Dynamic integration and excision of filamentous phage XacF1 in Xanthomonas citri pv. citri, the causative agent of citrus canker disease

Abdelmonim A. Ahmad1,2,3, Makoto Kawabe1, Ahmed Askora1,4, Takeru Kawasaki1, Makoto Fujie1 and Takashi Yamada1

1 Department of Molecular Biotechnology, Graduate School of Advanced Science of Matter, Hiroshima University, Higashi-Hiroshima, Japan
2 Department of Plant Pathology, Faculty of Agriculture, Minia University, El-minia, Egypt
3 Floral and Nursery Plants Research Unit, US National Arboretum USDA/ARS, BARC-West, Beltsville, MD, USA
4 Department of Microbiology, Faculty of Science, Zagazig University, Zagazig, Egypt

Keywords
biocontrol; citrus canker; filamentous phage; integration mechanism; XerC/D

Correspondence
T. Yamada, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan
Fax: +81-82-424-7752
Tel: +81-82-424-7752
E-mail: tayamad@hiroshima-u.ac.jp

(Received 24 March 2017, revised 26 August 2017, accepted 30 August 2017)
doi:10.1002/2211-5463.12312

Inovirus XacF1 (7325 nucleotides) is integrated into the genome of Xanthomonas citri pv. citri (Xcc) strains at the host dif site (attB) by the host XerC/D recombination system. The XacF1 attP sequence is located within the coding region of ORF12, a possible phage regulator. After integration, this open reading frame (ORF) is split into two pieces on the host genome. We examined dynamic integration/excision of XacF1 in Xcc strain MAFF 301080 and found that the integration started at 4 h postinfection (p.i.) and peaked at 12 h p.i. Thereafter, the ratio of integrated to free forms remained constant, suggesting equilibrium of integration and excision of XacF1 in the host genome. However, the integrated state became very unstable following a 5'-deletion of ORF12 in XacF1, suggesting that ORF12 plays a key role in the integration cycle of XacF1 in Xcc strains.

Asian citrus canker (ACC), caused by Xanthomonas citri pv. citri, Xcc (syn., Xanthomonas axonopodis pv. citri and Xanthomonas campestris pv. citri), is one of the most serious diseases in citrus-producing areas of the tropical and subtropical countries [1,2]. Typical symptoms of ACC include raised corky lesions on leaves, stems, and fruits, including general tree decline, defoliation, twig dieback, blemished fruit, and premature fruit drop in severely infected trees [3]. The causative agent Xcc encodes and expresses many genes involved in the pathogenesis and virulence [4]. Some relevant genes identified in Xcc are the ones coding for cell motility, plant cell wall-degrading enzymes, protein secretion systems, toxins, exopolysaccharide (EPS) synthesis enzymes, etc. [5]. Recently, a filamentous bacteriophage (inovirus) XacF1 infecting Xcc strains was isolated and characterized [6]. This phage encodes 13 open reading frames (ORF) and its genome is 7325 nucleotides in size (Fig. S1). In contrast to lytic phages, infection with filamentous phages does not cause host cell lysis, but frequently establishes a persistent association between the host and phage, producing and releasing phage particles from the growing and dividing host cells. After infection by XacF1, the bacterial host cells showed several cultural and physiological changes including lower levels of EPS production,

Abbreviations
ACC, Asian citrus canker; EPS, exopolysaccharide; MM, minimal medium; NA, nutrient agar; NB, nutrient broth; ORF, open reading frame; p.i., postinfection; Xcc, Xanthomonas citri pv. citri.
reduced motility, slower growth rate, and a dramatic reduction in the virulence [6]. This virulence-lowering effect of XacF1 infection suggests that XacF1 could be used as a biocontrol agent against citrus canker pathogens. However, it was also shown that XacF1 was frequently integrated in the host genome, potentially limiting the effect of XacF1 as a biocontrol agent if the phage needs to be present in the free-replicating state. This integration occurred at the host dif site (attB) like Vibrio cholerae phage CTXφ by the host XerC/D recombination system [6]. Some inoviruses, once integrated into the host genome via the host XerC/D, can be excised in the reverse direction through the same recombination system to be new extrachromosomal phage copies [7]. Because phage infection effects on the host (such as the virulence-lowering effect) may change depending on the phage states, namely a free-replicating state or a prophage state [8], we are interested to characterize the dynamics of XacF1 infection and integration in the Xcc host cells.

