A Novel Two-Component Response Regulator Links rpf with Biofilm Formation and Virulence of Xanthomonas axonopodis pv. citri

Tzu-Pi Huang*, Kuan-Min Lu, Yu-Hsuan Chen

Department of Plant Pathology, National Chung-Hsing University, Taichung, Taiwan

Abstract

Citrus bacterial canker caused by Xanthomonas axonopodis pv. citri is a serious disease that impacts citrus production worldwide, and X. axonopodis pv. citri is listed as a quarantine pest in certain countries. Biofilm formation is important for the successful development of a pathogenic relationship between various bacteria and their host(s). To understand the mechanisms of biofilm formation by X. axonopodis pv. citri strain XW19, the strain was subjected to transposon mutagenesis. One mutant with a mutation in a two-component response regulator gene that was deficient in biofilm formation on a polystyrene microplate was selected for further study. The protein was designated as BfdR for biofilm formation defective regulator. BfdR from strain XW19 shares 100% amino acid sequence identity with two-component response regulators in various pathogens and environmental microorganisms. The bfdR mutant strain exhibited significantly decreased biofilm formation on the leaf surfaces of Mexican lime compared with the wild type strain. The bfdR mutant was also compromised in its ability to cause canker lesions. The wild-type phenotype was restored by providing pbfdR in trans in the bfdR mutant. Our data indicated that BfdR did not regulate the production of virulence-related extracellular enzymes including amylase, lipase, protease, and lecithinase or the expression of hrpG, rfbC, and katF; however, BfdR controlled the expression of rpfF in XVM2 medium, which mimics cytoplasmic fluids in planta. In conclusion, biofilm formation on leaf surfaces of citrus is important for canker development in X. axonopodis pv. citri XW19. The process is controlled by the two-component response regulator BfdR via regulation of rpfF, which is required for the biosynthesis of a diffusible signal factor.

Introduction

Xanthomonas axonopodis pv. citri (syn. X. citri subsp. citri) affects most commercial citrus cultivars and causes citrus bacterial canker resulting in significant crop losses worldwide [1]. The bacteria are presumably considered as epiphytes on the plant surface before infection [2]. They infect leaves, stems, thorns and fruits and enter the citrus plant mainly through wounds and stomata [3]. Biofilms are formed when microorganisms attach to a surface and form a self-produced matrix of exopolysaccharides (EPS) that they embed themselves within [4]. Rigano et al. (2007) showed that biofilm formation is necessary for epiphytic fitness and canker development in X. axonopodis pv. citri [5]. Our previous study indicated that Bacillus subtilis TKS1-1 and Bacillus amyloliquefaciens WG6-14 can interfere with phytophthora biofilm formation by X. axonopodis pv. citri, which may contribute to the attenuation of citrus bacterial canker symptom development [6]. These results and findings from other plant-associated bacteria suggest that biofilm formation plays important roles in pathogenesis [5,6,7,8]. Thus, experiments were conducted to uncover genes and gene clusters that are involved in biofilm formation and virulence as well as to reveal potential interactions between these two processes in X. axonopodis pv. citri, which may aid in the development of a strategy for disease management.

Several approaches have been used to investigate and understand genes and gene clusters that govern mechanisms of pathogenesis and biofilm formation. These include comparative genome analyses, functional studies performed by mapping insertion sites within transposon-based mutant libraries [9,10], construction and use of macroarrays [11], and directed mutagenesis of genes that encode proteins with specific functions [12,13,14,15,16]. Comparative genome analysis of Xanthomonas campestris pv. campestris, which causes black rot of crucifers, and X. axonopodis pv. citri revealed that more than 80% of the genes are shared between the two species; however, subsets of genes are strain-specific and may be responsible for distinct host specificity and pathogenicity. These genes include avr, genes encoding members of the type III secretion system, rpf (regulation of pathogenicity factors), genes encoding type IV fimbriae, and lipopolysaccharide (LPS) O-antigen operons [17]. In a functional study, Gottig et al. (2010) suggested that X. axonopodis pv. citri uses several mechanisms to colonize and invade its host [18]. Citrus canker bacteria could attach to the host surface via adhesins such as the filamentous hemagglutinin-like protein FhaB [13].
bacteria may inject pathogenicity effectors into the host through the type III secretion system and modulate the plant’s defense mechanisms [19,20]. To prolong survival and colonization of the host, the bacteria form biofilms by producing EPS, xanthan which is synthesized by the gum operon [14], and FlhA [13]. Additionally, a single flagellum of *X. axonopodis* pv. *citri* was involved in the formation of mushroom-shaped structures in mature biofilms [13]. It was also found that the bacteria use a plant natriuretic peptide-like protein to modulate host homeostasis [12]. Several factors were shown to be relevant for host colonization or biofilm formation of favorable conditions for their survival [12]. Mutations in *X. axonopodis* pv. *citri rpfF*, rpfG and rpfC caused a reduction in bacterial attachment to grapefruit leaves and to abiotic surfaces in either XVM2 media or nutrient broth [27], which is in contrast to the findings in *X. campestris* pv. *campestris*. The rpfF and rpfGHC mutants of *X. campestris* pv. *campestris* showed increased initial attachment to plastic surfaces compared with the wild type [20]. By DNA microarray analysis of the RpfF, RpfG and RpfC regulons in *X. axonopodis* pv. *citri*, Guo et al. (2012) found a conserved group of genes that were regulated by all three proteins, suggesting a major role for RpfG and RpfC in the perception and transduction of signals in the rpf/DSF system [27]. However, some genes were controlled by only one of the three proteins, suggesting that the RpfG and RpfC TCS may regulate additional genes beyond those involved in the transduction of the DSF signal [27].

To understand the regulatory mechanisms of biofilm formation by *X. axonopodis* pv. *citri*, we subjected strain XW19 to transposon mutagenesis. One individual with a mutation in a two-component response regulator was identified that exhibited a defect in biofilm formation on polystyrene plates and on the leaf surfaces of citrus plants. Thus, the identified response regulator was designated as BfdR for plants. Thus, the identified response regulator was designated as BfdR for biofilm formation defective regulator, and its flanking two-component sensor was designated as BfdS. We also provide evidence that BfdR is involved in the pathogenesis and regulation of the rpf/DSF system.

