日植病報 59：128-134 (1993)
Ann. Phytopath. Soc. Japan 59：128-134 (1993)

# New Filamentous Phage Released from *Xanthomonas campestris* pv. *oryzae* Harbouring a Covalently Closed Circular DNA of 7.1 kb

Hirosi KAMIUNTEN*, Yasuhiro SUGA** and Tatuo ANDO*

**Abstract**

One of 88 strains of *Xanthomonas campestris* pv. *oryzae* harboured a covalently closed circular DNA (cccDNA) of 7.1 kb and released a filamentous phage into culture fluid. This strain M8883 easily lost the 7.1 kb cccDNA by subculturing five times, and the cured strains lost phage productivity. The filamentous phage released from strain M8883 was different from Xf and Xf2 phages in host range, particle length and molecular size of the replicative form DNA (RF), and was designated Xf8883. The electrophoretic pattern of the 7.1 kb cccDNA digested with *Hin*fI or *Msp*I was identical to that of Xf8883-RF isolated from M8819 infected with Xf8883. Furthermore, strain M8819 transformed with the 7.1 kb cccDNA acquired the productivity of a filamentous phage which was identical with Xf8883. From these results, it is concluded that the 7.1 kb cccDNA may be RF of a new filamentous phage, Xf8883. Electrophoretic analysis of the *Hae*III, *Rsa*I and *Hin*fI fragments of Xf8883-RF and Xf-RF suggested that the two phages, Xf8883 and Xf, were closely related but not identical.

(Received June 4, 1992)

**Key words:** *Xanthomonas campestris* pv. *oryzae*, covalently closed circular DNA, new filamentous phage.

## INTRODUCTION

*Xanthomonas campestris* pv. *oryzae* is the pathogenic bacterium causing bacterial leaf blight of rice. When we investigated the plasmid content of 88 strains of *X. campestris* pv. *oryzae*, one of the strains, M8883, harboured a covalently closed circular DNA (cccDNA) which was easily lost by subculturing. The centrifuged supernatant of a culture fluid of this strain produced plaques on the plate seeded with the indicator strain of *X. campestris* pv. *oryzae*. However, when the strain M8883 was cured of the cccDNA, its culture fluid did not produce plaques.

The present study was undertaken to determine whether or not the cccDNA in the strain M8883 was a replicative form DNA (RF) of the phage released from this strain.

## MATERIALS AND METHODS

***Bacteria and media.*** Eighty-eight strains of *X. campestris* pv. *oryzae* were isolated in 1987 from diseased rice leaves collected from various localities in Kyushu, Japan, and were preserved at −20°C after freeze-drying.

* Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, Japan 宮崎大学農学部
** Nagasaki Prefectural Agricultural and Forestry Experiment Station, Isahaya, Nagasaki 854, Japan 長崎県総合農林試験場

The bacteria were grown at 27°C in PS[1] and peptone-sucrose medium[5]. PS medium solidified with 0.6% agar was used for plaque formation. For single colony isolation, peptone-sucrose agar medium was employed.

***Curing of cccDNA by subculturing.*** Single colony isolation of the strain M8883 and M8853 was conducted three times. A colony which harboured cccDNA was transfered to 5 ml of peptone-sucrose medium and cultured for 2 days. Thereafter, 2% cultures were routinely transfered five times to 5 ml of fresh medium every two days. The culture fluid (0.1 ml) was taken out of each subculture, diluted in distilled water and plated on peptone-sucrose agar medium. After incubation at 27°C for 48 hr, 16 to 24 colonies were randomly selected and investigated for the presence of cccDNA.

