變。