### Materials and Methods

#### Bacterial strains and plant growth conditions

The *Xanthomonas* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *Xanthomonas* strains were routinely cultured on Trypircase™ Soy (TS) agar or in TS broth (Becton Dickinson, Franklin Lakes, NJ, USA) at 27°C unless otherwise stated. All *E. coli* strains were grown in Luria-Bertani (LB) broth (Becton Dickinson) at 37°C. When required, the medium was

| Table 1. Bacterial strains and plasmids used in this study. |
|-------------------------------------------------------------|
| **Strains and Plasmids** | **Relevant characteristics** | **Source** |
|-------------------------|-----------------------------|-----------|
| **E. coli**              |                             |           |
| DHSAx                    | λ phdI lacZΔM15 ΔlacZYA-argF u169 recA1 endA1 hsdR17(16 mcrB2 phoA supE44 thi-1) gyrA96 relA1 | Invitrogen |
| **X. axonopodis pv. citri** |                             |           |
| XW19                    | Wild type                   | [73]      |
| TPH1                    | Km<sup>+</sup>, two-component response regulator: EZ-TN transposon mutant, XW19 derivative | This study |
| TPH2                    | Gm<sup>+</sup>, XW19 harboring pBBR1MC55 | This study |
| TPH3                    | Gm<sup>+</sup>, TPH1 harboring pBBR1MC55 | This study |
| TPH4                    | Gm<sup>+</sup>, TPH1 harboring pBBF5R | This study |
| TPH5                    | Gm<sup>+</sup>, TPH1 harboring pBBFDR | This study |
| **Plasmids**            |                             |           |
| pBBR1MC55               | Gm<sup>+</sup>, broad-host range cloning vector | [74]      |
| pGTFKan                 | Gm<sup>+</sup>, 131 bp rpfII promoter driven gfp | [75]      |
| pBF5R                   | Gm<sup>+</sup>, 1954 bp promoters and coding regions of two-component sensor and response regulator | This study |
| pBBFDR                  | Gm<sup>+</sup>, 696 bp promoter and coding region of two-component response regulator | This study |

doi:10.1371/journal.pone.0062824.t001

The diffusible signal factor (DSF)-mediated cell-to-cell communication system [13,16] was first identified in *X. campestris* pv. *campestris*, and components of this system were shown to be synthesized and regulated by the rpf gene cluster [26]. rpfB and rpfF are responsible for the synthesis of the DSF cis-A2-11-methyl-dodecanoic acid, and the rpfGHC operon encodes for a TCS that is responsible for regulation [26]. Similar to the findings in *X. campestris* pv. *campestris*, the rpf/DSF system in *X. axonopodis* pv. *citri* was shown to regulate virulence factors such as extracellular cyclic β-(1,2)-glucan; proteases; endoglucanases; genes involved in flagella-dependent and independent motility, chemotaxis, and flagellar biosynthesis; genes involved in the TCA cycle and in the degradation of celluloses and glucans; the transcription factor σ54; and genes encoding hypothetical proteins [15,16,27]. Four of the hypothetical proteins share a high level of identity with XagA, XagB, XagC and XagD of *X. campestris* pv. *campestris*, which were found to be involved in biofilm formation and may contribute to adherence biosynthesis [27].
supplemented with gentamicin (Gm; Sigma-Aldrich, St. Louis, MO, USA; 10 μg/ml for Xanthomonas strains and 25 μg/ml for E. coli) or kanamycin (Km; Sigma-Aldrich; 50 μg/ml).

The citrus plants used in the study included navel orange (Citrus sinensis [L.] Osbeck), Mexican lime (Citrus aurantifolia [C.] Swingle) and Ruby grapefruit (Citrus paradisi Macfad). The navel orange was grafted onto a Cantonese lemon (C. limonia), which was used as the rootstock, whereas the Mexican lime was grafted onto a Swingle citrumelo. The plants were cultivated in potting mix (nacrite:vermiculite:loam:organic compost = 1:0.8:3:0.48) in 60 cm diameter pots and maintained in a greenhouse. For the pathogenicity assay, 30-day-old newly grown leaves were used.

**Table 2.** Primers used in this study.

| Gene          | Primer sequence (5'-3') | Protein/Source |
|---------------|-------------------------|----------------|
| Sequencing & complementation primers | | |
| KpnI-4-3D-F1 | F:CGGGGTCACCCGCAATGCGATTACCGAG | Two-component sensor and response regulator |
| KpnI-4-3D-F2 | F:CGGGGTAACCTAATTTGCGAGTTACCGAG | Two-component response regulator |
| KpnI-4-3D-R  | R:CGGGGTAACCTAATTTGCGAGTTACCGAG | |
| RAN-2 FP-1   | ACCTACAAAGAACCTCTACATACAC | Epicentre |
| R6KAN-2 RP-1 | CTACCCGTGGAAACCCCTACATCT | Epicentre |
| RT-PCR primers | | |
| hrpG         | F:GCCGTTCCATTCGCCGAGTTACCG | TTSS component regulator |
|             | R:CGCGCCGCGGGCTGAAAAAGA | |
| katE        | F:TCAATGAGAAAAGCGAGAGCACCT | Monofunctional catalase [21] |
|             | R:AGATCGCGAGCTGAAAAGTCTGAA | |
| rfbC        | F:ATCCATACACGACCTGTTGGTA | LPS O-antigen biosynthesis protein [21] |
|             | R:GAGATCCGCCGACTGCAAGACTG | |
| rpfF        | F:ATGAAACGATTTGAATATGTCCCCGTG | Regulation of pathogenicity factor and DSF biosynthesis |
|             | R:GCTACGGCCAGCAGTCCATGCCAGGC | |
| rpoD        | F:CATCAGGATGTGCTGTGTT | Sigma factor σ70 |
|             | R:TCAGCCAAATCTGGAAGGT | |

**Figure 1.** Schematic diagram of bfdS and bfdR and their homologues in X. axonopodis pv. citri strains XW19 and 306. The open arrows show the locations and orientations of the genes. The position of EZ-Tn5 in the mutant is indicated by an inverted red triangle. The construction of the complementation plasmids pbfdSR and pbfdR is described in Materials and Methods. The primers used to construct the plasmids for complementation are shown on the top of the solid arrows.

doi:10.1371/journal.pone.0062824.g001

Generation of transposon mutants and sequence analysis of inserted genes

X. axonopodis pv. citri strain XW19 transposon mutants were generated using EZ::TN <R6K\(\gamma\)ori/KAN-2> Tnp Transposome (Epicentre, Madison, WI, USA) as described by Huang et al. [29]. The transposon flanking regions were “rescue cloned” as described by the manufacturer and sequenced using the primers KAN-2 FP-1 and R6KAN-2 RP-1 (Table 2). Additional primers were used to fully sequence the genes and flanking regions. DNA sequencing was performed at the Automated DNA Sequencing Service Laboratory, National Chung-Hsing University, Taiwan. The sequences were compared with those in the GenBank nucleotide database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequences were translated into amino acid sequences using the ExPaSy translate tool (Bioinformatics Resource Portal, http://web.expasy.org/translate/SwissInstitutedBioinformatics) and compared with those in the GenBank database. The percent identity of protein sequences was analyzed using the FASTA program (Ver. 36.3.6) at the University of Virginia [30]. The amino acid sequences were aligned using the Pileup program, SeqWeb version 3.1.2 (GGG Wisconsin Package, Accelrys Inc., San Diego, CA, USA). The conserved domains were analyzed using the NCBI Conserved Domain Assay (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) and the simplified molecular architecture research tool (SMART) [31].