***Large-scale DNA isolation.*** *X. campestris* pv. *oryzae* M8883, harbouring a cccDNA of 7.1 kb, and M8819, infected with phage isolated from the culture fluid of M8883, were grown in 2 liters of PS medium with aeration. The bacteria were collected by centrifugation at $5,000 \times g$ for 10 min and washed two times with TE buffer[3]. The washed bacteria were suspended with 5 ml of TE buffer and lysed by adding 300 ml of TE buffer containing 3% SDS (pH 12.45). The lysate was incubated at 60°C for 60 min. Then, the pH was lowered to 8.0 by adding 2 M Tris (pH 4.0), and 5 M NaCl solution was added to adjust the final concentration at 1 M. After incubation on ice for 2 hr, the lysate was centrifuged at $10,000 \times g$ for 20 min at 4°C. Polyethylene glycol 6000 was added to the supernatant at a final concentration of 10% (w/v), and the mixture was incubated on ice for 2 hr. The mixture was centrifuged at $7,000 \times g$ for 10 min at 4°C. The pellet was dissolved in a minimal volume of TES buffer[3]. After the addition of an equal volume of phenol saturated with a solution of 3% NaCl, both phases were mixed. The clear phase, which contained DNA, was obtained by centrifugation at $7,000 \times g$ for 10 min. After removal of phenol by ethyl-ether extraction, a solution of 3 M sodium acetate was added at a final concentration of 0.3 M. The DNA was precipitated by adding two volumes of ethanol and kept at −20°C for overnight. The DNA was collected by centrifugation at $10,000 \times g$ for 20 min at 4°C, dried under vacuum and suspended in TES buffer. CsCl (8.6 g) was added to the DNA solution (8.6 ml) and completely dissolved. Two-tenth of a milliliter of ethidium bromide solution (10 mg/ml) was then added. The mixture was centrifuged at 45,000 rpm in a Hitachi RV65T rotor for 20 hr at 20°C. The lower DNA band was collected by side puncture of the tube with a 21-gauge needle. An equal volume of isoamylalcohol was added to the DNA solution and the extraction was repeated several times. After the last extraction, the DNA solution was dialyzed against 2 liters of TES buffer. The DNA was concentrated by ethanol precipitation.

***Cleavage of DNA with restriction endonucleases and electrophoresis.*** Restriction endonucleases that recognized four or six base were used in this experiment. The digestion of DNA (0.5-2 $\mu$g) with restriction endonucleases was carried out under the conditions recommended by manufacturer. The reaction was stopped by the addition of the stop solution containing 4 M urea, 50% sucrose, 50 mM EDTA, 0.1% bromophenol blue, pH 7.0 (digest mixture to stop solution ratio of 3 : 1). Electrophoresis was carried out in 6% polyacrylamide gel or 1-2% agarose gel in TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.2). After electrophoresis, DNA fragments were stained with ethidiuim bromide (0.5 $\mu$g/ml) and visualized on an UV transilluminator.

***Transformation.*** Transformation of M8819 with 7.1 kb of cccDNA were carried out by the same procedure of White *et al.*[6] The 7.1 kb of cccDNA solution (2 $\mu$l) was mixed with a suspension of the treated M8819 (60 $\mu$l), poured into a cuvette (1 mm gap) and subjected to a high voltage pulse (12 kV/cm for 5 msec). The mixture was took out from the cuvette, and the appropriate dilution was made. The pulsed bacterial cells (0.1 ml) and indicator bacteria M8819 (0.2 ml) were added to 5 ml of molten PS agar medium at 50°C and mixed. The mixture was poured into a Petri dish and incubated at 27°C. After 48 hr, the plaques produced by phage released from the transformed bacterial cells were counted.

## RESULTS

### *cccDNA isolated from X. campestris pv. oryzae and its curing by subculturing*

An attempt has been made to isolate plasmid DNA from 88 strains of *X. campestris* pv. *oryzae* by rapid procedure for isolation of cccDNA[4]. As shown in Fig. 1, two kinds of cccDNAs were isolated.

Strain M8883 harboured a cccDNA of 7.1 kb. Strain M8853 and 3 other strains (not shown in Fig. 1) harboured a 43.9 kb cccDNA.

The 7.1 kb cccDNA was easily lost from M8883 by subculturing five times, while the 43.9 kb cccDNA was retained in M8853 (Table 1).

***Filamentous phage isolated from culture fluid of M8883***

A phage was isolated from the culture fluid of M8883 which harboured the 7.1 kb cccDNA. The isolated phage produced tiny plaques on the lawn of indicator bacteria M8819 (Fig. 2-A). However, no phage was isolated from the culture fluid of M8883 cured of the 7.1 kb cccDNA. Electron microscopy showed that the phage isolated from M8883 was filamentous in shape, and the modal length of one hundred phage particles was estimated at 905 (±63) nm (Fig. 3).


Fig. 1. Agarose gel electrophoresis of cccDNA isolated from *X. campestris* pv. *oryzae*. (A) M8853, (B) M8883.

