Diffusible Signal Factor-Mediated Quorum Sensing Plays a Central Role in Coordinating Gene Expression of *Xanthomonas citri* subsp. *citri*

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Diffusible signal factor (DSF) family signal-mediated quorum sensing (QS) has been identified in many gram-negative bacteria. This QS pathway of *Xanthomonas* spp. consists of three major QS components: RpfF, RpfC, and RpfG. The *rpfF* gene encodes a putative enoyl-CoA hydratase that catalyzes the synthesis of the signal molecule. RpfC and RpfG serve as a two-component system for the perception and transduction of the extracellular DSF family signals. In order to further characterize the QS regulatory network in *Xanthomonas citri* subsp. *citri*, we investigated the RpfF, RpfC, and RpfG regulons by using transcriptome analyses. Comparison of the transcriptomes of the QS mutants (*rpfF*, *rpfC*, and *rpfG*) with that of the wild-type strain revealed a core group of genes controlled by all three QS components, suggesting that the RpfC-RpfG two-component system is a major and conserved signal perception and transduction system for DSF family signal-mediated QS in *X. citri* subsp. *citri*. The unique genes controlled by RpfF alone indicate the complexity of the QS pathway and the involvement of additional sensory mechanisms in *X. citri* subsp. *citri*. The unique genes controlled by RpfC and RpfG, respectively, support the possibility that RpfC and RpfG play broader roles in gene regulation other than transduction of DSF signals.

Quorum sensing (QS) is a sophisticated mechanism allowing bacteria to communicate via the exchange of chemical signals and to alter their behavior on a population-wide scale. It was first found in *Vibrio fischeri* as a mechanism that controls bioluminescence production based on the local bacterial density (Nealson and Hastings 1979). Since this discovery, QS has been identified in many gram-negative and gram-positive microorganisms (Waters and Bassler 2005). QS bacteria produce and release diffusible chemical signaling molecules into their environment. When the concentration of signaling molecules reaches a threshold, the bacteria detect and respond to these signals, altering their gene expression and thereby regulating a diverse range of physiological activities.

In gram-negative bacteria, besides the well-characterized AHL-type of QS signals and AI-2, the diffusible signal factor (DSF) family signals have recently been detected in a variety of bacterial species, including *Xanthomonas campestris* pv. *campestris*, *X. oryzae* pv. *oryzae*, *Xylella fastidiosa*, *Stenotrophomonas maltophilia*, and *Burkholderia cenocepacia* (Barber et al. 1997; Boon et al. 2008; Colnaghi Simionato et al. 2007; Fouhy et al. 2007; He et al. 2010). Distinct DSF family molecules can be produced by different bacterial species. For instance, a DSF from *Xanthomonas campestris* pv. *campestris* has been characterized as *cis*-11-methyl-2-dodecenoic acid while a DSF-like signal molecule, designated BDSF, has been characterized as *cis*-2-dodecenoic acid in *B. cenocepacia* (Boon et al. 2008; Wang et al. 2004). Notably, multiple DSF family molecules can also be produced by one organism; for example, *X. oryzae* pv. *oryzae* produces three DSF family signals: DSF, BDSF, and *cis,cis*-11-methyldodeca-2,5-dienoic acid (CDSF) in rich media (He et al. 2010).

DSF signal-mediated QS was first discovered in a mutagenesis analysis to identify genes involved in the regulation of the synthesis of extracellular enzymes in *X. campestris* pv. *campestris* (Barber et al. 1997). RpfF functioning as a putative enoyl-CoA hydratase is a key enzyme for DSF biosynthesis, while the two-component system RpfC-RpfG is involved in sensing and transduction of the DSF signal in *X. campestris* pv. *campestris* (Barber et al. 1997; He et al. 2006a; Slater et al. 2000). Recent studies revealed the presence of *rpfF* homologs in xanthomonads, *X. fastidiosa*, *S. maltophilia*, and *B. cenocepacia* (Boon et al. 2008; da Silva et al. 2002; Fouhy et al. 2007; Newman et al. 2004). RpfF from *S. maltophilia* is essential for the biosynthesis of DSF and seven structurally related fatty acids, and RpfF from *X. oryzae* pv. *oryzae* is required for the production of three DSF family signals (DSF, BDSF, and CDSF) (He et al. 2010; Huang and Wong 2007). In addition to RpfF, the well-characterized DSF sensory system RpfC-RpfG of *X. campestris* pv. *campestris* is also found in all xanthomonads.

Transcriptome analysis of the RpfF regulon has significantly advanced the understanding of the DSF-mediated QS regulons in bacteria. One pioneer study conducted by Zhang and colleagues (He et al. 2006b) investigated the QS regulon of *X. campestris* pv. *campestris* using whole-genome-wide microarray analysis to compare the gene expression patterns of the wild-type strain and its DSF-deficient mutant as well as those of the DSF-deficient mutant in the presence or absence of DSF signals. In that study, 165 genes were identified as belonging to the QS regulon; these were classified into 12 functional groups, includ-

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ing genes encoding extracellular enzymes and genes involved in exopolysaccharide production, flagellum synthesis, resistance to toxins and oxidative stress, and aerobic respiration (He et al. 2006b). A later study utilizing proteomic analysis of the wild-type and an *rpfF* mutant strain revealed that 48 proteins were differentially regulated by QS in *X. oryzae* pv. *oryzicola*, of which 18 proteins were identified by mass spectrometry as being putatively involved in nitrogen transfer, protein folding, resistance to oxidative stress, and flagellar synthesis (Zhao et al. 2011). However, considering the complicated QS signal transduction cascade, a comprehensive understanding of the RpfC and RpfG regulons is lacking. In this study, we aimed at further advancing our understanding of the QS regulatory network by characterizing the regulons of the DSF signaling pathway components RpfF, RpfC, and RpfG in the important model pathogen *X. citri* subsp. *citri* through transcriptome analyses.

X. citri subsp. *citri* (syn. *X. citri*, *X. campestris* pv. *citri*, or *X. axonopodis* pv. *citri*) is the causal agent of the Asiatic form of citrus canker (Cubero and Graham 2002; Schaad et al.

2006; Vauterin et al. 1995). The Asiatic form (or A type) of citrus canker is the most virulent of all of the strains that cause citrus canker, and it affects the widest range of hosts, including *Citrus* spp. and many closely related rutaceous plants. *X. citri* subsp. *citri* enters host plant tissues through stomata and wounds and grows in the intercellular spaces of the spongy mesophyll (Graham et al. 2004). Comparative genomic analysis has revealed that *X. citri* subsp. *citri* genome contains an *rpf* locus highly similar to that of *X. campestris* pv. *campestris*, including *rpfF*, *rpfC*, and *rpfG* (da Silva et al. 2002). Though the putative DSF-like signal produced by *X. citri* subsp. *citri* has not been yet characterized, several pieces of experimental evidence suggest that *X. citri* subsp. *citri* may employ a DSF signaling mechanism similar to that of *X. campestris* pv. *campestris*. i) Similar to the phenotypes of the *rpfF* and *rpfC* mutants of *X. campestris* pv. *campestris*, molecular genetic analysis revealed that the mutations of *rpfF* and *rpfC* in *X. citri* subsp. *citri* reduced the production of extracellular enzymes and compromised the virulence on the host lemon leaves

