

# Xanthomonas campestris pv. oryzae N 5850 の増殖, 微細構造および病原力に及ぼす繊維状ファージ Xf 感染の影響

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# Effect of Infection with Filamentous Phage Xf on the Growth, Ultrastructure and Virulence of *Xanthomonas campestris* pv. *oryzae* N 5850

Hiroshi KAMIUNTEN\* and Satoshi WAKIMOTO\*

上運天 博\*・脇本 哲\*: *Xanthomonas campestris* pv. *oryzae* N 5850 の増殖,  
微細構造および病原性に及ぼす繊維状ファージ Xf 感染の影響

## Abstract

Effect of infection with filamentous phage Xf on the growth, ultrastructure and virulence of *Xanthomonas campestris* pv. *oryzae* N 5850 was investigated. The growth of Xf-infected bacteria was considerably retarded at 30 C as compared with that of uninfected bacteria. The Xf-infected bacteria, however, rapidly multiplied from 36 hr after infection to almost the same level of concentration as reached by uninfected cells after 72 hr of incubation. The infected bacteria could grow even at 35 C to some extent, while uninfected bacteria could not. Electron microscopic observation of the ultrathin sectioned Xf-infected bacteria grown at 30, 33.8 and 35 C revealed that a number of vesicles exist on the cell surface. Some remarkable changes in the intracellular structures, viz., lack of uniformity in distribution of both ribosomes and fibrils in nucleoid area were observed in the Xf-infected bacteria. Xf-infection caused the bacteria to produce a greater amount of extracellular polysaccharide and to increase virulence.

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**Key Words:** *Xanthomonas campestris* pv. *oryzae*, filamentous phage.

## Introduction

Two filamentous phages, Xf and Xf 2, were isolated from culture fluids of *Xanthomonas campestris* pv. *oryzae* isolates and some of their properties were made clear<sup>2,3,6)</sup>.

In the previous paper<sup>4)</sup>, the effect of infection with Xf 2 phage on the properties of host bacteria was reported. The multiplication of *X. campestris* pv. *oryzae* N 5850 at 30 and 33.5 C was retarded by infection with Xf 2<sup>4)</sup>. The Xf 2-infected bacteria, however, reached the same or higher concentration level as compared with uninfected control later on<sup>4)</sup>. At 35 C, the Xf 2-infected bacteria could grow to some extent, while uninfected bacteria could not<sup>4)</sup>. The Xf 2-infected bacteria incited severe symptom on rice leaves earlier than uninfected bacteria<sup>4)</sup>. To make clear whether such changes in bacteriological properties of host bacteria caused by filamentous phage infection are specific for each kind of phage or not, similar experiments with another filamentous phage Xf were carried out.

\* Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan      九州大学農学部

## Materials

**Bacteria and phage.** *X. campestris* pv. *oryzae* N 5850 used in this experiment was furnished by the National Institute of Agricultural Sciences, Tsukuba, Ibaraki, Japan. This isolate was susceptible to both filamentous phages Xf and Xf2, and pathogenic to rice leaves<sup>2)</sup>. The filamentous phage Xf was originally isolated from *X. campestris* pv. *oryzae* N 5828 in our laboratory, and preserved at  $-40^{\circ}\text{C}$  after freeze-drying. To enrich phage Xf, the phage suspension was aseptically added to the medium inoculated with *X. campestris* pv. *oryzae* N 5850, and shake-cultured for 48 hr at  $30^{\circ}\text{C}$ . The culture fluid was centrifuged at  $10,000\times g$  for 20 min. The supernatant was treated at  $60^{\circ}\text{C}$  for 20 min to kill residual cells without killing Xf phage and used as enriched phage suspension. The phage titer of this suspension was about  $5\times 10^{10}$  PFU/ml.