Construction of plasmids and strains

To construct the plasmids for complementation, the bfdR and bfdS ribosomal binding sites, native promoters and coding regions were amplified from X. axonopodis pv. citri strain XW19 by PCR using the primers KpnI-4-3D-F1 and KpnI-4-3D-R; and bfdR ribosomal binding site, and the native promoter and coding region were amplified using the primers KpnI-4-3D-F2 and KpnI-4-3D-R (Table 2 and Figure 1). The products were subsequently cloned into pGEM-T Easy (Promega, Madison, WI). The fragment
containing bfdS and bfdR (1954 bp) was excised with KpnI and ligated into pBBR1MCS5 to generate pbfdSR, whereas bfdR (696 bp) was excised with KpnI and ligated into pBBR1MCS5 to generate pbfdR. For complementation, pbfdSR or pbfdR was electroporated (12.5 kV/cm, 25 μF, 400 V) into the bfdR mutant (TPH1). Electroporation, restriction endonuclease digestion, PCR, cloning, DNA extraction, and DNA purification were performed using standard procedures [32]. For the biofilm and pathogenicity assays, X. axonopodis pv. citri strain XW19 was electroporated into X. axonopodis pv. citri strain TPH2, TPH3 and TPH5.

**Biofilm formation assay**

Transposon mutants were screened for biofilm formation using a microplate assay adapted from Fletcher (1997) and O’Toole et al. (1993) [33,34]. Briefly, wells containing 2 ml of TS broth supplemented with 50 μg/ml kanamycin were inoculated with overnight bacterial cultures to an optical density at 620 nm (OD620) of 0.05 and incubated at 27°C with shaking (50 rpm) for 2 days. Biofilm cells were stained with 0.1% crystal violet and washed. Subsequently, the stain remaining in the cells was solubilized with 70% ethanol, and the OD620 was determined using a Tecan Infinite M200 plate reader (Tecan Austria GmbH, Groedig, Austria). The mutant TPH1 grew similarly to wild type but was deficient in biofilm formation; thus, TPH1 was selected for further characterization.
Epiphytic growth and pathogenicity assay

*X. axonopodis* pv. *citr* strains TPH2, TPH3 and TPH5 were cultured in TS broth supplemented with 10 μg/ml gentamicin at 27°C with shaking at 100 rpm for 2 days. The culture suspensions were diluted with media to an OD620 of 0.3 (1×10^9 cfu/ml) and subsequently sprayed on the leaves of Mexican lime (20 leaves per strain) to the point of runoff in the greenhouse. In planta growth assays were performed by grinding 0.5-cm diameter leaf discs (20 discs per leaf) from artificially inoculated leaves (3 leaves per strain) in 1 ml of sterile Milli-Q water followed by serial dilutions and plating onto *Xanthomonas* differential (Xan-D) medium [35]. Leaves were collected at 0, 1, 2, 4, and 6 days post-inoculation. Colonies were counted after 2 days of incubation at 27°C, and the results are presented as log cfu/cm² of leaf tissue. The same culture and inoculation conditions used for the in planta growth assays were used for the pathogenicity assay. At two months post-inoculation, cankers were counted on six leaves inoculated with different strains, and the areas of the counted leaves were measured on digital images using Adobe Photoshop software (Adobe Systems Inc, San Jose, CA, USA). The disease severity of citrus bacterial canker caused by the different strains was expressed as the number of cankers per cm².

Reverse transcription-PCR (RT-PCR) analysis of virulence-related genes

*X. axonopodis* pv. *citr* strains TPH2, TPH3 and TPH5 were cultured in TS broth and XVM2 medium [11] at 27°C for 2 days. The culture suspensions were diluted with media to an OD620 of 0.05 and incubated at 27°C with shaking at 100 rpm. Bacterial cells were collected after an 18 hr incubation period and subjected to RNA extraction using TRI Reagent® LS RNA Isolation Reagent (Molecular Research Center Inc, Cincinnati, OH, USA) according to the manufacturer’s instructions. Contaminating genomic DNA was removed using the TURBO DNA-96® Kit (Ambion, Austin, TX, USA). The RNA concentration was determined by measuring the absorbance at 260 nm with a Tecan Infinite M200 plate reader and adjusted to a concentration of 50 ng/ml. RT-PCR was performed with the Transcriptor One-Step RT-PCR Kit (Roche Applied Science, Indianapolis, IN, USA) in a 20 μl reaction mixture containing 50 ng of total RNA. Gene-specific primers (Table 2) were used for amplification of the virulence-related genes *rfbC* (113 bp), *hrpG* (747 bp), *vfF* (870 bp), and *katE* (127 bp). The *16S* rDNA (264 bp) gene encoding sigma factor σ^70^ [36] was used as a loading control.

Activity of extracellular enzymes

*X. axonopodis* pv. *citr* strains TPH2, TPH3, TPH4, and TPH5 were cultured in TS broth supplemented with 10 μg/ml Gm at 27°C with shaking at 100 rpm for 2 days. The culture suspension was diluted with TS broth to an OD620 of 0.05, and 10 μg of the diluted bacterial suspension was spotted onto starch agar for the detection of amylase [37], medium containing Tween 80 and skim milk for the detection of lipase and protease [35], and egg yolk agar for the detection of lecithinase [30]. The plates were incubated at 27°C for 2 days.

Statistical analysis

All experiments were performed at least three times. The data are presented as means and standard deviations obtained from at least four replicates of a single representative experiment. The significant difference between the treatments was analyzed by one-way ANOVA and Tukey’s honestly significant difference (HSD) test using SPSS 15.0 software (SPSS Inc, Chicago, IL, USA).

Nucleotide sequence accession numbers

The *vfF* and *hlySR* sequences of *X. axonopodis* pv. *citr* strain XW19 were deposited in the GenBank database (accession numbers JX987963 and JX987964).

Results

The transposon-inserted gene is homologous to a gene encoding a two-component response regulator

Biofilm formation by plant-associated bacteria was shown to be important for pathogenesis or symbiosis [3,7]. To understand the molecular mechanisms of biofilm formation and to elucidate the role of biofilm formation in symptom development, we subjected *X. axonopodis* pv. *citr* strain XW19 to transposon mutagenesis using the E-Z-Tn5<sup>TM</sup> <R6K<sub>K</sub> ori/KAN-2>P<sub>Tnp</sub> Transposome<sup>TM</sup> Kit. A total of 1710 transposon mutants were screened for defects in biofilm formation in 24-well polystyrene plates. One mutant had a growth rate that was similar to wild type in TS broth but was defective in biofilm formation. This mutant, TPH1, was selected...
for further investigation (data not shown). The transposon flanking regions were rescued by “rescue cloning” as described by the manufacturer (Epicentre) and sequenced using the primers KAN-2 FP-1 and R6KAN-2 RP-1 (Table 2). The transposon-inserted gene was homologous to the locus tag XAC1284, which encodes for a two-component response regulator in \textit{X. axonopodis pv. citri} strain 306 (GenBank accession no. NC_003919). The flanking sequences of the transposon-inserted genes were homologous to the locus tag XAC1282, which encodes for the two-component sensor (Figure 1). Both of the genes in strain XW19 share 100% identity with those of strain 306 (data not shown).