Fig. 1. Virulence of *Xanthomonas citri* subsp. *citri* in planta is impaired by mutations in *rpfF*, *rpfC*, and *rpfG*. **A**, Representative Duncan grapefruit leaf from four replicates was inoculated with bacterial solutions (10^8 CFU/ml) and photographed at 6 days postinoculation (DPI); **B**, representative Duncan grapefruit leaf from four replicates was inoculated with bacterial solutions (10^5 CFU/ml) and photographed at 18 DPI; **C**, representative Duncan grapefruit leaf from four replicates was inoculated with bacterial solutions (10^4 CFU/ml) and photographed at 18 DPI; **D**, quantification of canker lesions in Duncan grapefruit leaves inoculated with bacteria (10^4 CFU/ml) at 18 DPI. Error bars represent standard deviation. The PROC GLM procedure of SAS 9.2 was used to analyze the statistical significance of difference in virulence among the strains; *, significant difference ($P < 0.01$) in virulence between the mutants and the wild-type strain. The difference in virulence among the mutants was also tested. The *rpfG* mutant was significantly less virulent than the *rpfC* mutant ($P < 0.05$) and significantly less virulent than the *rpfF* mutant ($P < 0.01$). In contrast, the *rpfC* and *rpfF* mutants did not show difference in virulence from each other ($P < 0.05$). Three independent assays were performed with ten leaves each time. **E**, Spray inoculation of *X. citri* subsp. *citri* strains on the abaxial side of Duncan grapefruit leaves. Inoculated leaves were photographed at 20 DPI. A 10 mM $MgCl_2$ solution was used as the mock inoculation control. The assay was repeated three times with five plants each time with similar results, and only one representative result is presented.

(Siciliano et al. 2006). ii) The *rpfF* mutant of *X. citri* subsp. *citri* is DSF deficient whereas the *rpfC* mutant of *X. citri* subsp. *citri* is DSF hyperproducing, reminiscent of their counterparts in *X. campestris* pv. *campestris* (Siciliano et al. 2006). The yeast two-hybrid and in vitro biochemical assays showed the direct physical interaction of RpfF and RpfC in *X. citri* subsp. *citri* (Andrade et al. 2006). These findings suggest that *X. citri* subsp. *citri* uses an autoregulation mechanism similar to that of *X. campestris* pv. *campestris* to control DSF biosynthesis. iii) The protein–protein interaction analysis revealed that *X. citri* subsp. *citri* RpfC interacts with RpfG and the HD-GYP domain of RpfG directly interacts with a few GGDEF domain-containing proteins, which produce the second messenger molecule c-di-GMP, suggesting that the RpfC-RpfG two-component system links DSF signaling with the c-di-GMP signaling pathway (Andrade et al. 2006).

Though these genomic and functional genomic approaches advanced the studies of DSF family signal-mediated QS and provided detailed information about the signaling pathway, the newly discovered findings raise many fundamental questions. i) Because multiple DSF family signals can be produced by a single organism, are there DSF family synthases beyond RpfF? ii) Are there additional DSF signal perception and transduction systems in *X. citri* subsp. *citri*? iii) Does the RpfC-RpfG system sense and respond to signals other than DSF family signals?

In this study, a whole-genome microarray was employed to investigate the DSF family signal-mediated QS regulon of *X. citri* subsp. *citri* by characterizing the RpfF, RpfC, and RpfG regulons. We also investigated the roles of DSF family signal-mediated QS in the citrus canker disease cycle and its involvement in early attachment and in planta growth of *X. citri* subsp. *citri* in the citrus host.

RESULTS

Generation of the *rpfF*, *rpfC*, and *rpfG* mutants of *X. citri* subsp. *citri*.

To investigate the role of DSF family signal-mediated QS in citrus canker infection, deletion mutants of the three critical genes involved in DSF family signal synthesis, detection, and transduction—*rpfF*, *rpfC*, and *rpfG*—were generated using double cross-over recombination (Supplementary Fig. S1). The deletions were confirmed with sequence analysis of the corresponding polymerase chain reaction (PCR) products. The *rpfF*, *rpfC*, and *rpfG* mutants cannot form compact pellets, unlike the wild-type strain, when centrifuged from the liquid medium nutrient broth (NB) or XVM2 (Supplementary Fig. S2). To confirm the mutations in the QS pathway, the *rpfF*, *rpfC*, and *rpfG* mutants were assayed for motility and extracellular protease production, which are well-known phenotypes controlled by

Fig. 2. Venn diagrams showing the overlap of genes whose expression is influenced by RpfF, RpfC, and RpfG in different growth phases. **A**, Influence of growth phase on the RpfF, RpfC, and RpfG regulons. **B**, Comparisons of the three regulons in each growth phase. Ex, the exponential phase; St, the stationary phase. Numerals in gray represent the number of genes down-regulated by RpfF, RpfC, or RpfG; numerals in black indicate the number of genes up-regulated by RpfF, RpfC, or RpfG.

DSF-mediated QS (Barber et al. 1997; Ryan et al. 2006). The *rpfF*, *rpfC*, and *rpfG* mutants showed a significant decrease in the production of proteases (Supplementary Fig. S3) and had decreased motility (Supplementary Fig. S4). The phenotypes of all three mutants were restored to the wild-type level following complementation with the corresponding genes in trans.

QS is required for the full virulence of *X. citri* subsp. *citri* in the citrus host.

The role of QS in the growth of *X. citri* subsp. *citri* in planta was evaluated by monitoring the populations of the wild-type and *rpfF*, *rpfC*, and *rpfG* mutants in, as well as their effects on, Duncan grapefruit, which is a susceptible host for *X. citri* subsp. *citri*. The wild-type and *rpfF*, *rpfC*, and *rpfG* mutants were infiltrated into young leaves of Duncan grapefruit at the starting concentration of 10^8 CFU/ml. No difference was observed with

respect to the time to appearance of lesions or the magnitude of lesions (Fig. 1A). When 10^5 CFU/ml was used as the starting concentration, the *rpfF*, *rpfC*, and *rpfG* mutants produced fewer lesions than the wild-type (Fig. 1B). However, the cell densities of the mutants in planta were not significantly lower than those of the wild-type strain (data not shown). To quantify the difference in virulence between the *rpfF*, *rpfC*, and *rpfG* mutants and the wild-type, we used 10^4 CFU/ml to inoculate Duncan grapefruit leaves, photographed the inoculated leaves at 18 days post-inoculation (DPI), and then calculated the lesion number per square centimeter. The value of lesion numbers caused by the *rpfF*, *rpfC*, and *rpfG* mutants was 50, 32, and 20% of that caused by the wild-type strain, respectively, in an area of 1 cm² (Fig. 1C and D). Deletion of the *rpfF*, *rpfC*, and *rpfG* genes significantly reduced the number of lesions formed in Duncan grapefruit leaves ($P < 0.01$). Furthermore, based on these lesion

Table 1. Genes in the RpfF, RpfC, and RpfG regulons (core group) and in any two of the regulons in the exponential phase

