

Xanthomonas campestris pv, oryzaeの性状に及ぼす繊維状ファージXf2感染の影響

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Effect of the Infection with Filamentous Phage Xf 2 on the Properties of *Xanthomonas campestris* pv. *oryzae*

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上運天 博*・脇本 哲*: *Xanthomonas campestris* pv. *oryzae* の性状に
及ぼす繊維状ファージ Xf 2 感染の影響

Abstract

The effects of the infection with filamentous phage Xf 2 on the properties of *Xanthomonas campestris* pv. *oryzae* N5850 were investigated. The bacteria infected with Xf 2 released the phage into medium to reach the maximum concentration of about 1×10^{11} PFU/ml after 48 hr of shaking culture. The multiplication of bacteria was considerably retarded by infection with Xf 2 at temperatures lower than 33.5°C. The infected bacteria, however, reached the same or higher level as compared with uninfected control later on. The infected cells grew even at 35°C to some extent, while the uninfected cells did not. The Xf 2-infected bacteria grown at different temperatures of 30, 33.5 and 35°C for 72 hr were ultrathin sectioned and their structural change was investigated with electron microscope. Although no remarkable difference was observed between Xf 2-infected and uninfected cells in cytoplasmic structures, the cell wall of the former was vesiculated, denatured or partially stripped from the cells. The Xf 2-infected bacteria multiplied in the inoculated rice leaves more slowly than uninfected bacteria during the first few days after inoculation. The former's population, however, exceeded the latter later on. The Xf 2-infected bacteria incited symptoms on rice leaves earlier than uninfected bacteria. No difference was observed between Xf 2-infected and uninfected bacteria in activities of xylanase, cellulase and lipase in culture filtrates. The amount of extracellular polysaccharide produced by Xf 2-infected bacteria was higher than that produced by uninfected bacteria, suggesting some correlation with virulence.

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Introduction

The filamentous phage Xf 2 was released from *Xanthomonas campestris* pv. *oryzae* N5845 into culture medium at the maximum concentration of 1×10^4 PFU/ml¹⁰⁾. When the susceptible isolate *X. campestris* pv. *oryzae* N5850 was inoculated with Xf 2, however, the phage concentration increased remarkably. The phage Xf 2 was purified from enriched phage suspension. Some of its biological and physico-chemical properties were reported in the previous papers^{10,11,26)}.

Regarding to the effect of filamentous phage infection on the properties of host bacteria, Salivar *et al.*²¹⁾ reported that the growth rate of *Escherichia coli* was retarded by the infection with M13 phage. Kuo *et al.*¹⁶⁾ also reported almost the same results with the combination of *X. campestris* pv. *oryzae* and Xf phage.

In this experiment, the effects of Xf 2 infection on the properties of *X. campestris*

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pv. *oryzae* N5850 were investigated on cell structure, mode of growth under different temperature conditions and virulence to rice leaves.

Materials

Bacteria and phage. *X. campestris* pv. *oryzae* isolates N5845 and N5850 used in this experiment were furnished by the National Institute of Agricultural Sciences, Tsukuba, Ibaraki, Japan. The filamentous phage Xf2 was isolated from culture fluid of *X. campestris* pv. *oryzae* N5845 and enriched with *X. campestris* pv. *oryzae* N5850. To clarify the effect of phage infection on biological properties of host cells, the isolate N5850 was used because it was free from filamentous phages, susceptible to both filamentous phages Xf and Xf2 and pathogenic to rice leaves.

Media. Bacteria were grown in Suwa's synthetic medium²²⁾ (Na-glutamate 2g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1g, K_2HPO_4 0.1g, Fe as EDTA salt 1mg, sucrose 5g, peptone 10g, distilled water 1 liter, pH7.0). For colony formation, the same medium added with agar at a concentration of 1.5% was employed. PS medium²⁵⁾ (decoction of 300g potato 1 liter, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 2g, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 0.5g, peptone 5g, sucrose 20g, pH7.0) fortified with 0.6% agar was used for plaque formation.

Antiserum. Whole cell suspension of *X. campestris* pv. *oryzae* Q7472 was injected 6 times into the ear vein of an adult New Zealand white rabbit with doses increasing from 0.5 to 2 ml at 4-day intervals. Seven days after the final injection, the rabbit was bled and antiserum was prepared by the ordinary procedures. Antiserum thus obtained was used for modified rocket immunophoresis.