Materials and methods

Bacterial culture and phage infection

Xanthomonas citri pv. citri MAFF 301080 was obtained from the National Institute of Agrobiological Sciences, Japan. The cells were grown on nutrient agar (NA) medium (Difco, Cockeysville, MD, USA) at 28 °C. For the preparation of bacterial suspension, Xcc cells were cultured for 24 h at 28 °C with shaking at 220 r.p.m. in nutrient broth (NB) medium (BBL; Becton Dickinson and Co., Cockeysville, MD). Bacteriophage XacF1 was isolated as a biocontrol agent against citrus canker pathogens (such as the virulence-lowering effect) may change depending on the phage states, namely a free-replicating state or a prophage state [8], we are interested to characterize the dynamics of XacF1 infection and integration in the Xcc host cells.

Knockout of ORF12 and construction of ΔXacF1-mutant phages

A complete coding region of ORF12 was deleted from XacF1 DNA by PCR using a forward primer 5’ GAC GGG TTT TTC TCT TGT ACA AGG ACA GTG 3’ (corresponding to XacF1 DNA positions 6887–6920) and a reverse primer 5’ ATG ACG CTA GAC ACC TAC GAT CGC GTA GAC 3’ (reverse), corresponding to XacF1 DNA positions 6401–6430, were used to span the attP sequence. For quantitative comparison, Xcc 16S rDNA sequence was amplified with primers 5’ AAC GCC ACG AAC ACC TAC GAT CGC GTA GAC 3’ (forward) and 5’ TGC GGG ACT TAA CCC AAC ATC TCA 3’ (reverse; accession no. CP006857.1). Twenty-five rounds of PCR were performed under standard conditions in a LifePro Thermal Cycler (Bioer Technology Co., Ltd., Binjiang, China).

Preparation of Xcc competent cells

Electrocompetent cells of Xcc were prepared as previously described [10] with some modifications. Briefly, an overnight subculture of Xcc cells (MAFF 301080) was used to seed 500 mL of NB medium. The bacterial cells were
grown in NB medium with shaking (140 r.p.m.) at 28 °C to a density of OD600 = 0.6, harvested by centrifugation (4000 g), for 10 min, at 4 °C, washed twice under the same condition with 500 mL of distilled sterile water (0 °C), and resuspended in 500 μL of sterile 10% glycerol at 0 °C. Samples (50 μL aliquots) were kept in liquid nitrogen until use.

**Electroporation**

Electrocompetent cells (50 μL) were slowly thawed in an ice bath and mixed with 1–2 μL of transforming DNA solution. The mixture was immediately transferred to a chilled electroporation cuvette (0 °C). Electroporation was performed using a Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA) with a 2-mm cell at 2.5 kV, according to the manufacturer’s manual. Following the pulse, 1 mL of NB medium was immediately added to the cells and the bacterial suspension was transferred to a polypropylene tube. After a room temperature incubation of 1 h, the tube was shaken at 140 r.p.m. for 4 h at 28 °C. Then, the bacterial cells were subjected to plaque assay with MAFF 301080 cells as the host. Single plaques were isolated and phage-containing cells were cultivated to obtain the replicative-form phage DNA (RF).

**Biological and physiological assays**

To compare biological and physiological changes that occurred in the Xcc cells infected with wild-type or mutant XacF1 phages, several assays were conducted as follows: EPS production was determined according to Guo et al. [11]. Briefly, bacterial cells (Xcc strain MAFF 301080) were grown in NB supplemented with 2% glucose for 24 h at 28 °C with shaking at 200 r.p.m. A 10 mL portion of the culture was collected, and the cells were removed by centrifugation (5000 g) for 30 min. The dry weights of EPS were measured. Twitching motility was assayed as described previously [6]. Overnight bacterial cultures in NB were centrifuged at 8000 g for 2 min at 4 °C, washed twice with ddH2O, and resuspended in ddH2O. Two microlitre of the colony edge was observed under a culture microscope at 4×10× magnification (an Olympus CKX41, Tokyo, Japan). Virulence assay was performed with lemon leaves (immature fully expanded lemon leaves) as described before [6]. Needle-prick inoculation was performed by pricking the leaves and droplets (10 μL) of bacterial suspensions (109 colony-forming units·mL−1) were added to each inoculation site. Leaves were incubated in a growth chamber at 28 °C (12-h light and 12-h dark) for 4 weeks.