Table 1. Curing of cccDNA from *X. campestris* pv. *oryzae* by subculturing

| Bacterial strain and cccDNA | Numbers of subcultured | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| M8883 (7.1 kb cccDNA) | 6/16[a)] | 6/24 | 9/18 | 22/24 | 24/24 |
| | (60.0)[b)] | (25.0) | (50.0) | (91.7) | (100) |
| M8853 (43.9 kb cccDNA) | 0/16[a)] | 0/24 | 0/24 | 0/23 | 0/24 |
| | (0)[b)] | (0) | (0) | (0) | (0) |

a) No. of colonies which lost cccDNA/No. of colonies tested.
b) Curing frequency (%).

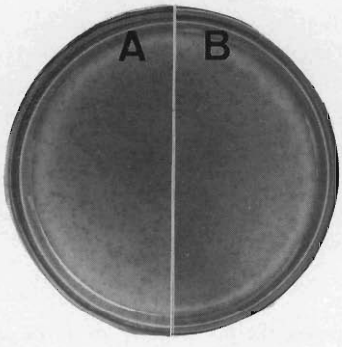


Fig. 2. Plaques produced by phages. (A) The phage isolated from the culture fluid of M8883 habouring the 7.1 kb cccDNA. (B) The phage released from the M8819 transformed with the 7.1 kb cccDNA.

The host range of the phage was tested by the drop method. Phage Xf and Xf2 were also used as controls. As shown in Table 2, the host range of the phage isolated from M8883 was different from that of Xf and Xf2. Strain M8858 was susceptible to the phage isolated from M8883 but resistant to Xf and

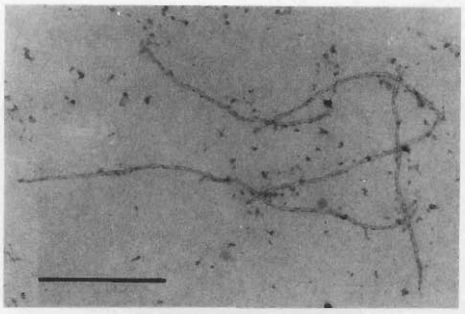

Fig. 3. Electron micrograph of phage Xf8883 isolated from the culture fluid of strain M8883. Bar marker represents 500 nm.

Table 2. Host range of the phage isolated from *X. campestris* pv. *oryzae* M8883 compared with Xf and Xf2 phage

| Bacterial strains | Phage Xf8883 isolated from M8883 | Xf | Xf2 |
|---|---|---|---|
| *X. campestris* pv. *oryzae* | | | |
| M8819 | + | + | + |
| M8841 | + | + | + |
| M8844 | + | + | − |
| M8847 | + | + | + |
| M8857 | − | − | − |
| M8858 | + | − | − |
| M8862 | + | + | + |
| M8865 | + | + | − |
| M8869 | + | + | + |
| N5850 | − | + | + |

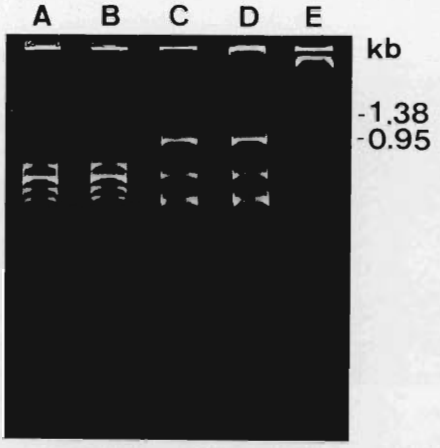


Fig. 4. Electrophoretic patterns of the 7.1 kb cccDNA and Xf8883-RF digested with restriction endonucleases. Electrophoresis was carried out in 6% polyacrylamide gel. (A) The 7.1 kb cccDNA digested with *Hin*fI. (B) Xf8883-RF digested with *Hin*fI. (C) The 7.1 kb cccDNA digested with *Msp*I. (D) Xf8883-RF digested with *Msp*I. (E) Lambda phage DNA digested with *Hin*dIII・*Eco*RI.

Xf2. Strain N5830, however, showed the opposite result. This new phage was designated Xf8883.

***Electrophoretic patterns of the 7.1 kb cccDNA and Xf8883-RF digested with endonuleases***

The relative mobility of Xf8883-RF in agarose gels was identical to that of the 7.1 kb cccDNA. The 7.1 kb cccDNA and Xf8883-RF were readily digested by *Hin*fI and *Msp*I. The molecular size of each fragment was smaller than 1.38 kb. The size and number of *Hin*fI and *Msp*I fragments of the two DNAs appeared identical (Fig. 4).

