Classification of strains of *Xanthomonas oryzae* on the basis of their susceptibility against bacteriophages.

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Introduction

The bacteriophage for *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson, the agent attacking the pathogen of the bacterial leaf blight disease of rice, was at first isolated by Yoshii and others¹⁷⁾ in 1953 from diseased paddy soil in Fukuoka prefecture, Japan. Some characteristics of this phage, named OP₁, were previously reported by the author¹³⁾. A new bacteriophage OP_{1h} was then isolated by Tagami and others in 1958, and Ikari and the author⁴⁾ confirmed that OP_{1h} had almost the same characteristics as OP₁ except host range. Recently, Yoshimura and others¹⁸⁾¹⁹⁾ reported that there were four kinds of phages, which differed from one another in host range or plaque shape, distributed in Hokuriku district, and that the host bacteria collected from the same district could be classified into 6 strains, A, B, C, D, E and AB according to the difference in susceptibility to these phages.

So as to contribute towards the practical applications¹⁴⁾¹⁵⁾ of the phages, the susceptibility of many isolates of *X. oryzae* collected from various places of Japan to the phage strain widely distributed was tested.

Material

For the purpose of finding new phages, diseased leaves of the rice plant affected with *X. oryzae* were collected during an epidemic period from various places of Japan, as shown in Table 1.

Bacterial strains *X. oryzae* 22-SR-2 and *X. oryzae* B were employed to isolate the phages from each sample, because these bacterial strains were known to show exactly opposite susceptibility pattern with each other. The strain 22-SR-2 is susceptible to OP₁ but not to OP_{1h}, while the strain B is susceptible to OP_{1h} but not to OP₁.

Every phages was isolated from single plaque produced on the culture plate of each bacterial strain. Many isolates of *X. oryzae* collected from various places of Japan were tested against the isolated phages as well as the standard phage strains of OP₁, and OP₂, in simultaneous tests. The OP₂ phage, which had been reported as SB phage by Yoshimura and others in 1959, was kindly furnished by him for this experiment.

The composition of the solid media used throughout the experiment was as follows ; potato decoction 300 gr., Na₂HPO₄·12H₂O 2 gr., Ca(NO₃)₂·4H₂O 0.5 gr., sucrose 20 gr., peptone 5 gr. : water to 1 l.

Experimental methods

Collected diseased leaves were cut finely by scissors, and 0.1 gr. of the sample was ground in a mortar with quartz sand and a few ml. of distilled water. The suspension of each of the samples was then centrifuged at 12,000 rpm. for 10 min. to remove the debris of leaf tissues and bacteria. The supernatant was diluted 100-fold with sterilized distilled water, and either 0.1 or 0.01 ml. aliquats were poured into ca. 2 ml. of dense suspension of host bacteria which had been prepared just before. Three ml. of agar media which had been melted and kept at about 45°C was poured into each tube, and then the whole content of the tube was plated in Petri dish. Plate dishes thus prepared were incubated at 25°C. Ten to 15 hours later, clear plaques appeared if phages in sufficient amount were contained in the original sample.

When plaques appeared on the plates of both B and 22-SR-2, the standard strains, it was concluded that the original sample contained either both OP₁ and OP_{1h}, or it contained a new phage which attacked both bacterial strains. Many of the samples, in practice, produced plaques on the culture plates and were shown with temporary abbreviations in Table 1.

The host range of the phages were determined by using 82 isolates of *X. oryzae*, according to the following procedure. Each isolate cultured on agar slant at 25°C for 3 days was suspended in

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Table 1. Distribution of *X. oryzae* phages in Japan.

Places from which diseased leaves were collected		Variety of rice plant collected	Phage isolate
Koga-gun	Shiga Pref.	Kinmaze	—
Gamoo-gun	Shiga Pref.	Kinmaze	B2
Aki-shi	Kōchi Pref.	Norin-mochi no. 5	C1
Gifu-shi	Gifu Pref.	?	D1
Iwami-gun	Tottori Pref.	Hōkiasahi	E1
Kamimasuki-gun	Kumamoto Pref.	Tyugoku-mochi no. 11	F1
Niitsu-shi	Niigata Pref.	Norin no. 43	G1, G2
Ōmagari-shi	Akita Pref.	Sasashigure	H1
Mii-gun	Fukuoka Pref.	Zikkoku	I1
Kanoya-shi	Kagoshima Pref.	Zuiho	J2
Kōfu-shi	Yamanashi Pref.	Wakabanishiki	K1
Gifu-shi	Gifu Pref.	?	L1, L2
Nangoku-shi	Kōchi Pref.	Kinmaze	M1, M2
Nangoku-shi	Kōchi Pref.	Kyushu no. 58	N1, N2
Aki-shi	Kōchi Pref.	Shimotsuki	O1, O2
Aki-shi	Kōchi Pref.	Zuikyū	P1, P2
Kumage-gun	Yamaguchi Pref.	Mihonishiki	Q1
Ayauta-gun	Kagawa Pref.	Akebono	R1

Fig. 1 Plaques of OP₁ phage produced on *X. oryzae* No. 49 incubated at 25°C for 15 hours. Fig. 2 Streak method to examine the reaction of an isolate of *X. oryzae* against a number of phage strains.