**Results**

**Effects of ORF12 mutation on XacF1 integration into the host genome**

XacF1 integration into the host genome was directly detected by PCR experiments. When wild-type XacF1 was infected with Xcc strain MAFF 301080, the integrated form was detected (a band of ca. 550 bp in size) as early as 4 h p.i., and increased its integrated amounts to a peak level at 12 h p.i. (Fig. 1A). In contrast, a deletion mutant of XacF1 (ΔXacF1) that lacks an entire region of ORF12 (including attP) could infect the host cells with forming plaques, but did not give any consistent integration bands in PCR (data not shown). This result agreed with the idea that XacF1 integrates into the host dif site by homologous recombination at the attP site. Meanwhile, another mutant of XacF1 (ΔXacF1′) that lacked a 5′ major portion of ORF12 but retained the attP site became ssDNA phages and its titer on MAFF 301080 as a host was approximately the same (or even higher) as that of wild-type XacF1. ΔXacF1′ showed a somewhat delayed integration (detected as a PCR product of ca. 270 bp in size) as shown in Fig. 1B. In this case, the integration signal was detected at 12 h p.i. and its peak was reached at 48–72 h p.i. This delayed integration could be caused by the function of ORF12 itself or by changing sequences flanking attP in the XacF1 genome.

**Effects of ORF12 mutation on the stability of integrated forms of XacF1 in the host genome**

Some inoviruses, once integrated into the host genome via the host XerC/D, can be excised in the reverse direction through the same recombination system to be new extrachromosomal phage copies [7,8]. This integration and excision should be dynamically regulated by some mechanism in the host cells. To evaluate the possible involvement of ORF12 in this mechanism, we compared stability of XacF1 prophage between the cells infected with wild-type XacF1 and with ΔXacF1′. A single colony containing a XacF1 prophage was picked and cultivated in NB for 48 h at 28 °C. The cells were then spread onto NA plates and colonies were formed. After 48-h incubation at 28 °C, 10 single colonies were picked and subjected to PCR for the detection of XacF1-integrated forms. As shown in Fig. 2A, XacF1 prophage states were very stable and all 10 colonies gave almost equivalent amounts of integration signals. However, ΔXacF1′-prophage states were relatively unstable and the prophage signals.
varied significantly among the colonies; a few showed only very faint signals (Fig. 2B). These results suggest that ΔXacF1, where almost the entire region of ORF12 was deleted, integrates into the host cells relatively inefficient and once integrated, its forms are maintained unstably and easily excised to be extrachromosomal. In other words, ORF12 possibly works as an integration-enhancing and integration-stabilizing element in XacF1.

**Effects of ORF12 mutation in XacF1 on the host virulence**

XacF1 infection caused drastic changes to host cells including lowering levels of EPS production, reducing motility, slowing growth rate, and dramatically reducing the virulence [6]. As shown above, XacF1 integrates into the host genome as early as 4 h p.i., and the prophage states are maintained stably thereafter. Here, the XacF1 mutants offered an opportunity to compare the effects of XacF1 infection on the host between the free forms and prophage forms.

When twitching motility was observed on assay plates, the colony margin of uninfected cells showed highly irregular shapes, indicating proficient twitching motility (Fig. 3A). This twitching state was indistinguishable from that of cells infected with ΔXacF1. Meanwhile, the colony edge of XacF1-integrated cells was smooth (Fig. 3B), suggesting a decrease or loss of twitching motility. ΔXacF1-infected cells showed reduced size, and the morphology of the colony edge was observed under increased magnification (Fig. 3C).

Exopolysaccharide production in XacF1-integrated cells showed a significant reduction (~0.6 ± 0.3 mg/10 mL culture, n = 3) compared to uninfected cells (6.7 ± 2.0 mg/10 mL culture, n = 3). Meanwhile, EPS levels were almost the same in the culture of cells infected with ΔXacF1, but were found to be significantly reduced in the ΔXacF1-infected cells (1.2 ± 1.0 mg/10 mL culture, n = 3).

*Xanthomonas citri* pv. *citri* strain MAFF 301080 virulence was observed after infection with XacF1 containing ORF12 mutations. In the virulence assay using the pricking method with lemon leaves, wild-type cells of strain MAFF 301080 caused infection symptoms after 3–4 days postinfection (d.p.i.) and formed clear canker symptoms 1 week after inoculation (Fig. 4A). On the other hand, the symptoms of XacF1-infected cells were very weak and no mature canker could be seen up to 4 weeks p.i., except for marginal lesions formed around pricking sites (Fig. 4B). When lemon leaves were pricked with cells infected with ΔXacF1, canker symptoms were obvious, appearing almost the
same as leaves inoculated with uninfected cells. Meanwhile, the symptoms of ΔXacF1'-infected cells were variable (Fig. 4C); some lesions were significantly large (almost the same as those formed by uninfected cells) and others were very small but with canker symptoms [6]. These results are summarized in Table 1.