**Medium.** Bacteria were grown in Suwa's synthetic medium<sup>5)</sup> (Na-glutamate 2g,  $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$  1g,  $\text{K}_2\text{HPO}_4$  0.1g, Fe as EDTA salt 1mg, sucrose 5g, peptone 10g, distilled water 1 liter, pH 7.0).

## Methods and Results

### Effect of Xf-infection on the growth of host bacteria

*X. campestris* pv. *oryzae* N 5850 was cultured in Suwa's synthetic medium overnight. The bacterial suspension was diluted with the same medium to adjust optical density at 450 nm to 0.02. Nine ml each of the diluted bacterial suspension was cultured with a temperature gradient incubator after addition of 1ml of the phage suspension.

Uninfected bacteria were also cultured simultaneously as a control. 0.4ml each of culture suspension was taken out at 12 hr intervals, diluted twice with distilled water to measure its O. D. at 450 nm.

The growth of bacteria infected with Xf phage was considerably retarded at  $30^{\circ}\text{C}$  as compared to that of uninfected bacteria. The infected bacteria, however, rapidly multiplied to a level close to the concentration of uninfected bacteria at 72 hr after infection. The Xf-infected bacteria could grow even at  $35^{\circ}\text{C}$  to some extent, while uninfected bacteria could not.

### Ultrastructure of the host bacteria infected with Xf

Xf-infected and uninfected bacteria were cultured simultaneously at 30, 33.8 and  $35^{\circ}\text{C}$  with a temperature gradient incubator. After 72 hr of incubation, the culture fluids were centrifuged at 8,000

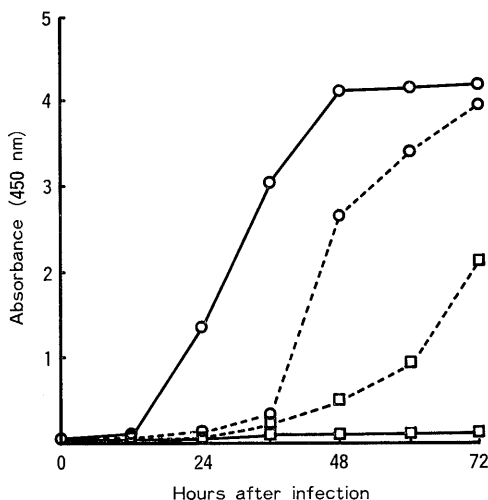


Fig. 1. Effect of Xf infection on the growth of *X. campestris* pv. *oryzae* N 5850 at different temperatures.

- Uninfected cells ( $30^{\circ}\text{C}$ )
- .....○..... Infected cells ( $30^{\circ}\text{C}$ )
- uninfected cells ( $35^{\circ}\text{C}$ )
- .....□..... Infected cells ( $35^{\circ}\text{C}$ )

$\times g$  for 10 min. The precipitated bacteria were resuspended with 2 % agar solution, fortified and cut into small pieces. These specimens were doubly fixed with buffered 2 % glutaraldehyde solution and 2 % osmium tetroxide solution. After dehydration with graded series of ethanol, they were embedded in Epon 812. Ultrathin sections prepared with Porter-Blum microtome were stained with uranyl acetate and lead citrate, and observed under the electron microscope JEM 100S.

Many vesicles different in size were observed on the cell surface of the Xf-infected bacteria grown at 30, 33.8 and 35 C (Plate I-1b, -1c, -2b, -2c, -3b, -3c). The envelope of large vesicles appeared to have originated from the cell wall of the infected bacteria (Plate I-1c) but the origin of the small vesicles was unknown. The structural changes in the cell wall of Xf-infected bacteria were severer than those in Xf 2-infected bacteria. In the sections of uninfected bacteria grown at 30 C, many fibrils were uniformly scattered in the electron less dense nucleoid (Plate I-1a), while in many Xf-infected bacteria the fibrils in nucleoid were assembled to form bundles (Plate I-1b, -1c). Electron density of cytoplasm of Xf-infected bacteria grown at 30 C and 33.8 C was ununiform, i.e., it was very high in some areas but extremely low in other areas (Plate I-1b, -1c, -2b, -2c). The structures with irregular shape of about 50–100 nm in diameter, which were composed of multiple layers, were observed frequently on or adjacent to the cell surface of Xf-infected bacteria grown at 35 C. Among these unusual structures, only small vesicles were also rarely observed in uninfected bacteria grown at each temperature.