Locus tag ^b	Log ₂ -fold change ^a			Gene	Description	JCVI ^c
	rpfC/W Ex	rpfF/W Ex	rpfG/W Ex			
Core group						
XAC1427	-1.57	-1.36	-1.36	<i>pru</i>	Protein U	Cell envelope
XAC3921	-1.24	-1.01	-1.29	<i>ugt</i>	Glucosyltransferase	Cell envelope
XAC0661	-1.05	-1.07	-1.99	<i>peh-1</i>	Endopolygalacturonase	Cell envelope
XAC3524	2.77	2.54	2.49	–	Hypothetical protein	Cell envelope
XAC1976	-2.50	-2.44	-1.15	<i>flgL</i>	Flagellar hook-associated protein FlgL	Cellular processes
XAC1931	-2.25	-1.57	-1.29	<i>cheZ</i>	Chemotaxis-related protein	Cellular processes
XAC1932	-2.14	-1.55	-1.42	<i>cheY</i>	Chemotaxis protein	Cellular processes
XAC2448	-2.11	-2.09	-2.00	<i>mcp</i>	Chemotaxis protein	Cellular processes
XAC2866	-2.10	-2.10	-2.19	<i>mcp</i>	Chemotaxis protein	Cellular processes
XAC1973	-1.84	-1.81	-2.10	<i>fliS</i>	Flagellar protein	Cellular processes
XAC2865	-1.77	-1.50	-1.87	<i>cheA</i>	Chemotaxis histidine protein kinase	Cellular processes
XAC1904	-1.77	-1.54	-1.90	<i>cheY</i>	Chemotaxis response regulator	Cellular processes
XAC2867	-1.75	-1.62	-1.80	<i>cheW</i>	Chemotaxis protein	Cellular processes
XAC1996	-1.73	-1.70	-1.98	<i>mcp</i>	Chemotaxis protein	Cellular processes
XAC1974	-1.73	-1.53	-1.92	<i>fliD</i>	Flagellar protein	Cellular processes
XAC3132	-1.71	-1.55	-2.01	<i>mcp</i>	Chemotaxis protein	Cellular processes
XAC2447	-1.67	-1.30	-1.60	<i>cheW</i>	Chemotaxis protein	Cellular processes
XAC1930	-1.66	-1.30	-1.03	<i>cheA</i>	Chemotaxis-related protein	Cellular processes
XAC4029	-1.65	-1.35	-1.78	<i>catB</i>	Catalase precursor	Cellular processes
XAC1987	-1.64	-1.44	-1.85	<i>cheV</i>	Chemotaxis protein	Cellular processes
XAC1893	-1.64	-1.70	-1.70	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC1950	-1.60	-1.58	-1.61	<i>fliJ</i>	Flagellar FliJ protein	Cellular processes
XAC1895	-1.57	-1.56	-1.83	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC1951	-1.54	-1.30	-1.47	<i>fliI</i>	Flagellar protein	Cellular processes
XAC1897	-1.53	-1.59	-1.65	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC1899	-1.44	-1.52	-1.58	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC1903	-1.42	-1.33	-1.69	<i>cheA</i>	Chemotaxis protein	Cellular processes
XAC1894	-1.42	-1.37	-1.66	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC0064	-1.41	-1.39	-1.13	–	Acetyltransferase	Cellular processes
XAC1907	-1.41	-1.11	-1.65	<i>paraA</i>	Chromosome partitioning protein	Cellular processes
XAC1953	-1.41	-1.24	-1.81	<i>fliG</i>	Flagellar protein	Cellular processes
XAC1902	-1.40	-1.51	-1.36	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC1952	-1.38	-1.24	-1.42	<i>fliH</i>	Flagellar protein	Cellular processes
XAC3213	-1.33	-1.39	-1.35	<i>mcp1</i>	Chemotaxis protein	Cellular processes
XAC1949	-1.33	-1.49	-1.71	<i>fliK</i>	Flagellar protein	Cellular processes
XAC1896	-1.25	-1.12	-1.24	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC1954	-1.23	-1.05	-1.74	<i>fliF</i>	Flagellar MS-ring protein	Cellular processes
XAC1900	-1.22	-1.26	-1.32	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC1891	-1.19	-1.29	-1.27	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC0934	-1.67	-1.32	-1.36	–	Truncated xylanase	Central intermediary
XAC0933	-1.42	-1.14	-1.34	–	Truncated xylanase	Central intermediary
XAC1890	-1.46	-1.43	-1.49	<i>cheR</i>	Chemotaxis protein methyltransferase	DNA metabolism
XAC0612	-4.38	-4.58	-4.29	<i>engXCA</i>	Cellulase	Energy metabolism
XAC1285	-3.34	-3.31	-2.56	<i>lamA</i>	Endo-1,3-β-glucanase	Energy metabolism
XAC0029	-3.19	-3.32	-3.85	<i>egl</i>	Cellulase	Energy metabolism

(continued on next page)

^a Log₂-fold change was derived from the mutant versus the wild-type strain. Ex, the exponential phase; NS = not significantly differentially expressed ($|\log_2$ -fold change| < 1 or false discovery rate [FDR] > 0.01).

^b Locus tags marked with * represent the extended members of the core group regulated by RpfC, RpfF, and RpfG. These genes showed altered expression with the less stringent cutoff values of $0.585 < |\log_2$ -fold change| < 1 and FDR < 0.05 in the *rpfC*, *rpfF*, and *rpfG* mutants as compared with the wild type.

^c J. Craig Venter Institute (JCVI) functional categories. Central intermediary = central intermediary metabolism; Transport and binding = transport and binding proteins.

numbers, the *rpfG* mutant was significantly less virulent than the *rpfC* mutant ($P < 0.05$) and the *rpfF* mutant ($P < 0.01$) while the *rpfC* and *rpfF* mutants were similar in virulence.

The *rpfF*, *rpfC*, and *rpfG* mutants also showed reduced virulence on spray-inoculated grapefruit compared with the wild-type strain. Although they still caused typical canker lesions, the numbers of lesions they caused were much fewer than the wild-type strain (Fig. 1E).

Microarray analysis indicates that RpfF, RpfC, and RpfG modulate the global gene expression profile of *X. citri* subsp. *citri*.

To investigate the role of RpfF, RpfC, and RpfG on the global gene expression profile of *X. citri* subsp. *citri*, XVM2 medium was used in this study to mimic the intercellular spaces of plant cells (Wengelnik et al. 1996). The growth of the QS

mutants (*rpfF*, *rpfC*, and *rpfG*) and the wild-type strain were monitored in XVM2 (Supplementary Fig. S5). Two time points, 11 and 25 h, were chosen to represent the exponential and stationary phases, respectively. In this study, a false discovery rate (FDR) of 0.01 and an absolute value of $\log_2$ -fold change of 1.0 (equivalent to fold change of 2.0) were used as the cut-off values. Loss of *rpfC* caused the differential expression of 160 genes at the exponential phase and 43 genes at the stationary phase (Fig. 2A; Supplementary Table S1). Loss of *rpfF* caused the alteration in expression of 120 genes at the exponential phase and 104 genes at the stationary phase (Fig. 2A). The deletion of *rpfG* caused the differential expression of 216 genes at the exponential phase and 214 genes at the stationary phase (Fig. 2A). The microarray data have been confirmed by quantitative reverse-transcription (QRT)-PCR with selected genes (Supplementary Table S2). Overall, 166, 180, and 388

Table 1. (continued from preceding page)