**Discussion**

Inoviruses coexist with their host cells, meaning that infection by these phages can influence host bacterial phenotypes in various ways. In pathogenic bacteria of

|                      | Twitching motility | EPS production | Virulence |
|----------------------|-------------------|----------------|-----------|
| No infection         | +                 | +              | +         |
| XacF1-lysogenic      | –                 | –              | –         |
| ΔXacF1               | +                 | +              | +         |
| ΔXacF1'              | –                 | ±              | ±         |

*a ‘+’, > 5 mg/10 mL culture; ±, 1–5 mg/10 mL culture; ‘−’, < 1 mg/10 mL culture.*

*b ‘+’, showing obvious canker symptoms; ±, showing canker symptoms, but very small in some cases; ‘−’, no canker symptoms.*
either animals or plants, virulence is frequently affected by phage infection. For example, infection of _X. campestris_ pv. _oryzae_ NP5850 by the filamentous phages Xf and XI2 enhanced virulence, possibly because of overproduction of extracellular polysaccharides (EPS) by the phage-infected bacterial cells [13]. In contrast with the virulence-enhancing effects of ϕRSS1 [14], phage-mediated loss of virulence was also reported in _Ralstonia solanacearum_. _Ralstonia solanacearum_ completely lost virulence through infection with two other filamentous phages ϕRSM1 and ϕRSM3 [12]. Many virulence factors were found to be significantly reduced in ϕRSM-infected cells. These opposing effects of different filamentous phages on _R. solanacearum_ virulence make it an ideal study model system for understanding the effect of filamentous phage on their hosts. In the same way with ϕRSM, XacF1 phage shows many significant effects on the physiological features of _Xcc_-infected cells. These effects include slower growth rate; lower EPS production; and reduced cells’ swimming, swimming, and twitching motility; and dramatic reduction in virulence [6]. This loss of virulence effect of XacF1 infection could be explained by the reduction in the growth rate, EPS production, and cell motility. It is also known that XacF1 can integrate into the host genome by site-specific recombination by the host XerC/D system [6]. This has raised a question of how those phage effects vary depending on the phage states, namely a free-replicating state or a prophage state. In this study, it was found that XacF1 starts to integrate into the host genome as early as 4 h p.i. and increased integrated forms to a peak level of 12 h p.i. (Fig. 1). However, in the form of ΔXacF1 where ORF12 was completely deleted, no integration occurred. This is because the _attrP_ was deleted in the XacF1 genome. As shown in Table 1, cells infected with this form (a free-replicating state) were not drastically different in physiological properties compared with noninfected cells. In contrast, in cells infected with ΔXacF1', where _attrP_ was intact and a 5' major portion of ORF12 was deleted, integration occurred but was inefficient and delayed compared with wild-type XacF1 (Fig. 1). Once integrated, the prophage state of ΔXacF1' was unstable and easily released from the host genome as shown in Fig. 2B. These results strongly suggest that ORF12 plays a key role to regulate (facilitate and stabilize) the prophage state of XacF1. Because the ΔXacF1'-infected cells showed very unstable features such as lowered twitching motility, lowered EPS production, and variable virulence (Figs 3 and 4, and Table 1), the virulence-lowering effect of XacF1 infection might be caused by stable integration of XacF1 into the host genome. In this sense, ORF12 containing _attrP_ and a regulatory function on the XacF1 genome may play an important role in regulating not only integration dynamics, but also virulence states of the host cells.

It is worth noting that the ORF12 region of XacF1 corresponds to ‘the primary immunity determinant’ of _Xcc_ phage Cf [15]. Cf is the first filamentous phage found to infect _Xcc_ strains [16] and was shown to be integrated into the host chromosome [17]. Cheng _et al._ [15] showed that mutations in the primary immunity determinant of Cf resulted in integration defect. Therefore, our observation that the integrated state became very unstable following a 5'-deletion of ORF12 in XacF1, suggesting that ORF12 plays a key role in the integration cycle of XacF1 in _Xcc_ strains, agrees with the Cf results.

**Acknowledgements**

This work was supported in part by JSPS Kakenhi (Grant Number 24380049) to TY. The authors thank Michael Stulberg (USDA-APHIS) for useful comments and critical reading of the manuscript.