#### ***Effect of Xf-infection on the virulence of host bacteria***

The Xf-infected and uninfected bacteria were shake-cultured simultaneously in Suwa's liquid medium for 3 days at 30 C. Both bacterial suspensions, the concentration of which was adjusted to show an O. D. (450 nm) value of 3.5, were inoculated to the central part of rice leaves (variey Kinmaze at six leaf stage) at both sides of midrib by single needle pricking. The inoculated plants were incubated in a greenhouse at 25–30 C. Length of the lesions was measured at 7, 9 and 13 days after inoculation.

As shown in Table 1, the mean length of the lesions caused by Xf-infected bacteria was always about two times greater than that caused by uninfected bacteria.

#### ***Effect of Xf-infection on the productivity of extracellular polysaccharide***

The Xf-infected and uninfected bacteria were inoculated to Suwa's medium to show O. D. (450 nm) value at 0.05, and shake-cultured at 30 C. Two ml of the culture suspension was taken out every day and centrifuged at  $10,000 \times g$  for 20 min. Ethanol (70% final concentration) was added to the supernatant. The resulting precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed against distilled water. Distilled water was added to the dialyzed crude extracellular polysaccharide (EPS) solution to fill up to the original volume. The amount of EPS was determined by phenol sulfuric acid on the basis of glucose standard. The

Table 1. Effect of Xf-infection on the virulence of *X. campestris* pv. *oryzae* N 5850

| Inoculum             | Days after inoculation |                    |                    |
|----------------------|------------------------|--------------------|--------------------|
|                      | 7                      | 9                  | 13                 |
| Xf-infected bacteria | 3.3 <sup>a)</sup>      | 5.7                | 23.5               |
| Uninfected bacteria  | 1.7                    | 2.5                | 12.3               |
| Ratio                | 1.94 <sup>b)</sup>     | 2.28 <sup>b)</sup> | 1.91 <sup>c)</sup> |

a) Mean length of the lesions

b) Significant at 1% level

c) Significant at 5% level

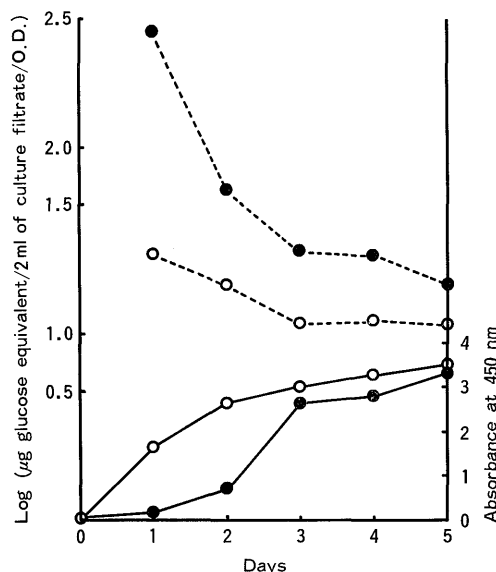


Fig. 2. Bacterial multiplication and production of extracellular polysaccharide (EPS) of Xf-infected and uninfected *X. campestris* pv. *oryzae* N 5850

- Xf-infected cells
- Uninfected cells
- .....●..... EPS of Xf-infected cells
- .....○..... EPS of uninfected cells

concentration of bacteria was expressed by O. D. (450 nm) of double diluted culture fluid.

EPS content per unit O. D. of culture suspension of Xf-infected bacteria was always higher than that of uninfected bacteria (Fig. 2).