The nucleotide sequence of the transposon-inserted gene was translated into an amino acid sequence using the ExPASy translate tool. The translated amino acid sequence in \textit{X. axonopodis pv. citri} strain XW19 was compared with sequences in \textit{Xanthomonas vesicatoria}, \textit{X. campestris pv. campestris}, \textit{Agrobacterium tumefaciens}, \textit{Pseudomonas putida}, \textit{Pseudomonas fluorescens}, \textit{Stenotrophomonas maltophilia} and \textit{Rhodopseudomonas palustris}. An alignment of the amino acid sequences is shown in Figure 2. The amino acid sequence of BfdR in \textit{X. axonopodis pv. citri} strain XW19 shares 89.3% identity with locus tag XVE_1034, which is a response regulator in \textit{X. vesicatoria} strain ATCC 35937 (GenBank accession no. ZP_08177161) with a CheY-like receiver domain, ATPase domain, and DNA-binding domain. BfdR also shares 89.7% identity with locus tag XCC1187, a two-component response regulator in \textit{X. campestris pv. campestris} strain ATCC33913 (GenBank accession no. NP_636561); 37.3% identity with locus tag PFVH6_4865, a response regulator in \textit{P. fluorescens} WH6 (GenBank accession no. ZP_07777428) that harbors a receiver domain; 30.8% identity with locus tag PputW619_2443, a PAS/PAC sensor hybrid histidine kinase in \textit{P. putida} strain W619 (GenBank accession no. YP_001749312); 35.1% identity with locus tag Atu3883, a chemotaxis response regulator in \textit{A. tumefaciens} strain C58 (GenBank accession no. NP_356752); 35.7% identity with locus tag Smal_3110, a response regulator receiver protein in \textit{S. maltophilia} strain R551-3 (GenBank accession no. YP_002029492); and 35.6% identity with locus tag RPD_0328, a response regulator receiver in \textit{R. palustris} strain BisB5 (GenBank accession no. YP_567467) (data not shown). According to the conserved domain assay, the amino acid sequence of the transposon-inserted gene in \textit{X. axonopodis pv. citri} strain XW19 contained a highly conserved CheY-homologous receiver domain, REC [39] (Figure 2). The REC domain in strain XW19 contains active sites at positions 11, 12, 65, 85, 101, 104, and 105; a

---

**Figure 4. Epifluorescence micrographs of \textit{Xanthomonas axonopodis pv. citri} biofilms on grapefruit, Mexican lime and navel orange leaf discs.** \textit{X. axonopodis pv. citri} TPH2, TPH3 and TPH5 were tagged with green fluorescent protein and expressed using the plasmid pGTKAn. The culture suspensions (OD$_{620}$ = 0.05) were inoculated in a 24-well polystyrene plate containing grapefruit, Mexican lime and navel orange leaf discs and incubated at 27°C with shaking at 50 rpm for 2 days. Scale bars, 10 μm. doi:10.1371/journal.pone.0062824.g004
phosphorylation site at position 56; and intermolecular recognition sites and dimerization interface sites at positions 104, 105, and 106. The active sites coordinate Mg²⁺ required for phosphorylation, the phosphorylation site functions in posttranslational modification, and the dimerization interface site (polypeptide binding site) allows homodimerization, which enhances binding to the target DNA [39]. Based on the above results, the transposon-inserted gene in X. axonopodis pv. citri strain TPH1 encodes for a two-component response regulator.

BfdR is involved in X. axonopodis pv. citri biofilm formation in polystyrene microplates and on the leaf surfaces of citrus plants

The mutant strain TPH1 exhibited significantly reduced biofilm formation on polystyrene plates compared with the wild type (data not shown). To determine whether deficient biofilm formation by X. axonopodis pv. citri strain TPH1 is due to a mutation in bfdR, bfdR and its predicted promoter were cloned into pBBR1MCS5 to generate pbfdR for complementation. Because the second and third start codons of BfdR and the first and second stop codons of BfdS are overlapping and the transposon is inserted in the second stop codon of BfdS (Figure 1), transposon insertion may also cause inactivation of BfdS in X. axonopodis pv. citri strain TPH1 that encodes a two-component response regulator.

The presence of BfdR in the transposon-inserted gene in X. axonopodis pv. citri was confirmed by PCR analysis using primers specific to the BfdR gene. The bacterial population density was determined by homogenizing the leaves in Milli-Q water followed by dilution and plating at 0, 1, 2, 4, and 6 days post-inoculation. All experiments were performed three times with similar results. The results shown are the means and standard deviations (error bars) of triplicates from one representative experiment. doi:10.1371/journal.pone.0062824.g006

citi strains. Biofilm formation by the bfdR mutant (TPH3/pGTKan) was significantly decreased on the leaf surfaces of grapefruit, Mexican lime, and navel orange compared with the wild type (TPH2/pGTKan) and the complemented strain (TPH5/pGTKan) (Figure 4). Additionally, the cells of the wild type (TPH2/pGTKan) and the complemented strain (TPH5/pGTKan) were observed to be clustered together, forming microcolonies and biofilm (Figure 5). The thickness of the biofilm was approximately 6 μm. However, only a few cells of the bfdR

Epifluorescence microscopy was used for observation of biofilms produced on the leaf surfaces of citrus plants by X. axonopodis pv.
mutant (TPH3/pTGKan) were clustered, and the thickness of the biofilm was approximately 1 μm. These results indicate that BfdR is involved in X. axonopodis pv. citri biofilm formation on abiotic surfaces (polystyrene microplates) as well as biotic surfaces (citrus leaf surfaces).