Methods and Results

Growth of and phage liberation from *X. campestris* pv. *oryzae* N5850 infected with Xf2

The fresh suspension 9.9 ml (1×10^7 cells/ml) of *X. campestris* pv. *oryzae* N5850 was

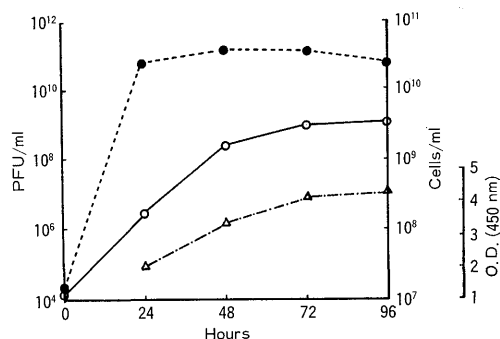


Fig. 1. The growth of Xf2-infected *X. campestris* pv. *oryzae* N5850 and phage liberation.

---●--- Xf2 phage
—○— *X. campestris* pv. *oryzae* N5850
--△-- Optical density

added with 0.1 ml of Xf2 phage suspension (2×10^6 PFU/ml) and shake-cultured at 30°C. Each 0.5 ml of culture suspension was taken out at 24 hr intervals, diluted with distilled water, and used for assaying bacterial and phage concentrations. Double diluted bacterial suspensions were used for measuring absorbance at 450 nm.

As shown in Fig. 1, the concentration of Xf2 phage in the medium increased rapidly for 24 hr and reached the maximum of about 1×10^{11} PFU/ml at 48 hr of incubation.

Viability of the host bacteria infected with Xf2

The fresh suspension of *X. campestris*

pv. *oryzae* N5850 was prepared and its concentration was adjusted to show a transmittance value of 60%. The bacterial suspension was divided into 3 parts. Two of them were infected with either Xf2 phage or virulent phage OP2 and the other one was not inoculated to serve as a control. All of them were simultaneously cultured with Bio-Log II. The results were shown in Fig. 2. Transmittance value was increased rapidly with OP2-infected cells, suggesting rapid lysis of host cells. With lapse of time, however, it decreased in the case of Xf2-infected cells, suggesting multiplication of bacterial cells. The culture suspension of uninfected cells decreased in transmittance value more quickly than that of infected cells.

Effect of Xf2 infection on the growth of host bacteria

Xf2 phage was enriched with *X. campestris* pv. *oryzae* N5850 in Suwa's synthetic medium for 48 hr. The enriched phage suspension was centrifuged at $10,000 \times g$ for 20 min to eliminate host cells. The supernatant was treated at 60°C for 20 min to kill residual cells without killing the phage Xf2. The phage suspension thus prepared was added to diluted suspensions of *X. campestris* pv. *oryzae* N5850 and cultured with a temperature gradient incubator. Uninfected bacteria were also cultured simultaneously as a control. A part of each suspension was periodically taken out, diluted twice with distilled water and O.D. was measured at 450 nm.

As shown in Fig. 3, the growth of Xf2-infected cells was retarded remarkably at 30°C for the first 60 hr of incubation, but finally it reached to the same level as that of uninfected cells. When the Xf2-infected and uninfected cells were cultured simultaneously at 33.5°C, growth

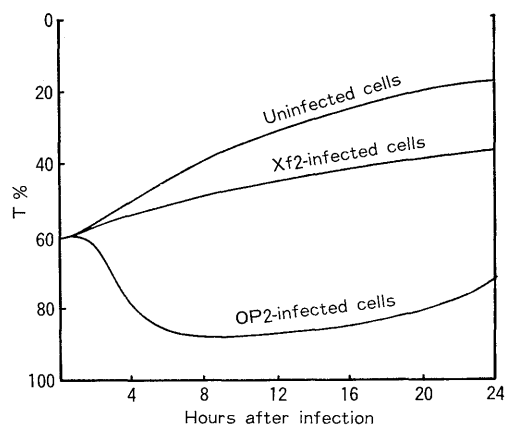


Fig. 2. Effect of Xf2 infection on the growth of *X. campestris* pv. *oryzae* N5850