***Transformation of the 7.1 kb cccDNA***

Strain M8819, transformed with the 7.1 kb cccDNA by electroporation, acquired the productivity of filamentous phage. The filamentous phage released from the transformed bacteria produced the same tiny plaques as Xf8883 (Fig. 2-B). The transformation efficiency was $2.4\times10^{6}$-$1.9\times10^{7}$ transformants/$\mu$g DNA. The particle length, host range and molecular size of RF of the filamentous phage released from M8819 transformed with the 7.1 kb cccDNA were identical to those of Xf8883.

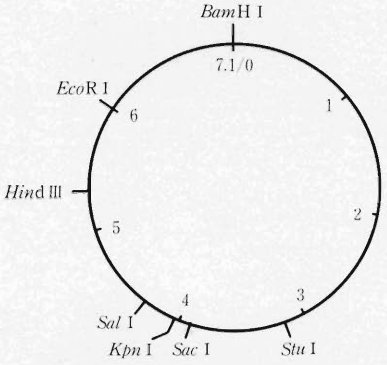


Fig. 5. Restriction map of Xf8883-RF. The *Bam*HI cleavage site was designated the zero point. The map is calibrated in kilobase (kb).

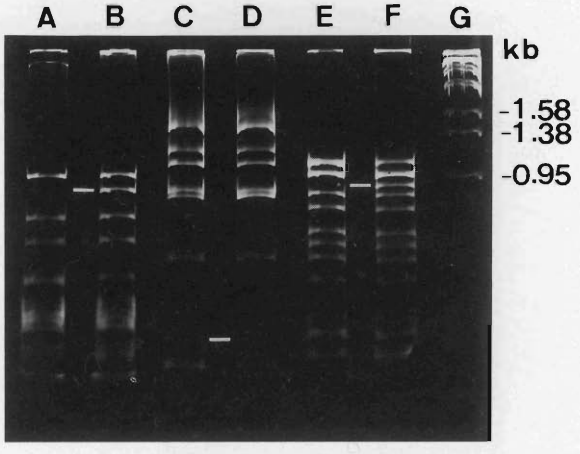


Fig. 6. Electrophoretic patterns of Xf8883-RF and Xf-RF digested with restriction endonucleases. Electrophoresis was carried out in 6% polyacrylamide gel. (A) Xf-RF digested with *Hae* III, (B) Xf8883-RF digested with *Hae* III, (C) Xf-RF digested with *Rsa*I, (D) Xf8883-RF digested with *Rsa*I, (E) Xf-RF digested with *Hin*fI, (F) Xf8883-RF digested with *Hin*fI, (G) Lambda phage DNA digested with *Hin*d III・*Eco*RI. The bars indicate DNA fragments which could be seen only in Xf8883-RF, or Xf-RF digest.

***Restriction map of Xf8883-RF***

The Xf8883-RF was cleaved with each restriction endonuclease *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, *Kpn*I, *Sac*I and *Stu*I to yield a single fragment. Based on the size and distribution pattern of the fragments generated by digestion with various combinations of these restriction endonucleases, a restriction map was constructed (Fig. 5).

***Electrophoretic patterns of Xf8883-RF and Xf-RF digested with endonucleases***

To determine phage similarity between Xf8883 and Xf, their RFs were digested with *Hae*III, *Rsa*I and *Hin*fI, and restriction fragments were electrophoresed on 6% polyacrylamide gel. As shown in Fig. 6, the electrophoretic patterns of restriction fragments resembled each other closely. However, a few characteristic differences were observed. The *Hae*III or *Hin*fI restriction fragment (*ca.* 0.8 kb) which was present in Xf8883-RF, but which was absent in the Xf-RF digest, could be seen. The *Rsa*I restriction fragment (*ca.* 0.2 kb), which was present in Xf-RF but which was absent in the digest of Xf8883, could also be seen.

## DISCUSSION

In a recent report, Xu *et al.*[7] have shown that 20 of 26 strains of *X. campestris* pv. *oryzae* isolated in the United States harboured indigenous plasmids (cccDNA). In this study, however, cccDNA was detected in only 5 of 88 strains of *X. campestris* pv *oryzae* isolated in Japan. An unexpectedly small number of Japanese strains harboured the cccDNA.

Strain M8883 harboured a cccDNA of 7.1 kb and released phage into the culture fluid. The 7.1 kb cccDNA was easily lost by subculturing and the cured strains lost phage productivity, we have reported previously that *X. campestris* pv. *oryzae* infected with filamentous phage lost phage productivity spontaneously at a high frequency[2]. From these facts, the cccDNA detected in the strain M8883 was suspected to be one of the filamentous phage RF.