Locus tag ^b	Log ₂ -fold change ^a			Gene	Description	JCVT ^c
	<i>rpfC</i> /W Ex	<i>rpfF</i> /W Ex	<i>rpfG</i> /W Ex			
XAC0028	-3.15	-2.51	-2.46	<i>egl</i>	Cellulase	Energy metabolism
XAC1927	-2.35	-2.51	-2.73	<i>aslB</i>	Fe-S oxidoreductase	Energy metabolism
XAC3507	-1.68	-2.03	-1.69	<i>celS</i>	Truncated cellulase S	Energy metabolism
XAC1975	-1.58	-1.79	-1.71	<i>fliC</i>	Flagellin	Energy metabolism
XAC0446	-1.26	-1.00	-1.19	<i>pdhA</i>	Pyruvate dehydrogenase E1 α subunit	Energy metabolism
XAC1905	-1.59	-1.43	-1.77	–	Hypothetical protein	Hypothetical proteins
XAC3763	-1.47	-1.33	-1.45	–	Hypothetical protein	Hypothetical proteins
XAC1021	-1.11	-1.39	-1.59	–	Hypothetical protein	Hypothetical proteins
XAC1926	-2.28	-2.43	-2.56	–	Hypothetical protein	Not in JCVI
XAC1452	-2.15	-2.11	-2.51	–	Hypothetical protein	Not in JCVI
XAC4026	-1.91	-1.74	-2.29	–	Hypothetical protein	Not in JCVI
XAC4030	-1.81	-1.68	-1.86	<i>catB</i>	Catalase	Not in JCVI
XAC1034	-1.80	-2.06	-1.76	–	Peptidyl-Asp metalloendopeptidase	Not in JCVI
XAC3219	-1.56	-1.77	-2.06	–	Hypothetical protein	Not in JCVI
XAC0543	-1.51	-1.19	-1.95	–	Hypothetical protein	Not in JCVI
XAC1928	-1.49	-1.32	-1.66	–	Hypothetical protein	Not in JCVI
XAC3506	-1.49	-1.47	-1.41	–	Truncated cellulase S	Not in JCVI
XAC4091	-1.40	-1.66	-1.56	–	Hypothetical protein	Not in JCVI
XAC3787	-1.33	-1.56	-1.53	–	Hypothetical protein	Not in JCVI
XAC2268	-1.31	-1.23	-1.08	–	Hypothetical protein	Not in JCVI
XAC3533	-1.31	-1.63	-2.18	–	Hypothetical protein	Not in JCVI
XAC1634	-1.27	-1.08	-1.49	–	Hypothetical protein	Not in JCVI
XAC3018	-1.24	-1.17	-1.08	–	Hypothetical protein	Not in JCVI
XAC1972	-1.22	-1.21	-1.19	–	Hypothetical protein	Not in JCVI
XAC0817	-1.20	-1.23	-1.26	–	Hypothetical protein	Not in JCVI
XAC1971	-1.15	-1.21	-1.17	–	Hypothetical protein	Not in JCVI
XAC1778	-1.15	-1.18	-1.34	–	Sensor kinase	Not in JCVI
XAC3523	2.06	2.00	2.33	–	Hypothetical protein	Not in JCVI
XAC3522	2.19	1.97	2.10	–	Hypothetical protein	Not in JCVI
XAC3525	2.98	2.65	2.54	–	Hypothetical protein	Not in JCVI
XAC2151	-4.76	-4.60	-4.40	<i>yapH</i>	YapH protein	Protein fate
XAC4182	-3.16	-2.88	-2.87	–	Cytochrome C biogenesis protein	Protein fate
XAC0465	-2.88	-3.00	-2.94	–	Metalloproteinase	Protein fate
XAC0540	-1.76	-1.90	-1.58	–	Ribonuclease	Protein fate
XAC2763	-1.75	-2.32	-2.39	–	Extracellular protease	Protein fate
XAC2831	-1.46	-1.62	-1.27	–	Extracellular serine protease	Protein fate
XAC2992	-2.36	-2.54	-2.69	–	Endoproteinase Arg-C	Protein synthesis
XAC2868	-1.68	-1.44	-1.82	<i>vieA</i>	Response regulator	Regulatory functions
XAC3922	-1.36	-1.15	-1.28	<i>entF</i>	ATP-dependent serine activating enzyme	Regulatory functions
XAC3273	-1.14	-1.05	-1.35	–	Histidine kinase-response regulator hybrid protein	Regulatory functions
XAC3769	-2.37	-2.82	-2.59	<i>nucA</i>	Endonuclease precursor	Transcription
XAC1969	-1.31	-1.21	-1.11	<i>rpoN</i>	RNA polymerase σ^{54} factor	Transcription
XAC2864	-2.42	-2.16	-2.50	–	Hypothetical protein	Transport and binding
XAC2853	-2.98	-2.99	-3.80	–	Cysteine protease	Unclassified
XAC3868	-2.51	-2.47	-2.31	<i>ylil</i>	Dehydrogenase	Unclassified
XAC3014	-1.59	-1.62	-1.27	<i>rebB</i>	RebB protein	Unclassified
XAC0066	-1.56	-1.76	-1.33	–	Microcystin dependent protein	Unclassified
XAC0067	-1.46	-1.66	-1.27	<i>mdpB</i>	Microcystin dependent protein	Unclassified
XAC0065	-1.39	-1.60	-1.33	–	Microcystin dependent protein	Unclassified
XAC0346	-1.31	-2.00	-1.88	–	Degenerated cellulase	Unclassified
XAC3016	-1.29	-1.36	-1.19	<i>rebA</i>	RebA protein	Unclassified
XAC3015	-1.24	-1.31	-1.27	<i>rebB</i>	RebB protein	Unclassified

(continued on next page)

genes were differentially expressed in the *rpfC*, *rpfF*, and *rpfG* mutants, respectively, when compared with the wild-type strain (Fig. 2A). Notably, 22.3% (37 genes), 24.4% (44 genes), and 10.8% (42 genes) were found to be regulated in both growth phases by RpfC, RpfF, and RpfG, respectively.

The majority of altered genes were up-regulated by RpfF, RpfC, and RpfG in both growth phases (Fig. 2) based on their lower transcript levels in the mutants compared with the wild-type strain. Functional analysis revealed that the RpfF, RpfG, and RpfC regulons each contained genes in the majority of J. Craig Venter Institute (JCVI)-defined functional categories (Supplementary Fig. S6), indicating that the three QS regulatory components have global effects on gene expression of *X. citri* subsp. *citri*.

Comparison of the RpfF, RpfC, and RpfG regulons in the exponential phase.

Comparison of the RpfF, RpfC, and RpfG regulons in the exponential phase of growth yielded a comprehensive picture of the DSF family signal-mediated QS pathway. The analysis revealed a core group of 102 genes regulated by RpfF, RpfC,

and RpfG (Fig. 2B; Table 1). Two-way comparisons showed further overlap in the repertoire of RpfF-, RpfC-, and RpfG-regulated genes, with 12 genes regulated by both RpfC and RpfF, 25 by both RpfC and RpfG, and 1 by RpfF and RpfG in addition to the core group (Table 1). For each regulon, certain genes were regulated by only one of the three proteins: five genes were regulated by RpfF, 21 by RpfC, and 88 by RpfG (Fig. 2B).