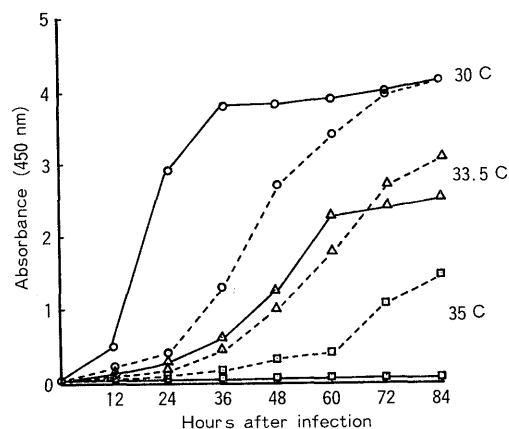


Fig. 3. Effect of Xf2 infection on the growth of *X. campestris* pv. *oryzae* N5850 at different temperatures.
— Uninfected
- - - Infected

Table 1. Temperature effect on the growth of Xf2-infected and uninfected *X. campestris* pv. *oryzae* N5850^{a)}

Temperature	Bacteria	
	Uninfected	Infected
20°C	3.3 ^{b)}	3.1
22	3.5	3.4
23-32	3.5-3.7	3.5-3.7
33	2.6	3.2
34	0.3	2.6
35	0.09	0.92

a) Initial concentration of both infected and uninfected bacteria was adjusted at O.D. (450 nm) 0.05.

b) O.D. (450 nm) after 72 hr of incubation.

inhibition was observed in the Xf2-infected cells for the first 60 hr of incubation. However, the concentration of Xf2-infected cells exceeded that of uninfected cells at 72 hr of incubation. At 35 C, uninfected cells did not multiply while Xf2-infected cells multiplied to some extent. The effects of different temperatures on the growth of Xf2-infected and uninfected cells were comparatively investigated more in detail by using a temperature gradient incubator. After 72 hr of incubation, O.D. (450 nm) of culture suspension was measured.

As shown in Table 1, both infected and uninfected bacteria multiplied well within the temperature range from 23 to 32 C. At lower temperatures such as 20 or 22 C, the growth of the Xf2-infected cells was suppressed to some degree. At higher temperatures such as 33, 34 or 35 C, the Xf2-infected cells multiplied to a higher level than that of uninfected cells.

Electron microscopic observation

The ultrastructure of Xf2-infected bacteria in a plaque was investigated. The enriched Xf2 phage suspension was dropped on the plate seeded with *X. campestris* pv. *oryzae* N5850. Pieces of the plaque were taken out every 6 hr during 36 hr after plating, and doubly fixed with buffered 2 % glutaraldehyde solution and 2 % osmium tetroxide solution. The pieces were then dehydrated in a series of ethanol and embedded in Epon 812. Sections prepared with Porter-Blum microtome were stained with uranyl acetate and lead citrate. Observation was made with the electron-microscope JEM 100S. Not any structural disorder was observed in Xf2-infected cells (Plate I-1, 2).

The ultrastructures of Xf2-infected and uninfected bacteria cultured in liquid medium under shake condition at different temperatures of 30, 33.5 and 35 C for 72 hr were also compared. The bacteria were collected by centrifugation, resuspended into dissolved 2 % agar solution, fortified and cut into small pieces. These specimens were thin sectioned and observed by electron microscope.

The Xf2-infected cells grown at each temperature revealed remarkable change in cell wall structure, showing vesiculation, denaturation and partial stripping (Plate I-3B, 4B, 5B).

Effect of Xf2 infection on virulence of host bacteria

The Xf2-infected and uninfected cells were cultured for 2 or 3 days at 30 C. Both suspensions of the infected and uninfected bacteria were adjusted to show the O.D. (450 nm) value of 3.5, and inoculated to the leaves of rice variety Kinmaze at eight leaf stage. Inoculation was made on both sides of midrib by single needle pricking.

As shown in Table 2 and Fig. 4, the mean length of the lesions caused by Xf2-infected cells was always longer than that caused by uninfected cells. The ratio of the former to the latter, however, became smaller with the lapse of time

Table 2. Effect of Xf2-infection on virulence of *X. campestris* pv. *oryzae* N5850

Inoculum	Days after inoculation		
	10	13	18
Xf2-infected bacteria	2.0 ^{a)}	3.9	5.9
Uninfected bacteria	0.4	1.1	1.8
Ratio	5.0 ^{b)}	3.5 ^{b)}	3.3 ^{b)}

a) Mean length of the lesions.

b) Significant at 1 % level.

after inoculation.