2 ml. of sterilized water. The bacterial suspensions thus prepared were mixed with ca. 3 ml. of melted (45°C) potato semi-synthetic agar media, and poured into Petri dishes. After the plated agar settled, phage suspensions to be tested were streaked using a sterilized loop along the lines marked in parallel on the under surface of the sterilized dishes. After incubating the dishes at 25°C for 15 hours, clear streaks having some width appeared, if the plated bacteria are susceptible to the phages.

Results

1. Distribution of isolates of the bacteriophage for *X. oryzae* in Japan.

Most of the diseased leaves collected during an epidemic period contained some phages. The phages could be classified on the basis of their host range, as was shown in Table 2.

Table 2. Classification of *X. oryzae* phages from Japan.

Strain of <i>X. oryzae</i> phage	<i>X. oryzae</i> phage isolate
OP ₁	E1, F1, G1, H1, I1, K1, Q1, R1
OP _{1h}	B2
OP _{1h2}	C1, D1, G2, J2, L1, L2, M1, M2, N1, O1, O2, P1, P2, (N2)
OP ₂	

Probable reason for the higher frequency of isolation of OP_{1h2} than that of OP₁ was that the diseased leaves employed in isolating the phages in this experiment were mostly collected from places from where OP₁-resistant bacteria had been collected in preliminary experiments. Though OP₂ phage was isolated by Yoshimura and others in 1958 from Hokuriku district, it was considered that the range of distribution of this phage was rather limited from the results of this experiment in which OP₂ was not found.

Whatever may be the reason, it seems likely that OP₁ and OP_{1h2} have the more extensive distribution in Japan than OP_{1h} or any other phages.

2. Susceptibility of the isolates of *X. oryzae* to the phages.

Eighty-two isolates of *X. oryzae* were tested for their susceptibility to each of the phages OP₁, OP_{1h}, OP_{1h2}, and OP₂. The results were given in Table 3.

Table 3. Reaction of isolate of *X. oryzae* against phages.

Isolate	Pref. from which the diseased leaves were collected	Strain of phage				Strain classification of <i>X. oryzae</i>
		OP ₁	OP _{1h}	OP _{1h2}	OP ₂	
N 5801	Akita	—	—	—	+	E
N 5802	Akita	—	+	+	+	B
N 5803	Akita	+	—	+	+	A
N 5804	Akita	—	+	+	+	B
N 5805	Yamagata	—	+	+	+	B
N 5806	Fukushima	+	—	+	+	A
N 5807	Fukushima	+	—	+	+	A
N 5808	Miyagi	+	—	+	+	A
N 5809	Miyagi	+	—	+	+	A
N 5810	Miyagi	—	+	+	+	B
N 5811	Miyagi	—	—	—	—	C
N 5812	Miyagi	+	—	+	+	A
N 5813	Ibaragi	+	—	+	+	A
N 5814	Ibaragi	+	—	+	+	A
N 5815	Ibaragi	—	+	+	+	B
N 5816	Ibaragi	—	+	+	+	B
N 5817	Gunma	+	—	+	+	A
N 5818	Kanagawa	+	—	+	+	A
N 5819	Kanagawa	+	—	+	+	A
N 5820	Saitama	+	—	+	+	A
N 5821	Saitama	+	—	+	+	A
N 5822	Saitama	+	—	+	+	A
N 5823	Chiba	+	—	+	+	A
N 5824	Tokyo	+	—	+	+	A
N 5825	Niigata	+	—	+	+	A