A mutation in BfdR affects the epiphytic growth of X. axonopodis pv. citri on the leaf surfaces of Mexican lime

Previous results from Rigano et al. (2007) indicate that biofilm formation is important for epiphytic survival and canker development in X. axonopodis pv. citri [5]. To evaluate the role of X. axonopodis pv. citri BfdR in epiphytic growth, the population density of the bfdR mutant (TPH3), wild type (TPH2) and the complemented strain (TPH5) were quantified in Mexican lime leaves. At all times examined, the number of bfdR mutant bacteria recovered from inoculated leaves was significantly lower than the numbers of wild type and complemented bacteria (Figure 6). The number of bfdR mutant bacteria isolated from inoculated leaves was especially low at one day post-inoculation; specifically, 1.6±1.1×10^4 cfu/cm^2 (2.25±0.17 log cfu/cm^2) and 0.9±1.3×10^6 cfu/cm^2 (3.00±0.31 log cfu/cm^2) for the bfdR mutant and wild type bacteria were isolated from leaves, respectively, at one day post-inoculation. At six days post-inoculation, 5.7±2.0×10^5 cfu/cm^2 (3.07±0.07 log cfu/cm^2) and 4.5±1.1×10^6 cfu/cm^2 (3.54±0.05 log cfu/cm^2) for the bfdR mutant and wild type bacteria were isolated from leaves. For the complemented strain TPH5, the number of bacteria recovered at one day post-inoculation (2.8±0.7×10^6 cfu/cm^2 = 2.92±0.05 log cfu/cm^2) was slightly lower than the number of bacteria in the initial inoculum. At four days post-inoculation, 1.9±0.5×10^6 cfu/cm^2 (3.35±0.06 log cfu/cm^2) TPH5 bacteria were recovered from leaves. The number of bacteria recovered from leaves inoculated with the complemented strain was lower than the number of wild type bacteria recovered at all times examined, which may have occurred because the plasmid pbfdR was provided in trans of the bfdR mutant or may be due to the loss of antibiotic selection in planta. These results suggest that BfdR plays an important role in regulating the epiphytic growth of X. axonopodis pv. citri.

BfdR is involved in regulating canker development in X. axonopodis pv. citri

To determine whether BfdR plays a role in symptom development and virulence, TPH2, TPH3 and TPH5 strains were artificially inoculated on the leaves of Mexican lime plants. After spray inoculation, the number of cankers that had developed on both sides of the leaves inoculated with the bfdR mutant (TPH3) was 4.6 times lower than that observed on leaves inoculated with wild type (Figure 7) at two months post-inoculation. Complementation partially restored the phenotype of the wild type bacteria. These results indicate that BfdR plays a role in canker development in X. axonopodis pv. citri strain XV19. However, if the strains were applied by wound-inoculation, no significant difference in canker development was observed among TPH2, TPH3 and TPH5 strains. All inoculated leaves showed yellowing and developed necrotic lesions at two weeks post-inoculation. At five weeks post-inoculation, typical canker lesions with water-soaked margins were visible, and the disease incidence rate reached 100% (data not shown). These data suggest that BfdR may be involved in the early stages of leaf surface colonization.

BfdR positively regulates the transcription of rpfF in X. axonopodis pv. citri

Our results showed that symptom development and virulence are controlled by BfdR in X. axonopodis pv. citri strain XV19. We performed RT-PCR to determine whether the regulation of symptom development and virulence by BfdR is associated with expression regulation of virulence-related genes. We examined gene expression levels in TS broth and XVM2 medium, which mimics cytoplasmic fluids in planta [11]. Our results showed that the expression of rpfF, which is required for the biosynthesis of a diffusible signal factor, was upregulated by BfdR in XVM2 medium but not in TS broth after an 18 hr incubation period (Figure 8). However, the expression levels of genes involved in the synthesis of LPS O-antigen (rfbC), key regulator of type III secretion system (hrpG), and catalase (katE) were not affected by the mutation in bfdR in TS broth or XVM2 medium. rpoD, which encodes for sigma factor 70, was constitutively expressed in both TS broth and XVM2 medium and was used as a loading control.

Known pathogenicity factors in Xanthomonas include regulation of pathogenicity factor (puf) [40]; xanthan [40,41]; LPS [41]; extracellular enzymes such as esterase [42]; mannanase [26], endoglucanases [41], and protease [43]; PthA [44]; HrpX [20,41]; and catalase [45]. To understand whether a mutation in X. axonopodis pv. citri strain XV19 BfdR affects the production of extracellular enzymes or the activities of amylase, lipase, lecithinase and protease activities when compared with the wild type and complemented strains (Suppl. Figure S1).

Discussion

The TCS consists of a histidine kinase (HK) and a response regulator (RR) and plays a major role in a prokaryote’s ability to sense and respond to environmental stimuli [39]. Although each Xanthomonas genome contains approximately 92-121 TCS genes, the biological functions of the majority of these TCS genes remain unknown [46]. In genome of X. axonopodis pv. citri strain 306, there

![Figure 8. Virulence-related gene expression in Xanthomonas axonopodis pv. citri measured by RT-PCR analysis. RNA was isolated from cultures of strains TPH2, TPH3 and TPH5 in TSB or XVM2 medium, the latter of which mimics cytoplasmic fluids in planta, at 27°C for 18 hr with shaking at 100 rpm. RT-PCR was performed with primers specific for rfbC (113 bp), hrg (747 bp), rpfF (870 bp), katE (127 bp) and rpoD (263 bp). The mRNA level of rpoD was used as loading control. The experiments were performed three times with similar results, and representative results from one experiment are shown.](image-url)
are 35 orthodox HKs, 21 hybrid HKs, and 58 RRs [46]. In X. axonopodis pv. citri XW19, we identified a histidine kinase (BfdS) flanking BfdR, and we found that this kinase possesses a conserved asparagine at position four downstream from histidine (unpublished data); thus, BfdS is classified as a group II HK. The BfdR in X. axonopodis pv. citri strain XW19 shares 100% identity with locus tag XAC1284 in X. axonopodis pv. citri strain 306 and 99% identity with both locus tag XVE_1054 (which is a response regulator in X. vesicatoria strain ATCC 33937 with CheY-like receiver, ATPase, and DNA-binding domain) and locus tag XCC1187 (which is a two-component regulatory protein in X. campestris pv. campestris strain ATCC33913). BfdR in X. axonopodis pv. citri strain XW19 contains a conserved REC domain including active sites, a phosphorylation site, and dimerization sites similar to Escherichia coli CheY, which is involved in direction switching in the flagellar motor [47,48], and OmpR, which controls expression of outer membrane proteins in response to osmotic stress [49,50]. BfdR is also similar to Pseudomonas aeruginosa PhoB [51]. In addition, the phosphorylated OmpR and PhoB homologues usually function to stimulate the transcription of many genes in E. coli and are essential for virulence or biofilm formation in numerous pathogens [52,53,54,55]. However, in contrast to E. coli CheY [56], a mutation in bfrR of X. axonopodis pv. citri strain XW19 did not affect swimming or swarming motility (unpublished data).