**Author contributions**

TY and AAA conceived the studies and designed the experiments that were performed by AAA, MK, and AA. TK and MF contributed to the construction of deletion mutants and plant assay, respectively. TY wrote the manuscript together with AAA, TK, and MF.

**References**

1 Civerolo EL (1984) Bacterial canker disease of citrus. _J Rio Grande Val Hort Soc_ 37, 127–145.
2 Gottwald TR, Graham JH and Schubert TS (2002) Citrus canker: the pathogen and its impact. _Plant Health Prog_. https://doi.org/10.1094/PHP-2002-0812-01-RV.
3 Graham J, Gottwald T, Cubero J and Achor D (2004) _Xanthomonas axonopodis_ pv. _citri_: factors affecting successful eradication of citrus canker. _Mol Plant Pathol_ 5, 1–15.
4 da Silva AC, Ferro JA, Reinach FC, Farah CS, Furlan LR, Quaggio RB, Monteiro-Vitorello CB, Van Sluys MA, Almeida NF, Alves LM _et al._ (2002) Comparison of the genomes of two _Xanthomonas_ pathogens with differing host specificities. _Nature_ 417, 459–463.
5 Van Sluys MA, Monteiro-Vitorello CB, Camargo LE, Menck CF, da Silva AC, Ferro JA, Oliveira MC, Setubal JC, Kitajima JP and Simpson AJ (2002) Comparative genomic analysis of plant-associated bacteria. _Annu Rev Phytopathol_ 40, 169–189.
6 Ahmad AA, Askora A, Kawasaki T, Fujie M and Yamada T (2014) The filamentous phage XacF1 causes loss of virulence in Xanthomonas axonopodis pv. citri, the causative agent of citrus canker disease. Front Microbiol 5, 321.

7 Das B, Bischerour J and Barre F-X (2011) VGJφ integration and excision mechanisms contribute to the genetic diversity of Vibrio cholerae epidemic strains. Proc Natl Acad Sci USA 108, 2516–2521.

8 Yamada T (2013) Filamentous phages of Ralstonia solanacearum: double-edged swords for pathogenic bacteria. Front Microbiol 4, 325.

9 Kawasaki T, Nagata S, Fujiwara A, Satsuma H, Fujie M, Usami S and Yamada T (2007) Genomic characterization of the filamentous integrative bacteriophage ΦRSS1 and ΦRSM1, which infect Ralstonia solanacearum. J Bacteriol 189, 5792–5802.

10 do Amaral AM, Toledo CP, Baptista JC and Machado MA (2005) Transformation of Xanthomonas citri pv. citri by electroporation. Fitopatol Bras 30, 292–294.

11 Guo Y, Sagaram US, Kim JS and Wang N (2010) Requirement of the galU gene for polysaccharide production by and pathogenicity and growth in planta of Xanthomonas citri subsp. citri. Appl Environ Microbiol 76, 2234–2242.

12 Addy HS, Askora A, Kawasaki T, Fujie M and Yamada T (2012) Loss of virulence of the phytopathogen Ralstonia solanacearum through infection by ΦRSM filamentous phages. Phytopathology 102, 469–477.

13 Kamiunten H and Wakimoto S (1982) Effect of the infection with filamentous hage Xf-2 on the properties of Xanthomonas campestris var oryzae. Ann Phytopathol Soc Japan 47, 627–636.

14 Addy HS, Askora A, Kawasaki T, Fujie M and Yamada T (2012) The filamentous phage ΦRSS1 enhances virulence of phytopathogenic Ralstonia solanacearum on tomato. Phytopathology 102, 244–251.

15 Cheng C-M, Wang H-J, Bau H-J and Kuo T-T (1999) The primary immunity determinant in modulating the lysogenic immunity of the filamentous bacteriophage cf. J Mol Biol 287, 867–876.

16 Dai H, Chiang KS and Kuo TT (1980) Characterization of a new filamentous phage cf from Xanthomonas citri. J Gen Virol 46, 277–289.

17 Kuo TT, Chao YS, Lin YH, Lin BY, Liu LF and Feng TY (1987) Integration of the DNA of filamentous bacteriophage Cf1t into the chromosomal DNA of its host. J Virol 61, 60–65.

18 Stoddard SF, Smith BJ, Hein R, Roller BRK and Schmidt TM (2015) rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. Nucleic Acids Res 43, D593–D598, Database issue.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Genomic organization of bacteriophage XacF1.

Fig. S2. A model for integration/excision of XacF1 in Xanthomonas citri.