Isolate	Pref. from which the diseased leaves were collected	Strain of phage				Strain classifi- cation of <i>X.</i> <i>oryzae</i>
		OP ₁	OP _{1h}	OP _{1h2}	OP ₂	
N 5826	Fukui	—	+	+	+	B
N 5827	Fukui	—	+	+	+	B
N 5828	Fukui	—	+	+	+	B
N 5829	Fukui	+	—	+	+	A
N 5830	Fukui	—	+	+	+	B
N 5831	Ishikawa	—	+	+	+	B
N 5832	Ishikawa	—	—	+	+	D
N 5833	Toyama	+	—	+	+	A
N 5834	Nagano	+	—	+	+	A
N 5835	Nagano	+	—	+	+	A
N 5836	Nagano	+	—	+	+	A
N 5837	Yamanashi	+	—	+	+	A
N 5838	Yamanashi	+	—	+	+	A
N 5839	Yamanashi	—	+	+	+	B
N 5840	Shiga	—	—	+	+	D
N 5841	Shiga	—	+	+	+	B
N 5842	Shiga	—	—	—	+	E
N 5843	Gifu	—	—	+	+	D
N 5844	Gifu	—	—	+	+	D
N 5845	Shizuoka	—	+	+	+	B
N 5846	Nara	+	—	+	+	A
N 5847	Hyogo	+	—	+	+	A
N 5848	Hyogo	+	—	+	+	A
N 5849	Osaka	+	—	+	+	A
N 5850	Okayama	+	—	+	+	A
N 5851	Okayama	+	—	+	+	A
N 5852	Okayama	+	—	+	+	A
N 5853	Hiroshima	—	+	+	+	B
N 5854	Yamaguchi	+	—	+	+	A
N 5855	Yamaguchi	—	+	+	+	B
N 5856	Yamaguchi	—	+	+	+	B
N 5857	Yamaguchi	+	—	+	+	A
N 5858	Yamaguchi	+	—	+	+	A
N 5859	Shimane	+	—	+	+	A
N 5860	Kagawa	—	—	—	—	C
N 5861	Tokushima	—	+	+	+	B
N 5862	Tokushima	—	+	+	+	B
N 5863	Kōchi	+	—	+	+	A
N 5864	Kōchi	—	—	+	+	D
N 5865	Kōchi	—	—	—	—	C
N 5866	Kōchi	—	—	+	+	D
N 5867	Kōchi	—	—	+	+	D
N 5868	Fukuoka	—	—	+	+	D
N 5869	Fukuoka	+	—	+	+	A
N 5870	Fukuoka	+	—	+	+	A
N 5871	Saga	+	—	+	+	A
N 5872	Saga	+	—	+	+	A
N 5873	Saga	—	—	—	+	E
N 5874	Saga	—	—	—	—	C
N 5875	Saga	+	—	+	+	A
N 5876	Saga	+	—	+	+	A
N 5877	Saga	+	—	+	+	A
N 5878	Saga	+	—	+	+	A
N 5879	Kumamoto	+	—	+	+	A
N 5880	Kumamoto	+	—	+	+	A
N 5881	Miyazaki	—	+	+	+	B
N 5882	Kagoshima	—	—	+	+	D

* 82

46

20

75

78

* Nos. of bacterial isolates, total and affected with the phage strains.

X. oryzae could be divided into 5 strains A, B, C, D, and E, as was shown in Table 4. Most of the 82 isolates tested in this experiment belonged to A strain, and only a small number of them belonged to B, D, C, or E, in frequency in this order.

Discussion

Presence of several phage strains attacking the same phytopathogenic bacterial species was reported by several workers. Fulton³⁾ demonstrated that there are two *P. tabaci* phages, phage 1 and phage 2, attacking *P. tabaci*, and that these phages showed different host range and different particle shape. Okabe and others¹⁰⁾¹¹⁾¹²⁾ and Matsui⁷⁾ also reported similar phenomena with *P. solanacearum* phages.

Present studies showed that the phages for *X. oryzae* distributed in Japan belonged either to OP₁ group or to OP₂ group. The OP₁ group comprised several strains, OP₁, OP_{1h}, OP_{1h2}, and OP_{1t}, while the OP₂ group comprised only one strain. From the results of morphological studies using electronmicroscope, it was found that phages of both these group had tadpole shape, but they differ in the size of tail: the former had a head of 70 mμ in diameter with a tail of 150 mμ in length and 15 mμ in width, while the latter had a head of the same diameter with a tail of 95 mμ in length and 25 mμ in width. Furthermore some differences in serological properties was found between OP₁ group and OP₂ by Yoshimura and others¹⁹⁾.

Classification of the strains of phytopathogenic bacteria on the basis of the susceptibility to phage together with some biological characteristics was first reported by Okabe and others in 1953. Recently, Yoshimura¹⁹⁾ reported that *X. oryzae* collected from Hokuriku district could be classified into 6 strains, A, B, C, D, E, and, AB, based on the susceptibility to 4 strains of phage. I phage which was a plaque shape mutant of OP₂, used in his experiment instead of OP_{1h2}, seemed to be questionable, inasmuch as the more confusion of the classification was resulted.

Random distribution of various phage strains which differ in host range, in a company with that of various strains of *X. oryzae* which differ in susceptibility to the phages, present a problem in the practical use of the phages, particularly for the diagnosis, bacterial leaf blight of rice, or for the forecasting of the disease outbreaks. Practically, it will be necessary to select the phage and the bacterial strains, for the purpose of determining bacterial population, or assaying phage titre, in the field.