TCs in X. axonopodis pv. citri have been functionally characterized including RpC/RpfG [57], HrpG [20] and CoR/CoS [21]. RpC is a hybrid protein consisting of N-terminal transmembrane, histidine kinase, response-regulator and C-terminal histidine phosphotransfer domains that interacts with both RpfG and RpfF [57]. RpfG consists of an N-terminal REC domain and a C-terminal HD-GYP domain, the latter of which was demonstrated to exhibit 3,5-cyclic diguanylic acid (c-di-GMP) phosphodiesterase activity [57,58]. Furthermore, RpfG was shown to interact with diguanylate cyclase GGDEF domain-containing proteins, which are responsible for the production of c-di-GMP. C-di-GMP is an important second messenger that was shown to regulate an array of bacterial processes including biofilm formation, virulence and motility [57,59]. The OmpR family regulators HrpG and CoR contain REC domains at their N-termini and DNA-binding motifs at their C-termini [20,21]. In contrast, BfdR in X. axonopodis pv. citri strain XW19 contains only the REC domain. The function of a BfdR homologue (XAC1284) in X. axonopodis pv. citri strain 306 has not been reported. Here, we have demonstrated the involvement of X. axonopodis pv. citri BfdR in biofilm formation and virulence and showed that BfdR positively regulates RpfF.

HrpG, CoR, and RpfG in X. axonopodis pv. citri were shown to play roles in coordinating the expression of multiple genes that are critical for pathogenicity [20,21,57]. Based on microarray analyses, HrpG was shown to regulate 24 type III secretion system genes, 23 type III secretion system effector genes, and 29 type II secretion system substrate genes in addition to genes related to chemotaxis, flagellar biosynthesis, and transport as well as regulatory genes such as RpfG, fsgM (encodes for a flagellar protein), phoU (encodes for a phosphoglucomutase regulatory protein), and rggV (a two-component system sensor) [20]. CoR is thought to play multiple roles in the pathogenicity of citrus canker bacteria [21]. It regulates not only virulence but also growth in planta, biofilm formation, catalase activity, LPS production, and resistance to environmental stress [21]. Based on quantitative RT-PCR assays, CoR positively regulated the expression of virulence-related genes including components of the type III secretion system (hrpD6 and hpaF7), the type III secretion system effector gene pthA, the LPS O-antigen synthesis gene fbsC, and the catalase gene katE [21]. Mutations in rpfG of X. axonopodis pv. citri were shown to reduce endoglucanase and protease activities, the production of cyclic β-(1,2)-glucan and xanthan, bacterial motility and attachment to the surface of Duncan grapefruit leaves and virulence on lemon leaves in addition to increasing the level of DSF [16,60]. In this study, we identified a novel two-component system response regulator, BfdR, in X. axonopodis pv. citri strain XW19 and demonstrated its involvement in biofilm formation on the leaf surfaces of citrus plants, epiphytic growth, and canker development as well as its ability to regulate the expression of RpfF. Mutation of bfrR in X. axonopodis pv. citri strain XW19 did not affect amylase, protease, lipase or lecithinase activities. Semi-quantitative RT-PCR analysis indicated that the transcript levels of rpsC, hpfG (encodes for a master regulator of type III secretion system components), katE (encodes for catalase) were similar in the bfrR mutant compared with the wild type in XVM2 medium. In the complemented strain, the RpfF transcript was restored back to wild type levels. These results suggest that BfdR in X. axonopodis pv. citri XW19 was not involved in the synthesis of known virulence-associated extracellular enzymes (including amylase, protease, lipase, or lecithinase) or genes including rpsC, hpfG and katE; however, BfdR positively regulated the transcription of RpfF. Our results showed that the expression of RpfF was only regulated by BfdR in XVM2 medium and not in TS broth. It is plausible that RpfF may be differentially expressed in XVM2 medium and TS broth. Similarly, data from Astua-Monge et al. (2005) suggested that the expression levels of rpsC and rpsE were decreased in XVM2 medium compared with their levels in nutrient broth [11].

RpfF encodes for an enoyl CoA hydratase and is partially dependent on RpfB, a long chain fatty acyl CoA ligase, for the synthesis of DSF [61]. Comparative genomic analyses have revealed that the rpf gene cluster is found in plant and human pathogens closely related to X. campestris including X. axonopodis pv. citri, Xylella fastidiosa, S. maltophila, and Burkholderia cenocepacia [17,62,63,64,65]. rpf/DSF signalling has been shown to contribute to virulence, biofilm formation, interaction with insect vectors or antibiotic tolerance in these pathogens [26,64,66,67,68]. The findings in X. campestris and B. cenocepacia suggest that the two-component regulator RpfG (with HD-GYP domain) and cis-2-dodecenoic acid receptor RpfR, respectively, link the rpf/DSF quorum sensing system with virulence regulation via c-di-GMP turnover [69,70]. At high cell densities, RpfC binds to DSF and phosphorylates RpfG, leading to phosphodieserstase activation and a decrease in c-di-GMP levels in X. campestris [58,71]. The decrease in c-di-GMP levels activates the cNMP-binding transcription regulator Clp, which induces the expression of virulence-related genes such as those involved in the synthesis of EPS, extracellular enzymes, membrane proteins, flagella, and components of the Hrp system; iron uptake; multidrug resistance; detoxification; and biofilm dispersal [72]. Here, we identified a two-component regulator, BfdR, in X. axonopodis pv. citri XW19 that is located far from the rpf gene cluster. We have shown that BfdR regulates RpfF expression, virulence and biofilm formation. These results suggest that BfdR may be connected to virulence and biofilm formation in X. axonopodis pv. citri XW19 through the regulation network of rpf/DSF. It remains to be determined whether the BfdR regulation network is also linked with c-di-GMP. Additionally, the results from the wound-inoculation pathogenicity assay indicated no significant differences in canker development between the wild type strain and the bfrR mutant.
suggesting that BdIR may be involved in the early stages of leaf surface colonization.

In conclusion, our results demonstrate that BdIR in X. axonopodis pv. citri WX19 plays a critical role in colonization, biofilm formation and virulence on the leaf surfaces of citrus plants. BdIR did not regulate the production of virulence-regulate extracellular enzymes including amylase, lipase, protease and lecinthinase or the expression of genes involved in the synthesis of type III secretion system components, O-antigen LPS, or catalase. However, BdIR controlled the expression of gfp/E, a gene involved in DSF synthesis, in a medium that mimics cytoplasmic fluids in planta.

Supporting Information

Figure S1 Xanthomonas axonopodis pv. citri wild type, bdIR mutant and complemented strains showed similar activities of amylase, lipase and lecinthinase. 10 μl bacterial suspensions of X. axonopodis pv. citri strains WX19, TPH1, TPH2, TPH3, TPH4 and TPH5 (OD620 = 0.3) were spotted on medium as the sequence shown in (A). Activities of extracellular enzymes for amylase (B), lipase (C), and lecinthinase (D) by the strains were shown. (TIF)

Acknowledgments

We thank Drs. A. S. Hwang and T. H. Hung for generous gifts of Mexican lime plants, and Dr. K. C. Tzeng for providing Xanthomonas axonopodis pv. citri strain WX19. We appreciate “The Wisconsin Package” (GCG/SeqWeb, http://bioinfo.nhri.org.tw) services provided by the National Health Research Institutes (NRHI), Taiwan.