Population changes of the Xf 2-infected and uninfected bacteria in the inoculated rice leaves

The seedlings of the variety Kinmaze were grown in a greenhouse. At five leaf stage, the third and fourth leaves were used for inoculation. Each of Xf2-infected and uninfected bacteria, of which concentration was adjusted to the same O.D. (450 nm) value at 3.5, was inoculated to the rice leaves at symmetrical positions on both sides of midrib by single needle pricking. The inoculated seedlings were incubated in a greenhouse at 30 C. Five leaves were detached, inoculated parts were scissored, surface sterilized with 70 % of ethanol, washed, and ground with 5ml of sterilized distilled water in mortar. The suspensions thus prepared were serially diluted, 0.1ml of each dilution was spread on Suwa's synthetic agar medium and incubated at 30 C. Colonies were counted after 5 days of incubation.

The population of Xf2-infected cells was lower than that of uninfected cells during few days after inoculation, however, the tendency reversed afterwards (Fig. 5).

Comparison of the activities of hydrolytic enzymes

The activities of seven hydrolytic enzymes which were suspected to be related to pathogenicity were measured. The Xf2-infected and uninfected bacteria were cultured in Suwa's synthetic medium for 3 to 4 days. The culture fluids were centrifuged at $8,000 \times g$ for 20 min. The supernatants were dialysed against distilled water for 4 to 6 hr. The culture solutions thus obtained were used for measuring enzyme activities at optimum pH, according to the methods used for *X. campestris* pv. *oryzae* by Miyazaki *et al.*¹⁹⁾. No remarkable difference was

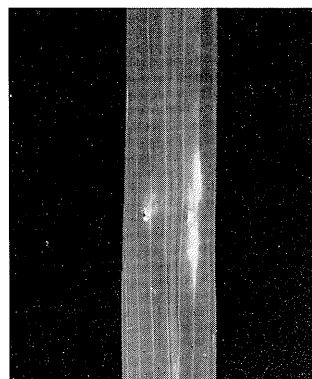


Fig. 4. Symptoms incited by Xf 2-infected *X. campestris* pv. *oryzae* N 5850 (right) and uninfected control (left) on a rice leaf

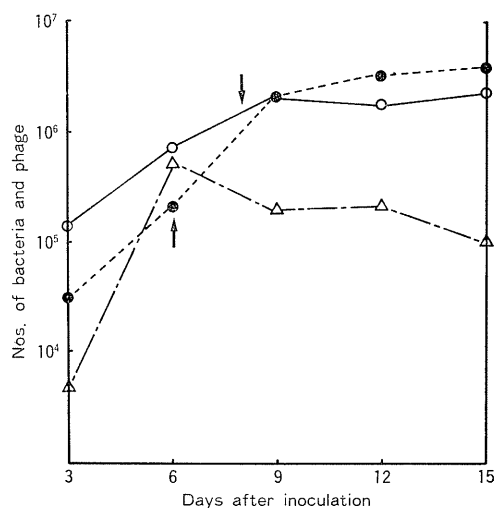


Fig. 5. Multiplication of the Xf2-infected and uninfected *X. campestris* pv. *oryzae* N 5850 in rice leaves.

---●--- Xf 2-infected cells
—○— Uninfected cells
---△--- Phage population in the leaves inoculated with Xf 2-infected cells
→ Symptom appearance

Table 3. Comparison of hydrolytic enzyme activities in the culture filtrates of Xf 2-infected and uninfected *X. campestris* pv. *oryzae* N 5850

Enzyme	Ratio of enzyme activity (Infected cells/Uninfected cells)		
	Exp. I	Exp. II	Average
Xylanase	0.83	1.00	0.92
Cellulase (Cx)	0.94	0.94	0.94
Lipase	1.00	1.00	1.00

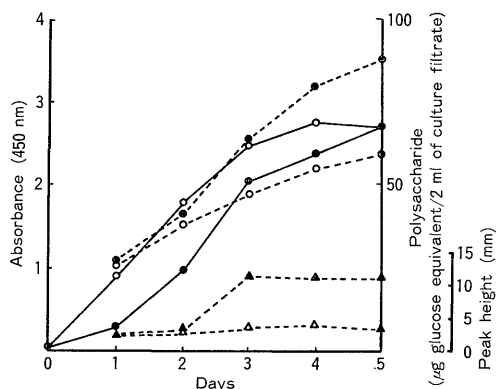


Fig. 6. Bacterial multiplication and production of extracellular polysaccharide (EPS) of Xf2-infected and uninfected *X. campestris* pv. *oryzae* N 5850.