Between the pathogenicity of the host bacterial strains and their phage-susceptibility, relationship was not found according to this results joined with that obtained from the infection experiments⁹⁾ carried out simultaneously.

Summary

Xanthomonas oryzae bacteriophage collected from various places of Japan could be classified into 4 strains, OP₁, OP_{1h}, OP_{1h2} and OP₂ on the basis of their host range. It appeared that OP₁ and OP_{1h2} were the ones more extensively distributed than the other phage strains in Japan.

On the basis of susceptibility to these phage strains, 82 isolates of *X. oryzae* collected from various places of Japan were classified into 5 strains as follows;

- A strain : Susceptible to OP₁, OP_{1h2} and OP₂ but not to OP_{1h}
- B strain : Susceptible to OP_{1h}, OP_{1h2} and OP₂ but not to OP₁
- C strain : Resistant to all of these phages
- D strain : Susceptible to OP_{1h2} and OP₂ but not to OP₁ and OP_{1h}
- E strain : Susceptible to OP₂ but not to OP₁, OP_{1h} and OP_{1h2}

Of these bacterial strains, A strain was the one most widely and commonly distributed in Japan, followed by B, D, C, and E strains in decending order. There was found no specific rule in the distribution pattern of these strains.

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References

- 1) Adams, M. H. (1950). Method of Study of Bacterial Viruses, in "Method in Medical Research" 2, The Year Book Publishers, Cicago.
- 2) Adams, M. H. (1959). Bacteriophages, Interscience Publishers, Inc., New York.
- 3) Fulton, R. W. (1950). Phytopath., 40 : 936~949.

- 4) Ikari, H. and. Wakimoto S. (1958). Proc. Assoc. Plant Protection, Kyushu, 4 : 38~40.
- 5) Klement, Z. and Lovas, B. (1960). Phytopath. Zeitsch., 37 : 321~329.
- 6) Klement, Z. (1959). Nature, 184 : 1248~1249.
- 7) Matsui, C. (1953). Sci. Bul. Fac. Agr., Kyushu Univ., 14 : 43~49.
- 8) Matsui, C. (1955). Sci. Bul. Fac. Agr., Kyushu Univ., 15 : 333~338.
- 9) Mukoo, H., Kusaba, T., Watanabe, M., Wakimoto, S., and Yamazaki, Y. (unpublished)
- 10) Okabe, N. and Goto, M. (1952). Rept. Fac. Agr., Shizuoka Univ., 2 : 64~93.
- 11) Okabe, N. and Goto, M. (1953). Rept. Fac. Agr., Shizuoka Univ., 3 : 52~80.
- 12) Okabe, N. and Goto, M. (1953). Rept. Fac. Agr., Shizuoka Univ., 3 : 81~100.
- 13) Wakimoto, S. (1954). Sci. Bul. Fac. Agr., Kyushu Univ., 14 : 485~493.
- 14) Wakimoto, S. (1954). Sci. Bul. Fac. Agr., Kyushu Univ., 14 : 495~498.
- 15) Wakimoto, S. and Yoshii, H. (1955). Sci. Bul. Fac. Agr., Kyushu Univ., 15 : 161~169.
- 16) Wakimoto, S. (1955). Sci. Bul. Fac. Agr., Kyushu Univ., 15 : 151~160.
- 17) Yoshii, H., Yoshida, T. and Matsui, C. (1953). Ann. Phytopath. Soc., Japan, 17 : 117 (abst.).
- 18) Yoshimura, S., Morihashi, T. and Nakaomi, Y. (1959). Proc. Assoc. Plant Protection, Hokuriku, 7 : 53~55.
- 19) Yoshimura, S. Personal communication.

和文摘要

バクテリオファージに対する感受性による イネ白葉枯病菌の分類

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日本の各地から収集したイネ白葉枯病菌ファージは、それらの寄主範囲から、OP₁, OP_{1h}, OP_{1h2}, OP₂ の4系統に分類できる。これらの内、OP₁ と OP_{1h2} とが最も広く分布しているようである。

また日本の各地から集めたイネ白葉枯病菌の分離地別 82 系統菌は、ファージに対する感受性から次の5系統に分類できる。

系統 A : OP₁, OP_{1h2}, OP₂ に感受性で OP_{1h} に抵抗性

系統 B : OP_{1h}, OP_{1h2}, OP₂ に感受性で OP₁ に抵抗性

系統 C : すべてのファージに抵抗性

系統 D : OP_{1h2}, OP₂ に感受性で OP₁, OP_{1h} に抵抗性

系統 E : OP₂ に感受性で OP₁, OP_{1h}, OP_{1h2} に抵抗性

これらのイネ白葉枯病菌系統の内、日本における分布は系統Aが最も広く、ついで B, D, C, E の順である。しかしながら、これらの系統の分布模様には規則性は認められない。

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