Author Contributions

Conceived and designed the experiments: TPH. Performed the experiments: TPH KML YHC. Analyzed the data: TPH KML. Contributed reagents/materials/analysis tools: TPH. Wrote the paper: TPH.

References

1. Schaad NW, Postnacka E, Lacy GH, Secler A, Agarkova I, et al. (2005) Reclassification of Xanthomonas campestris pv. citri (ex Hasse 1915) Dye 1978 forms A, B/C/D, and E as X. simiae subsp. citri (ex Hasse) sp. nov. nom. rev. comb. nov.; X. fuscans subsp. unamunisfiae (ex Gabriel 1989) sp. nov. nom. rev. comb. nov.; and X. alfalfae pv. citri subsp. citri (ex Rieter and Jones) Gabriel, et al., 1989 sp. nov. nom. rev. comb. nov.; X. campestris pv. malvacearum (ex Simon 1901) Dye 1978 as X. simiae subsp. simiae nov. comb. nov. nom. nov.; X. campestris pv. alfalfae (ex Rieter and Jones, 1935) dype 1978 as X. alfalfae subsp. alfalfae (ex Rieter, et al., 1935) sp. nov. nom. rev.; and "var. fuscans" of X. campestris pv. phascoli (ex Smith, 1987) Dye 1978 as X. fuscans subsp. fuscans sp. nov. Syt Appl Microbiol 28: 494–518.

2. Ryan RP, Vorholt FH, Pomis N, Jones JB, Van Shus M-A, et al. (2011) Pathogenomics of Xanthomonas: understanding bacterium-plant interactions. Mol Plant-Microbe Interact 24: 354–355.

3. Brunings AM, Gabriel DW (2003) Xanthomonas citri: breaking the surface. Mol Plant Pathol 4: 141–157.

4. Charakis WG, Marshall KC (1996) Biofilms: a basis for an interdisciplinary approach. In: Charakis WG, Marshall KC, editors. Biofilms. New York: A Wiley-Interscience Publication, John Wiley & Sons. Inc. 3–16.

5. Rigano LA, Siciliano F, Enrique R, Sendin L, Filippone P, et al. (2007) Biofilm formation, epiphytic fitness, and canker development in Xanthomonas axonopodis pv. citri. Mol Plant-Microbe Interact 20: 1222–1230.

6. Huang TP, Tseng DD-S, Wong ACL, Chen C-H, Lu K-M, et al. (2012) DNA polymorphisms and biocorrol of Bacillus anthracis to citrus bacterial canker with indication of the interference of phylosephorobiophila. PLoS One 7: e11219.

7. Danhorn T, Fuqua C (2007) Biofilm formation by plant-associated bacteria. Annu Rev Microbiol 61: 401–422.

8. Parsek MR, Singh PK (2003) Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 57: 677–701.

9. Laia M, Moreira L, Dezaacayo J, Brigati J, Ferreira C, et al. (2009) New genes of Xanthomonas citri subsp. citri involved in pathogenesis and adaptation revealed by a transposon-based mutant library. BMC Microbiol 9: 12.

10. Yan Q, Wang N (2012) High-throughput screening and analysis of genes of Xanthomonas citri subsp. citri involved in citrus cancer symptom development. Mol Plant-Microbe Interact 25: 69–84.

11. Astua-Monge G, Freitas-Astua J, Bacocina G, Roncoletta J, Carvalho SA, et al. (2011) The LOV protein modulates the physiological attributes of Xanthomonas axonopodis pv. citri relevant for host plant colonization. PLoS ONE 7: e38226.

12. Liu, J, Wang N (2011) The wzcO gene of Xanthomonas citri sp. citri encodes a protein with a role in lipopolysaccharide biosynthesis, biofilm formation, stress tolerance and virulence. Mol Plant Pathol 12: 381–396.

13. Kraiefiburd I, Alet AI, Tondo ML, Petrocelli S, Dauroedo LD, et al. (2012) A LOV protein modulates the physiological attributes of Xanthomonas axonopodis pv. citri relevant for host plant colonization. PLoS ONE 7: e33822.

14. da Silva AC, Ferro JA, Reinaich FC, Farah CS, Farlan LR, et al. (2002) Comparison of the genomes of two Xanthomonas pathogens with differing host specificities. Nature 417: 459–463.

15. Gottig N, Garavaglia BS, Garofalo CG, Zimaro T, Sgro GG, et al. (2010) Mechanisms of infection used by Xanthomonas axonopodis pv. citri incitus canker disease. In: Mendz-Vilas A, editor. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. 196–204.

16. Dunger G, Arbalolaza AL, Gottig N, Orellano EG, Ottado J (2005) Participation of Xanthomonas axonopodis pv. citri hsp cluster in citrus canker and nonhost plant responses. Plant Pathol 54: 781–788.

17. Guo Y, Figueredo F, Jones J, Wang N (2011) HrpG and HrpX play global roles in coordinating different virulence traits of Xanthomonas axonopodis pv. citri. Mol Plant-Microbe Interact 24: 649–661.

18. Yan Q, Wang N (2011) The Goe-Col/Sis two-component system plays multiple roles in the pathogenicity of the citrus canker pathogen Xanthomonas citri subsp. citri, J Bacteriol 193: 1590–1598.

19. Guo Y, Sagaras U, Kit-JS, 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.

20. Guo Y, Zhang Y, Li J-L, Wang N (2011) Diffusible signal factor-mediated quorum sensing plays a central role in coordinating gene expression of Xanthomonas citri subsp. citri relevant for host plant colonization. PLoS ONE 7: e33822.

21. Liu, J, Wang N (2011) Genome-wide mutagenesis of Xanthomonas axonopodis pv. citri reveals novel genetic determinants and regulation mechanisms of biofilm formation. PLoS ONE 6: e21004.

22. Dow JM, Crossman L, Findlay K, He-Y-Q, Feng JX, et al. (2003) Biofilm dispersal in Xanthomonas campestris is controlled by cell-cell signaling and is required for full virulence to plants. Proc Natl Acad Sci USA 100: 10993–11000.

23. Guo Y, Zhang Y, Li J-L, Wang N (2011) Diffusible signal factor-mediated quorum sensing plays a central role in coordinating gene expression of Xanthomonas citri subsp. citri relevant for host plant colonization. PLoS ONE 7: 165–179.

24. Crossman L, Dow JM (2004) Biofilm formation and dispersal in Xanthomonas campestris. Microbes Infect 6: 623–629.

25. Huang TP, Somers EB, Wong AC (2006) Differential biofilm formation and motility associated with lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes in Stenotrophomonas maltophilia. J Bacteriol 188: 3116–3120.

26. Crossman L, Findlay K, He-Y-Q, Feng JX, et al. (2003) Biofilm formation in Xanthomonas campestris is controlled by cell-cell signaling and is required for full virulence to plants. Proc Natl Acad Sci USA 100: 10993–11000.

27. Schultz J, Milepsz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: Identification of signaling domains. Proc Natl Acad Sci USA 95: 5857–5864.

28. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

29. Fletcher M (1977) The effect of culture concentration, age, time and temperature on bacterial attachment to polystyrene. Can J Microbiol 23: 1–6.

30. O’Toole GA, Pratt LA, Wintz MK, Newman KK, Weaver VB, et al. (1999) Genetic approaches to study of biofilms. In: Doyle RJ, editor. Methods in Enzymology Topics in Applied Microbiology and Microbial Biotechnology. 196–204.

31. Savil H, Karadenizli A, Kollay F, Gundes S, Ozbek U, et al. (2003) Expression stability of six housekeeping genes: a proposal for resistance gene quantification.
studies of Pseudomonas aeruginosa by real-time quantitative RT-PCR. J Med Microbiol 52: 403–408.
37. Stark E, Wellerston RJ, Tettayt PA, Kossack CF (1953) Bacterial alpha amylase paper disc tests on starch agar. Appl Environ Microbiol.
38. Colmer AR (1948) The action of Bacillus cereus and related species on the lecithin complex of egg yolk. J Bacteriol 55: 777–785.
39. Muller-Dieckmann HJ, Grantz AA, Kim S-H (1999) The structure of the signal receiver domain of the Arabesbus thaliana ethylene receptor ETR1. Structure: 7: 1347–1356.
40. Tang JL, Liu YN, Barber CE, Dow JM, Wootton JC, et al. (1991) Genetic and molecular analysis of a cluster of gff genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in Xanthomonas campestris pv. campestris. Mol Gen Genet 226: 409–417.
41. Dow JM, Daniels MJ (1994) Pathogenicity determinants and global regulation of pathogenicity of Xanthomonas campestris pv. campestris. Curr Top Microbiol Immunol 192: 29–41.
42. Aparna G, Chatterjee A, Sonti RV, Sankaranarayanan R (2009) A cell wall-degrading esterase of Xanthomonas oryzae requires a unique substrate recognition module for pathogenesis on rice. Plant Cell 21: 1860–1873.
43. Kim J-G, Taylor KW, Hostom A, Keegan M, Schmelz EA, et al. (2000) XopD, a Xanthomonas oryzae pv. oryzae protease affects host transcription, promotes pathogen growth, and delays symptom development in Xanthomonas-infected tomato leaves. Plant Cell 20: 1915–1929.
44. Duan YP, Castañeda A, Zhao G, Erdos G, Gabriel DW (1999) Expression of a novel repressor involved in virulence of Pseudomonas aeruginosa. J Biol Chem 274: 33414–33421.
45. Tondo ML, Petrocelli S, Ottado J, Orellano EG (2010) The monofunctional OmpR of Pseudomonas aeruginos indicates the role of OmpR in the expression of virulence factors. Mol Microbiol 78: 5034–5040.
46. Qian W, Han Z-J, He C (2008) Two-component signal transduction systems of Stenotrophomonas maltophilia. Mol Microbiol 62: 537–551.
47. Newman KL, Chatterjee S, Ho KA, Lindow SE (2008) Virulence of plant pathogenic bacteria attenuated by degradation of fatty acid cell-to-cell signaling factors. Molecular Plant-Microbe Interactions 21: 326–334.
48. Ryan RP, Fouhy Y, Lucey JF, Dow JM (2006) A two-component system involving an HD-GYP domain protein links cell-to-cell signaling to pathogenicity gene expression in Xanthomonas campestris. Mol Microbiol 58: 986–1003.
49. Fouhy Y, Scanlon K, Schouest K, Spillane C, Grossman I, et al. (2007) Diffusible signal factor-dependent cell-cell signaling and virulence in the nosocomial pathogen Stenotrophomonas maltophilia. J Bacteriol 189: 4964–4968.
50. Huang TP, Wong AC (2007) A cyclic AMP receptor protein-regulated cell-cell communication system mediates expression of a FecA homologue in Stenotrophomonas maltophilia. Appl Environ Microbiol 73: 5034–5040.
51. Anba J, Bidaud M, Vasil ML, Lazdunski A (1990) Nucleotide sequence of the rpfB gene of Xanthomonas campestris pv. oryzae: Regulation of pathogenicity on rice. J Bacteriol 55: 777–785.
52. Dorman CJ, Chatfield S, Higgins CF, Gordon PI, Dow JM (2006) Characterization of atypical symptoms on citrus leaves in Taiwan. Plant Pathol Bull 14: 227–238.
53. Robins-Kelly KP, Cotty PJ (1997) Cyclic di-GMP signaling in pathogenic bacteria: recent advances and new puzzles. J Bacteriol 188: 8327–8334.
54. Ryan RP, Fouhy Y, Lucey JF, Dow JM (2006) Cyclic di-GMP signaling in bacteria: recent advances and new puzzles. J Bacteriol 188: 8327–8334.
55. Anba J, Bidaud M, Vasil ML, Lazdunski A (1990) Nucleotide sequence of the rpfB gene of Xanthomonas campestris pv. oryzae: Regulation of pathogenicity on rice. J Bacteriol 55: 777–785.
56. Chatterjee S, Newman KL, Lindow SE (2008) Cell-to-cell signaling in Xylella fastidiosa suppresses movement and xylem vessel colonization in grape. Molecular Plant-Microbe Interactions 21: 1309–1315.
57. Paul K, Brouns H, Tan H, Blair DF (2011) A molecular mechanism of direction switching in the flagellar motor of Escherichia coli. Proc Natl Acad Sci USA 108: 17171–17176.
58. Wurtzel ET, Chou MY, Inouye M (1982) Osmoregulation of gene expression. I. DNA sequence of the ompF gene of the ompF operon of Escherichia coli and characterization of its gene product. J Biol Chem 257: 13685–13691.
59. Tao F, He Y-W, Wu D-H, Swarup S, Zhang L-H (2010) The cyclic nucleotide communication system mediates expression of a FecA homologue in Stenotrophomonas maltophilia. Mol Microbiol 78: 5034–5040.
60. Pradhan A, Zacchei D, Leveau JH, Lindow SE (2000) Improved gfp and maeG broad-host-range promoter-probe vectors. Mol Plam Microbe Interact 13: 1243–1250.
61. Slater H, Alvarez-Morales A, Barber CE, Daniels MJ, Dow JM (2000) A two-component system involving an HD-GYP domain protein links cell-to-cell signaling to pathogenicity gene expression in Xanthomonas campestris. Mol Microbiol 38: 986–1003.
62. Paul K, Brouns H, Tan H, Blair DF (2011) A molecular mechanism of direction switching in the flagellar motor of Escherichia coli. Proc Natl Acad Sci USA 108: 17171–17176.
63. Pradhan A, Zacchei D, Leveau JH, Lindow SE (2000) Improved gfp and maeG broad-host-range promoter-probe vectors. Mol Plam Microbe Interact 13: 1243–1250.