Filamentous phage Xf8883 isolated from the culture fluid of M8883 was different from Xf and Xf2 in host range and particle length[1]. The molecular size of Xf8883-RF was the same size as the 7.1 kb cccDNA, but was different from that of Xf-RF[3] and Xf2-RF[3]. The electrophoretic pattern of the 7.1 kb cccDNA digested with *Hin*fI or *Msp*I was identical to that of Xf8883-RF. This suggested that the 7.1 kb cccDNA was identical to Xf8883-RF. Furthermore, M8819, transformed with the 7.1 kb cccDNA, acquired the productivity of the filamentous phage. The phage released from the transformants was identical with Xf8883 in host range, particle length and molecular size of RF. From these results, it is concluded that the 7.1 kb cccDNA may be RF of a new filamentous phage Xf8883.

The relative positions of *Bam*HI, *Stu*I and *Hin*dIII cleavage sites on the restiction maps of Xf8883-RF and Xf-RF[3] were almost the same. Therefore, restriction fragment patterns of Xf8883-RF and Xf-RF were compared to investigate the homology between two phages. The electrophoretic patterns resembled each other, but a few characteristic differences were observed, this suggested that Xf8883 and Xf were closely related but were not identical.

We additionally attempted to reveal the genetic function of the 43.9 kb cccDNA, but could not find any phenotype encoded in this cccDNA.

### Literature cited

1. Kamiunten, H. and Wakimoto, S. (1979). Biological properties of the filamentous phages released from *Xanthomonas oryzae* isolates. Ann. Phytopath. Soc. Japan. 45 : 174-181.
2. Kamiunten, H. and Wakimoto, S. (1987). Elimination of Xf phage productivity from *Xamthomonas campestris* pv. *oryzae* by acridine orange treatment and its effect on the host bacteria. Ann. Phytopath. Soc. Japan. 53 : 262-265.
3. Kamiunten, H. (1988). Restriction maps of the filamentous phage Xf and Xf2 DNA. Ann. Phytopath. Soc. Japan. 54 : 571-576.
4. Kamiunten, H. (1990). Loss of a plasmid in *Pseudomonas syringae* pv. *eriobotryae* is correlated with change of symptoms. Ann. Phytopath. Soc. Japan. 56 : 645-650.

5. Tsuchiya, K., Mew, T.W. and Wakimoto, S. (1982). Bacteriological and pathological characteristics of wild types and induced mutant of *Xanthomonas campestris* pv. *oryzae*. Phytopathology 72 : 43-46.
6. White, T.T. and Gonzalez, C.F. (1991). Application of electroporation for efficient transformation of *Xanthomonas campestris* pv. *oryzae*. Phytopathology 81 : 521-524.
7. Xu, G.W. and Gonzalez, C.F. (1991). Plasmid, genomic, and bacteriocin diversity in U.S. strains of *Xanthomonas campestris* pv. *oryzae*. Phytopathology 81 : 628-631.

## 和 文 摘 要

上運天博・菅 康弘・安藤達男：7.1 kb の 2 本鎖閉環状 DNA を有するイネ白葉枯病菌から放出される新しい繊維状ファージ

イネ白葉枯病菌 88 菌株のうち，1 菌株が 7.1 kb の 2 本鎖閉環状 DNA (cccDNA)を有しており，この菌株 M8883 は培養液中にファージを放出していた。この 7.1 kb cccDNA は 5 回の継代液体培養で容易に欠落し，欠落株は同時にファージ産生能を失った。M8883 株から放出される繊維状ファージは，その宿主域，粒子の長さおよび複製型 DNA(RF)の分子量において Xf および Xf2 ファージとは異なっており，これを Xf8883 ファージと命名した。7.1 kb cccDNA の制限酵素 *Hin*fI または *Msp*I による切断パターンは Xf8883 を感染させた M8819 株から分離した Xf8883-RF の切断パターンと同一であった。さらに，7.1 kb cccDNA を M8819 株に導入すると Xf8883 と同じファージを産生するようになった。これらの結果から，7.1 kb cccDNA は新しい繊維状ファージ Xf8883 の RF であると結論した。Xf8883-RF と Xf-RF の *Hae*III, *Rsa*I および *Hin*fI による切断パターンの分析により，両ファージは近縁のファージであることが示唆された。