- Xf2-infected cells
- Uninfected cells
- EPS produced by Xf2-infected cells (phenol-sulfuric acid)
- EPS produced by uninfected cells (phenol-sulfuric acid)
- ▲--- EPS of Xf2-infected cells (modified Laurell rocket assay)
- △--- EPS of uninfected cells (modified Laurell rocket assay)

precipitate was collected by centrifugation, dissolved in a small quantity of distilled water, and dialyzed against distilled water. The dialyzed crude extracellular polysaccharide (EPS) solution was added with distilled water to fill up to the original volume. The amount of EPS was determined by phenol-sulfuric acid on the basis of the glucose standard.

A modified Laurell rocket assay method¹⁾ was also applied to compare the amounts of EPS. In the gel diffusion test carried out as a preliminary experiment, it was clarified that there was no difference in serological reactions between the supernatant of culture suspension of Xf2-infected bacteria and that of uninfected bacteria against anti-Q 7472 serum. It was also made clear that the purified EPS obtained from *X. campestris* pv. *oryzae* N 5850 precipitated with anti-Q 7472 serum showing quantitative linear correlation. These results of the preliminary experiments indicated the applicability of the rocket assay method for comparing the amounts of polysaccharide produced by Xf2-infected and uninfected bacteria.

Five μ l of each supernatant containing EPS antigen was electrophoresed at 10V/cm in agarose gel added with anti-Q 7472 serum.

As shown in Fig. 6, EPS content in the culture fluid of Xf2-infected cells was always higher than that of uninfected cells, though the concentration of Xf2-infected cells was lower than that of uninfected cells.

observed between Xf2-infected and uninfected bacteria in the activities of xylanase⁶⁾, cellulase (Cx)¹⁹⁾, and lipase¹⁹⁾ (Table 3). Pectinmethylesterase⁸⁾, polygalacturonase⁷⁾, lecithinase²³⁾ and protease¹⁹⁾ were not detected in our experiment.

Production of extracellular polysaccharide by Xf2-infected and uninfected bacteria

Culture suspension of *X. campestris* pv. *oryzae* N 5850 was diluted with Suwa's fresh medium to adjust O. D. (450 nm) value at 0.05 and incubated at 30C after adding with Xf2 phage suspension. The centrifuged supernatant of culture suspension of uninfected bacteria was added instead of phage suspension to serve as a control. Five ml of the culture suspension was taken out every 24 hr and centrifuged at $10,000 \times g$ for 20min. Each supernatant was passed through Millipore filter (0.45 μ m). Ethanol was then added at a final concentration of 70 %. The resulting

Discussion

X. campestris pv. *oryzae* isolates resistant to either filamentous phage Xf or Xf2 are not suitable for testing the effect of phage infection, because such isolates are possibly infected with either Xf or Xf2 and releasing phages continuously. Therefore, the isolate N5850 susceptible to both phages Xf and Xf2 was selected for use.

The filamentous phage Xf2 was released from *X. campestris* pv. *oryzae* N5845 in the medium at the maximum concentration of about 1×10^4 PFU/ml¹⁰⁾. When *X. campestris* pv. *oryzae* N5850 was infected with Xf2, however, the phage concentration increased up to 1×10^{11} PFU/ml in the medium 48 hr after infection. Although the bacterial growth was inhibited by Xf2 infection to some degree, the transmittance decreased gradually, suggesting bacterial multiplication. In the case of cells infected with virulent phage OP2, the transmittance increased rapidly.

Electron microscopic observation revealed that Xf2 phage did not lyse the host cells as was reported with other filamentous phages^{12,13,18)}.