The conserved core group of genes regulated by all three proteins showed that the alteration of expression was influenced in the same direction by the mutations of QS components. The core group includes 35 genes in cellular processes, with 32 genes involved in chemotaxis and flagellar biosynthesis, such as *fliDFGHIJKS*. The core group also contains eight genes in energy metabolism, which includes genes involved in the TCA cycle and biosynthesis and degradation of polysaccharides (cellulase and glucanase); six genes involved in determining protein fate (protease); three regulatory genes; the transcription factor σ^{54} ; and a large number of genes encoding hypothetical proteins (Table 1). Among these hypothetical genes, four genes (XAC3522–XAC3525) that were down-regulated

Table 1. (continued from preceding page)

Locus tag ^b	Log ₂ -fold change ^a			Gene	Description	JCVI ^c
	rpfc/W Ex	rpfF/W Ex	rpfG/W Ex			
XAC1906	–1.21	–1.13	–1.40	<i>cheW</i>	Chemotaxis protein	Unclassified
XAC2832	–1.21	–1.44	–1.19	–	Hypothetical protein	Unclassified
XAC3017	–1.08	–1.21	–1.11	<i>rebB</i>	RebB protein	Unclassified
XAC3314	–1.84	–1.75	–1.32	–	Hypothetical protein	Unknown function
XAC1909	–1.04	–1.00	–1.10	<i>motC</i>	Flagellar motor protein	Cellular processes
RpfC and RpfF only						
XAC0611*	–1.28	–1.04	NS	<i>tsr</i>	Chemotaxis protein	Cellular processes
XAC1982*	–1.68	–1.32	NS	<i>flgF</i>	Flagellar basal body rod protein FlgF	Cellular processes
XAC1983*	–2.04	–1.64	NS	<i>flgE</i>	Flagellar hook protein FlgE	Cellular processes
XAC0798*	–1.17	–1.21	NS	<i>amy</i>	α -Amylase	Energy metabolism
XAC1287*	–1.40	–1.42	NS	<i>galM</i>	Aldose 1-epimerase	Energy metabolism
XAC3562*	–1.35	–1.22	NS	<i>pel</i>	Pectate lyase	Energy metabolism
XAC4321*	–1.20	–1.08	NS	–	Hypothetical protein	Not in JCVI
XAC2600	–1.06	–1.09	NS	<i>btuB</i>	TonB-dependent receptor	Transport and binding
XAC2830	–1.04	–1.15	NS	<i>fhuA</i>	TonB-dependent receptor	Transport and binding
XAC0330	–1.15	–1.22	NS	<i>cmfA</i>	Conditioned medium factor	Unclassified
XAC3019*	–1.20	–1.10	NS	–	Hypothetical protein	Unclassified
XAC2986*	–1.40	–1.19	NS	<i>pelB</i>	Pectate lyase II	Energy metabolism
RpfC and RpfG only						
XAC1984*	–1.99	NS	–1.76	<i>flgD</i>	Flagellar basal body rod modification protein	Cell envelope
XAC0443*	–1.20	NS	–1.09	<i>pdhB</i>	Branched-chain α -keto acid dehydrogenase subunit E2	Cellular processes
XAC1978*	–1.81	NS	–1.55	<i>flgJ</i>	Flagellar rod assembly protein/muramidase FlgJ	Cellular processes
XAC1979	–1.84	NS	–1.87	<i>flgI</i>	Flagellar basal body P-ring protein	Cellular processes
XAC1980	–1.84	NS	–1.83	<i>flgH</i>	Flagellar basal body L-ring protein	Cellular processes
XAC1981	–1.91	NS	–1.94	<i>flgG</i>	Flagellar basal body rod protein FlgG	Cellular processes
XAC1985	–1.81	NS	–1.65	<i>flgC</i>	Flagellar basal body rod protein FlgC	Cellular processes
XAC2869*	–1.27	NS	–1.34	<i>cheR</i>	Response regulator for chemotaxis	DNA metabolism
XAC0445*	–1.27	NS	–1.21	<i>pdhB</i>	Pyruvate dehydrogenase E1 β subunit	Energy metabolism
XAC4079*	1.40	NS	1.06	<i>ecaA</i>	A-type carbonic anhydrase	Energy metabolism
XAC0753	–1.31	NS	–1.63	–	Hypothetical protein	Hypothetical proteins
XAC1210*	–1.78	NS	–1.12	–	Hypothetical protein	Not in JCVI
XAC1810*	1.23	NS	2.35	–	Hypothetical protein	Not in JCVI
XAC1898*	–1.12	NS	–1.36	–	Hypothetical protein	Not in JCVI
XAC1990*	–1.36	NS	–1.65	–	Hypothetical protein	Not in JCVI
XAC2786*	–1.32	NS	–1.65	–	Hypothetical protein	Not in JCVI
XAC3926*	1.24	NS	1.57	–	Hypothetical protein	Not in JCVI
XAC1328*	–1.09	NS	–1.29	–	Hypothetical protein	Regulatory functions
XAC1812*	1.23	NS	2.53	<i>hmsF</i>	HmsF protein	Regulatory functions
XAC1989*	–1.27	NS	–1.63	<i>flgM</i>	Flagellar protein	Regulatory functions
XAC1993*	–1.08	NS	–1.34	–	Hypothetical protein	Regulatory functions
XAC2382*	1.10	NS	1.34	–	GGDEF family protein	Regulatory functions
XAC0435*	–1.28	NS	–1.61	<i>virK</i>	VirK protein	Unclassified
XAC0350*	–1.01	NS	–1.30	–	Hypothetical protein	Unknown function
XAC1934*	–1.70	NS	–1.78	<i>fleN</i>	Flagellar biosynthesis switch protein	Unknown function
RpfF and RpfG only						
XAC1995*	NS	–1.07	–1.20	–	Hypothetical protein	Not in JCVI

share high identities at the amino acid sequence level with the *xagA*, *xagB*, *xagC*, and *xagD* genes of *X. campestris* pv. *campestris*. The *xagA*, *xagB*, *xagC*, and *xagD* genes have been characterized as being involved in biofilm formation and may contribute to the synthesis of polysaccharide adhesin because they share high similarity to the *pgaA*, *pgaB*, *pgaC*, and *pgaD* locus of *Escherichia coli*, which is responsible for adhesin biosynthesis (Tao et al. 2010). The functions of the genes in the conserved core group regulated by the three QS components explained the observation of the alterations in motility and protease production and the pellet phenotype caused by the mutations of *rpfC*, *rpfF*, and *rpfG*. The findings further supported that *X. citri* subsp. *citri* uses a DSF family signal-mediated QS mechanism similar to that of *X. campestris* pv. *campestris*.

We examined the expression of the genes altered in two of the three regulons for alterations in the third based on a less stringent cut-off value; namely, a fold-change of 1.5 ($|\log_2\text{-fold change}| = 0.585$) and an FDR of 0.05. Most of these genes could be grouped into the core group when using this cut-off value, including 9 of 12 genes shared by the RpfF and RpfC regulons, 20 of 25 genes shared by the RpfC and RpfG regulons, and the 1 gene shared by the RpfF and RpfG regulons (Table 1). The expression of all of these genes was altered in the same direction in all three mutants compared with the wild-type. Therefore, we designated these genes as the extended members of the core group, which includes eight genes associated with chemotaxis and flagellar biosynthesis, six genes involved in energy metabolism (sugar metabolism and pectate lyases for polysaccharides degradation), five regulatory genes, and genes encoding hypothetical proteins. The biological functions of the extended core group are consistent with the functions of genes in the conserved core group, further supporting the idea that the extended members are true members of the core group.