## Discussion

The properties of *X. campestris* pv. *oryzae* N 5850 were remarkably changed by infection with Xf phage. Although the bacterial growth at 30 C was inhibited initially by Xf-infection, the infected bacteria rapidly multiplied to reach almost the same concentration as that of uninfected bacteria by 72 hr incubation. Thus the effects of Xf-infection on the growth of the bacteria at different temperatures were almost similar to those of Xf 2-infection<sup>4)</sup>. Besides, the bacteria acquired tolerance to higher temperatures, viz., Xf-infected bacteria could multiply even at 35 C. The fact that the growth retardation

at 30 C caused by Xf-infection was more intense than that caused by Xf 2-infection may explain the phenomenon that plaques produced by Xf on the plate seeded with *X. campestris* pv. *oryzae* N 5850 were clearer than those produced by Xf 2.

The intracellular changes caused by Xf-infection were more remarkable than those caused by Xf 2-infection. The vesicles were observed with higher frequency on the cell surface of the Xf-infected bacteria than on that of Xf 2-infected bacteria. The size of the vesicles was quite different from one another. The large vesicles having a diameter of more than 0.5  $\mu$ m were observed frequently in Xf-infected bacteria, but not in Xf 2-infected bacteria. The envelopes of large vesicles were originated from the cell wall of the infected bacteria but the origin of the envelope of small vesicles was still unknown. The development of mesosomes and multiple membrane structures inside bacterial cells was reported with *E. coli* infected with ZJ/2 phage<sup>1)</sup>, but not in *X. campestris* pv. *oryzae* infected with Xf. Thus, *X. campestris* pv. *oryzae* N 5850 changed in its properties to be able to grow at higher temperatures, to produce greater amount of EPS and to show higher virulence by infection with Xf or Xf 2 phage. Similar changes in virulence were also confirmed with two other isolates of *X. campestris* pv. *oryzae* which were susceptible to Xf and Xf 2 phages. These results suggest that at least some of the changes in properties of host bacteria associated with phage infection are a common phenomenon caused by infection, multiplication and release of filamentous phages.

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## 和文摘要

*Xanthomonas campestris* pv. *oryzae* N 5850 の増殖、微細構造および  
病原性に及ぼす繊維状ファージ Xf 感染の影響

上運天 博・脇本 哲

繊維状ファージ Xf の感染が *X. campestris* pv. *oryzae* N 5850 株の増殖、微細構造および病原性に及ぼす影響について検討した。Xf に感染した菌は非感染菌に比べて30℃における初期増殖がかなり抑制された。しかし、Xf 感染菌は感染後36時間目から急に増殖し、72時間後には非感染菌の菌濃度と同程度にまで達した。感染菌は35℃でもある程度増殖することができたが、非感染菌はほとんど増殖できなかった。30、33.8および35℃で増殖させた Xf 感染菌と非感染菌の超薄切片を作成し、電顕観察を行った結果、Xf 感染菌では非感染菌とは異なり、菌体表面に多数の小胞が認められ、またリボソームや核内繊維状物質の不均一な分布が観察された。さらに *X. campestris* pv. *oryzae* N 5850株は Xf 感染により菌体外多糖類の産生量が多くなり病原性も強まった。

## Explanation of Plate

## Plate I.

Electron micrographs of the ultrathin sectioned Xf-infected and uninfected cells of *X. campestris* pv. *oryzae* N 5850 cultured at different temperatures for 72 hr.

- 1 a) Uninfected bacteria cultured at 30℃.
- 1 b) Xf-infected bacteria cultured at 30℃.
- 1 c) Do.
- 2 a) Uninfected bacteria cultured at 33.8℃.
- 2 b) Xf-infected bacteria cultured at 33.8℃.
- 2 c) Do.
- 3 a) Uninfected bacteria cultured at 35℃.
- 3 b) Xf-infected bacteria cultured at 35℃.
- 3 c) Do.

(Bar: 250 nm)

Plate I