The growth rate of host cells was reported to be decreased by infection with filamentous phages^{4,14,15,18)}. In these reports, however, the investigation was carried out only for 16 hr after infection at the longest. In our experiment, the growth of Xf2-infected bacteria was tested as long as 84 hr at different temperatures. The growth of Xf2-infected bacteria was retarded as compared with that of uninfected bacteria for the first 60 hr at 30 C. At 33.5 C, the growth of the infected bacteria was also inhibited to some degree for the first 60 hr but after that it exceeded that of uninfected bacteria. At 35 C, Xf2-infected bacteria could grow while uninfected one could not. The reasons why Xf2-infected bacteria grow better than uninfected bacteria at higher temperatures are not known.

Ultrathin section of the Xf2-infected bacteria shake cultured for 72 hr at different temperatures showed the cell walls protruded, vesiculated or partially separated from the cells. The appearance of mesosomes and multiple layers in membraneous structure was reported with 2J/2 phage infected *E. coli*²⁾. However, we could not observe such changes in Xf2-infected bacteria.

Some workers^{9,20,24)} reported that temperate phage infection caused little change of pathogenicity in plant pathogenic bacteria. Under the present experimental conditions, Xf2-infected *X. campestris* pv. *oryzae* N5850 propagated more slowly than control in the tissue of rice leaves. Nevertheless, the symptoms developed earlier by Xf2-infected bacteria and the mean length of the lesions was longer than that caused by uninfected bacteria.

It has been reported that bacterial EPS is associated with virulence in several plant pathogenic bacteria^{1,3,5,15,17)}. A significant difference was observed in the amount of EPS production between Xf2-infected and uninfected bacteria, which might be responsible for the difference in symptom expression.

There was no difference in the activities of some hydrolytic enzymes between Xf2-infected and uninfected cells.

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和 文 摘 要

Xanthomonas campestris pv. *oryzae* の性状に及ぼす
繊維状ファージ Xf 2 感染の影響

上運天 博・脇本 哲

繊維状ファージ Xf 2 の感染が *X. campestris* pv. *oryzae* N 5850 菌株の性状に及ぼす影響について検討した。*X. campestris* pv. *oryzae* N 5850 に Xf 2 ファージを感染させて、振盪培養すると、培養液中に放出されるファージ濃度は感染48時間後に最高の約 1×10^{11} PFU/ml に達した。33.5°C よりも低い温度では Xf 2 感染菌の増殖は非感染菌よりもかなり遅れた。しかし、その後、非感染菌と同じか、あるいはそれ以上の濃度に達した。Xf 2 感染菌は35°C 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, 33.5および35°C の各温度で72時間振盪培養した Xf 2 感染菌と非感染菌の超薄切片を作成し、Xf 2 感染による構造的変化を電顕により調べた結果、細胞質では両者の間に著しい差が認められなかったが、Xf 2 感染菌では菌体表面における小胞の形成または細胞壁の一部が変性あるいは剥離した状態が観察された。イネ葉組織中における増殖については接種後、初期数日間は Xf 2 感染菌の方が非感染菌に比べて遅かったが、その後、菌数は逆転した。また、同濃度の菌液を接種した場合、Xf 2 感染菌の方が非感染菌よりも早く病徴を現わし、病斑の長さも大であった。培養液中に含まれるキシラナーゼ、セルラーゼ、リパーゼの活性については Xf 2 感染菌および非感染菌の間にほとんど差が認められなかったが、菌体外多糖類の産生量は非感染菌よりも Xf 2 感染菌の方が多く、この産生量と病原性との間の相関が示唆された。

Explanation of Plate**Plate I.**

- 1-2. Electron micrographs of the ultrathin sectioned *X. campestris* pv. *oryzae* N 5850 infected with Xf 2 phage (plate culture).
 - 1: At 6 hr after infection.
 - 2: At 18 hr after infection.
- 3-5. Electron micrographs of the ultrathin sectioned Xf 2-infected and uninfected cells of *X. campestris* pv. *oryzae* N 5850 cultured for 72 hr at different temperatures (shake culture).
 - 3A: Uninfected bacteria cultured at 35 C.
 - 3B: Xf 2-infected bacteria cultured at 35 C.
 - 4A: Uninfected bacteria cultured at 33,5 C.
 - 4B: Xf 2-infected bacteria cultured at 33,5 C.
 - 5A: Uninfected bacteria cultured at 30 C.
 - 5B: Xf 2-infected bacteria cultured at 30 C.

(Bar : 500 nm)

Plate I