The genes controlled by only one of the three QS components were generally similar regardless of whether the cut-off criteria were an FDR of 0.01 and twofold change or an FDR of 0.05 and a 1.5-fold change (Supplementary Table S3). The genes controlled only by RpfF include *fimA* encoding type IV pilin, *cirA* encoding a TonB-dependent receptor, and *uahA* (XAC4326 and XAC4327) involved in energy metabolism. In addition to the hypothetical genes controlled by RpfC, the unique genes in the RpfC regulon included genes involved in adaptation to the environment, such as *katE* encoding a catalase, *czcD* encoding a heavy metal transporter, and genes encoding oxidoreductases (XAC1178 and XAC3738). RpfG alone regulated 88 genes, including 19 genes involved in transport; four genes encoding a type III secretion system (T3SS) translocon (*hrcC*, *hrpB2*, *hrcJ*, and *hpaB*); seven genes associated with energy metabolism; and five genes encoding cell surface appendages such as a type V secretion system adhesin (*xadA*), PGA adhesin (*hmsHR*), and fimbria (*pilM*). Among the genes encoding transporters controlled by RpfG, 14 genes involved in ferrous transport were down-regulated by RpfG.

Comparison of the RpfF, RpfC, and RpfG regulons in the stationary phase.

The comparison of the RpfF, RpfC, and RpfG regulons in the stationary phase identified a conserved core group of 33 genes showing differential expression in all three mutants compared with the wild-type (Fig. 2B; Table 2). A two-way comparison revealed an overlap of 5 genes by the RpfC and RpfF regulons, 1 gene by the RpfC and RpfG regulons, and 24 genes by the RpfF and RpfG regulons (Fig. 2B; Table 2). Most of the genes shared by only two regulons could fall into the third regulon based on the less stringent cut-off values described above; these included 4 of 5 genes shared by the RpfF

and RpfC regulons, the 1 gene shared by the RpfC and RpfG regulons, and 16 of 24 genes shared by the RpfF and RpfG regulons (Table 2). The expression of all of these genes was altered in the same direction compared with the wild-type in all three mutants. Therefore, we considered these genes as the extended members of the core group in the stationary phase. The genes regulated by only one of the three QS components included 4 genes controlled by RpfC, 42 genes by RpfF, and 156 genes by RpfG (Fig. 2B; Supplementary Table S4).

The conserved core group consists of two flagellar genes, one gene encoding catalase (*katE*), four genes in energy metabolism including two genes encoding cellulases, one regulatory gene, and genes encoding hypothetical proteins (Table 2). In addition to the hypothetical genes, the extended members of the core group include genes involved in flagellar biosynthesis, genes encoding adhesins (*HmsFH* and XAC3523-3524), and the gene XAC1969 encoding factor σ^{54} .

Similar to the genes altered in the exponential phase, the genes controlled by only one of the three QS components in the stationary phase were generally similar based on both the low- and high-stringency cut-off criteria described above. The unique genes in the RpfC regulon included two genes involved in energy metabolism and two genes associated with protein fate. The genes regulated only by RpfG included genes associated with the cell envelop such as outer membrane proteins, genes involved in energy metabolism, regulatory genes, genes involved in transcription, genes encoding transporters, and a large number of genes encoding hypothetical proteins. The genes regulated only by RpfF include genes involved in flagellar biosynthesis, virulence genes, regulatory genes, and genes encoding hypothetical proteins.

The attachment of *X. citri* subsp. *citri* was reduced by the mutations of *rpfF*, *rpfC*, and *rpfG*.

Given the significant effect of QS on expression of chemotaxis and flagellar genes as well as genes encoding membrane surface components such as the type IV pilus, we investigated whether QS mediates the bacterial attachment in pathogenesis. First, the wild-type and *rpfF*, *rpfC*, and *rpfG* mutants were tested for their early attachment ability on abiotic surfaces (hydrophobic). For all of the mutants, attachment to a hydrophobic surface was significantly reduced compared with the wild-type strain, with the level of crystal violet stain retained similar to that of the negative buffer control (Fig. 3A and B). In addition, the wild-type strain performed better in the attachment assay when grown in XVM2 than in NB.

We also investigated the bacterial attachment to host leaves based on attachment of cells in suspensions to the abaxial surface of Duncan grapefruit leaves. Similar to the results of the attachment to the abiotic surface, the wild-type strain grown in either XVM2 or NB attached to grapefruit leaves and retained crystal violet (CV) staining, whereas the *rpfF*, *rpfC*, and *rpfG* mutants were not able to attach to the leaf, and there was no significant difference for the mutants in CV staining compared with the negative buffer control (Fig. 3C).

To further examine the difference in attachment to leaves by the wild-type and the *rpfF*, *rpfC*, and *rpfG* mutants, we used confocal laser-scanning microscopy to observe the green fluorescent protein (GFP)-labeled bacterial cells attached to the leaves. The GFP plasmid did not affect the attachment to abiotic surface and biotic surfaces or the virulence in planta (data not shown). Given the better performance of bacteria grown in XVM2, the GFP-labeled strains were grown in XVM2 prior to the leaf attachment assay. After 1 h of incubation, the wild-type cells started to aggregate to microcolonies (Fig. 4A). In contrast, a few individual cells of the *rpfF*, *rpfC*, and *rpfG* mutants attached to the leaves (Fig. 4A). After 6 h of

incubation, many more wild-type cells attached to the leaves and formed a matrix on the leaves (Fig. 4B and C), whereas the *rpfF*, *rpfC*, and *rpfG* mutants formed microcolonies on the leaves (Fig. 4B). Each leaf was scanned at different depths and showed bacteria primarily located in the depression formed by the anticlinal wall of epidermal cells and around stomata, rather than on the top of the periclinal wall of epidermal cells (Fig. 4C). To confirm this observation, we used a scanning electron microscope to examine leaves incubated with bacterial suspensions for 6 h. More wild-type cells than QS mutant cells occupied the depressions between epidermal cells and around stomata (Fig. 4D).

DISCUSSION

DNA microarrays have been widely used to study the transcriptional responses of many organisms to genetic and environmental perturbations (Dharmadi and Gonzalez 2004; Ye et al. 2001). In the present study, we studied the QS regulon using a whole-genome DNA microarray for *X. citri* subsp. *citri* 306. The reliability and robustness of this Agilent array have been confirmed in the transcriptome analyses of the HrpG and HrpX regulons in our previous study (Guo et al. 2011). They were further validated in the present study using QRT-PCR.

Transcriptome analysis identified that 180 genes were under the control of RpfF under the conditions tested. Genes in the RpfF regulon include those involved in chemotaxis and motility, adhesion, stress tolerance, regulation, transport, and detoxifi-

cation, which is consistent with the previous findings on the DSF regulon in *X. campestris* pv. *campestris* (He et al. 2006b). The comparison of the RpfF regulon in the exponential phase and stationary phase revealed that 44 genes overlapped in both phases; these composed only 24.6% of the RpfF regulon. A similar growth-phase-dependent regulation pattern was shown for the RpfC and RpfG regulons, indicating that regulation of QS in *X. citri* subsp. *citri* is growth phase-dependent (Fig. 2A), as was shown previously for QS in *Pseudomonas aeruginosa* (Schuster et al. 2003; Wagner et al. 2003). In *P. aeruginosa*, some QS-regulated genes showed increased expression in the early exponential or stationary phase but most showed increases in the late exponential to early stationary phase (Schuster et al. 2003). The mechanism for the growth-phase-dependent QS regulation remains to be determined.

Notably, the numbers of genes in the core group controlled by all three QS components varied from the exponential phase to the stationary phase (Fig. 2B). The fewer genes in the core group in the stationary phase compared with in the exponential phase (33 versus 102) may be due to the fewer genes in the RpfC regulon in the stationary phase. Because the *rpfC* mutant is a DSF family signal-overproducing strain, we hypothesize that the accumulated DSF family signals might bypass the RpfC-RpfG system and be sensed by other two-component systems for gene regulation. This hypothesis is supported by the regulation patterns of RpfF, RpfC, and RpfG. The expression of 40% (42 genes) of genes in the RpfF regulon was not regulated by RpfC and RpfG in the stationary phase (Fig. 2B),

Table 2. Genes in the RpfF, RpfC, and RpfG regulons (core group) and in any two of the regulons in the stationary phase

Locus tag ^b	Log ₂ -fold change ^a			Gene	Description	JCVI ^c
	rpfC/W St	rpfF/W St	rpfG/W St			
Core group						
XAC3524	1.24	2.01	1.14	–	Hypothetical protein	Cell envelope
XAC1178	–1.89	–1.95	–2.63	–	Oxidoreductase	Cellular processes
XAC1950	–1.34	–1.83	–1.35	<i>fliJ</i>	Flagellar FljI protein	Cellular processes
XAC1949	–1.24	–1.52	–1.29	<i>fliK</i>	Flagellar protein	Cellular processes
XAC1211	–1.44	–1.51	–2.20	<i>katE</i>	Catalase	Cellular processes
XAC3738	–1.23	–1.16	–1.23	–	Oxidoreductase	Central intermediary
XAC0029	–3.96	–4.52	–3.80	<i>egl</i>	Cellulase	Energy metabolism
XAC0612	–2.06	–3.09	–1.89	<i>engXCA</i>	Cellulase	Energy metabolism
XAC1927	–1.67	–2.65	–3.51	<i>aslB</i>	Fe-S oxidoreductase	Energy metabolism
XAC3747	–1.27	–1.29	–1.85	<i>ybdR</i>	Zn-dependent alcohol dehydrogenase	Energy metabolism
XAC1021	–1.36	–1.59	–1.57	–	Hypothetical protein	Hypothetical proteins
XAC0543	–2.50	–2.80	–2.59	–	Hypothetical protein	Not in JCVI
XAC1926	–1.50	–2.55	–3.52	–	Hypothetical protein	Not in JCVI
XAC3219	–1.71	–2.18	–2.11	–	Hypothetical protein	Not in JCVI
XAC2786	–1.75	–2.14	–1.97	–	Hypothetical protein	Not in JCVI
XAC3533	–1.88	–1.74	–2.11	–	Hypothetical protein	Not in JCVI
XAC1210	–1.26	–1.25	–1.34	–	Hypothetical protein	Not in JCVI
XAC3513	–1.36	–1.20	–1.38	–	Hypothetical protein	Not in JCVI
XAC3745	–1.12	–1.08	–1.90	–	Hypothetical protein	Not in JCVI
XAC3746	–1.07	–1.05	–1.89	–	Hypothetical protein	Not in JCVI
XAC3522	1.11	1.77	1.10	–	Hypothetical protein	Not in JCVI
XAC3525	2.04	2.47	1.55	–	Hypothetical protein	Not in JCVI
XAC2992	–1.97	–2.58	–2.55	–	Endoproteinase Arg-C	Protein synthesis
XAC3273	–1.31	–1.38	–1.16	–	Histidine kinase-response regulator hybrid protein	Regulatory functions
XAC3856	–1.53	–1.49	–1.84	–	Hypothetical protein	Transport and binding
XAC2853	–3.40	–4.20	–4.02	–	Cysteine protease	Unclassified
XAC0346	–1.84	–2.42	–2.07	–	Degenerated cellulase	Unclassified
XAC3868	–1.86	–2.21	–2.05	<i>ylil</i>	Dehydrogenase	Unclassified
XAC0435	–1.77	–2.14	–2.03	<i>virK</i>	VirK protein	Unclassified
XAC1177	–1.69	–1.79	–1.83	–	Hypothetical protein	Unclassified

(continued on next page)

^a Log₂-fold change was derived from the mutant versus the wild-type strain. St, the stationary phase; NS = not significantly differentially expressed ($|\log_2\text{-fold change}| < 1$ or false discovery rate [FDR] > 0.01).

^b Locus tags marked with * represent the extended members of the core group regulated by RpfC, RpfF, and RpfG. These genes showed altered expression with the less stringent cutoff values of $0.585 < |\log_2\text{-fold change}| < 1$ and FDR < 0.05 in the *rpfC*, *rpfF*, and *rpfG* mutants as compared with the wild type.

^c J. Craig Venter Institute (JCVI) functional categories. Central intermediary = central intermediary metabolism, Transport and binding = transport and binding proteins, and Mobile = mobile and extrachromosomal element functions.

suggesting that DSF family signals might be sensed and transduced by additional sensing systems other than the RpfC-RpfG system. McCarthy and associates (2010) found that the novel sensor of the DSF family signal BCAM0227 controls only a subset of the genes in the BDSF regulon in *B. cenocepacia* and proposed the existence of further sensing mechanisms for BDSF.

Although additional sensing mechanisms might be involved in the perception of DSF family signals, the RpfC-RpfG system serves as a major sensing system for DSF family signals in *X. citri* subsp. *citri*. The comparison of the RpfF, RpfC, and RpfG regulons revealed the conserved core gene group controlled by RpfF, RpfC, and RpfG in the exponential and stationary phases (Fig. 2B). The expression of the genes in the core group exhibits alterations in the same direction in all three mutants compared with the wild-type strain (Tables 1 and 2). The finding indicates that RpfF, RpfC, and RpfG exert the same effects on gene expression and are involved in the same signaling pathway. Furthermore, the majority of the genes that belonged to any two of the three regulons also belonged to the third regulon based on the application of a slightly relaxed stringency in the cut-off determination and showed altered expression in the same direction in all three mutants. Thus, we extended the core group to include these genes and designated it as an extended core group. The extended core group is controlled by all three QS components and includes genes involved in chemotaxis and flagellar biosynthesis, genes encoding extracellular enzymes, and genes encoding adhesins (Tables 1 and 2). The similar motility, protease production, and pelleting phenotypes of the *rpfF*, *rpfC*, and *rpfG* mutants compared with the

wild-type further supported the regulation of these functions by RpfF, RpfC, and RpfG. Overall, these findings indicate that RpfC-RpfG is the major sensory system of DSF family signal-mediated QS in *X. citri* subsp. *citri*.

How does DSF family signal-mediated QS regulate diverse biological functions? Recent studies have advanced our understanding of this QS regulatory pathway. The interaction of the HD-GYP domain of RpfG with a subset of GGDEF domain-containing proteins, which generally influence c-di-GMP levels, suggests the involvement of a few different downstream pathways in controlling subsets of QS-regulated functions. One pathway was shown to involve binding of c-di-GMP to the protein Clp and preventing it from regulating a subset of the DSF regulon genes (He et al. 2007). Another pathway was shown to involve RpfG interacting with two GGDEF proteins, XAC0258 and XAC0424, in *X. citri* subsp. *citri* (Andrade et al. 2006) and their homologues XC_0249 and XC_0420 in *X. campestris* pv. *campestris* (Ryan et al. 2010). XC_0249 and XC_0420 have been revealed to control motility but not other DSF-mediated phenotypes (Ryan et al. 2010). Notably, the expression of XAC0424 was up-regulated by RpfG in the exponential phase in this study. In addition to these two pathways, our data support the interaction of RpfC-RpfG with the NtrB-NtrC complex, as previously demonstrated in *X. citri* subsp. *citri* (Andrade et al. 2006). NtrC contains ATP-dependent σ^{54} -activating and DNA-binding domains which interact with the RpfG HD-GYP domain. In *V. harveyi* and *V. cholera*, the NtrC-like σ^{54} activator (LuxO) and σ^{54} are involved in QS (Lenz et al. 2004). In this study, the expression of σ^{54} (XAC1969) was up-regulated by all three QS components in both growth

Table 2. (continued from preceding page)

Locus tag ^b	Log ₂ -fold change ^a			Gene	Description	JCVI ^c
	rpfC/W St	rpfF/W St	rpfG/W St			
XAC3726	-2.20	-1.56	-1.03	–	Hypothetical protein	Unclassified
XAC0067	-1.45	-1.47	-1.13	<i>mdpB</i>	Microcystin dependent protein	Unclassified
XAC0066	-1.27	-1.46	-1.18	–	Microcystin dependent protein	Unclassified
RpfF and RpfG only						
XAC2922	NS	-1.32	-1.53	<i>hrpW</i>	HrpW protein	Cell envelope
XAC1813	NS	1.29	1.38	<i>hmsH</i>	HmsH protein	Cell envelope
XAC3354*	NS	2.03	1.74	<i>ompW</i>	Outer membrane protein W	Cell envelope
XAC1951*	NS	-1.46	-1.11	<i>fliI</i>	Flagellar protein	Cellular processes
XAC1988	NS	1.55	1.44	<i>flgA</i>	Flagellar basal body P-ring biosynthesis protein FlgA	Cellular processes
XAC1955	NS	1.94	1.78	<i>fliE</i>	Flagellar protein	Cellular processes
XAC1948	NS	2.16	1.81	<i>fliH</i>	Flagellar protein	Cellular processes
XAC2028*	NS	-1.11	-1.68	<i>fdh</i>	Glutathione-dependent formaldehyde dehydrogenase	Energy metabolism
XAC2336	NS	1.72	1.57	<i>cydA</i>	Cytochrome D ubiquinol oxidase subunit I	Energy metabolism
XAC3776	NS	-1.10	-2.16	–	Hypothetical protein	Hypothetical proteins
XAC2026*	NS	-1.20	-1.67	–	Hypothetical protein	Not in JCVI
XAC2027*	NS	-1.12	-1.61	–	Hypothetical protein	Not in JCVI
XAC3715*	NS	-1.12	-1.53	–	Hypothetical protein	Not in JCVI
XAC1928*	NS	-1.49	-1.53	–	Hypothetical protein	Not in JCVI
XAC2787*	NS	-1.60	-1.32	–	Hypothetical protein	Not in JCVI
XAC3085*	NS	-1.03	-1.23	–	Hypothetical protein	Not in JCVI
XAC3787*	NS	-1.40	-1.16	–	Hypothetical protein	Not in JCVI
XAC2370*	NS	-1.42	-1.08	–	Hypothetical protein	Not in JCVI
XAC3523*	NS	1.88	1.58	–	Hypothetical protein	Not in JCVI
XAC1512*	NS	-1.20	-1.41	–	Serine peptidase	Protein fate
XAC2151*	NS	-1.79	-1.08	<i>yapH</i>	YapH protein	Protein fate
XAC1328*	NS	-1.17	-1.18	–	Hypothetical protein	Regulatory functions
XAC1812	NS	1.33	1.40	<i>hmsF</i>	HmsF protein	Regulatory functions
XAC1969*	NS	-1.07	-1.11	<i>rpoN</i>	RNA polymerase σ^{54} factor	Transcription
RpfC and RpfF only						
XAC4199*	-1.47	-1.46	NS	–	Polyvinylalcohol dehydrogenase	Unclassified
XAC1953*	-1.09	-1.45	NS	<i>fliG</i>	Flagellar protein	Cellular processes
XAC0661*	-1.18	-1.38	NS	<i>peh-1</i>	Endopolygalacturonase	Cell envelope
XAC3727	-1.27	-1.24	NS	–	Hypothetical protein	Not in JCVI
XAC3725*	-1.97	-1.22	NS	–	Hypothetical protein	Mobile
RpfC and RpfG only						
XAC3966*	-1.09	NS	-1.50	–	Hypothetical protein	Not in JCVI

phases. Furthermore, insertion mutation of σ^{54} (XAC1969) affected the biofilm formation of *X. citri* subsp. *citri* (Li and Wang 2011), suggesting that the NtrB-NtrC complex and σ^{54} might regulate QS-regulated biofilm formation in *X. citri* subsp. *citri*.

Comparison of the three regulons revealed that there are a large number of unique genes in the RpfG regulon that were not regulated by RpfC and RpfF, including genes encoding transporters such as iron transporters, genes encoding outer membrane proteins such as type V secretion system adhesins, genes involved in energy metabolism, genes encoding T3SS translocon, and regulatory genes (Fig. 2B). The partial overlap of the RpfG regulon with the RpfF regulon suggests that the regulatory role of RpfG in *X. citri* subsp. *citri* is not restricted to the transduction of the DSF family signals. In addition to the GGDEF proteins and NtrC mentioned above, RpfG also physically interacts with other proteins, such as a general stress protein XAC2369, an Hpr kinase XAC2975, and a putative polysaccharide deacetylase HmsF (Andrade et al. 2006). These protein–protein interactions are another line of evidence indicating that RpfG might be involved in other regulatory pathways. The influence on the expression of genes encoding transporters suggests that RpfG may coordinate with unknown sensors or other proteins in the transduction of external environmental signals. This may be related to the adaption to environmental changes, such as iron concentration and nutrient availability. Our previous transcriptome study of the HrpG regulon revealed that the expression of *rpfG* is repressed by the virulence regulator HrpG at the stationary phase, suggesting the involvement of RpfG in adaptation to the different infection stages (Guo et al. 2011). It is not clear how *X. citri* subsp. *citri* coordinates RpfG and HrpG during citrus canker infection. However, the few genes encoding the T3SS translocon and effectors (*hrcC*, *hrcJ*, *hrpB1*, *hrpB2*, *hrpB4*, *hrpB5*, *hpa1*, *hpaB*, and *avrXacE1*) up-regulated only by RpfG may account

for the lower virulence of the *rpfG* mutant than that of the *rpfF* and *rpfC* mutants in citrus canker infection (Fig. 1).

RpfC may play a role in the regulatory pathway other than transduction of DSF signals. Interestingly, the LuxR-type receptors were reported to be activated by diketopiperazines in addition to AHL family signals in *P. aeruginosa* and multiple gram-negative bacteria (Holden et al. 1999). In total, the RpfC regulon includes only 21 and 4 genes in the exponential and stationary phases, respectively, that are distinct from in the other regulons (Fig. 2). The unique genes controlled by RpfC alone include *czcD* encoding a heavy metal transporter, genes associated with energy metabolism, and genes involved in protein fate. This may suggest that the RpfC participates in bacterial adaptation to the environment through an unknown pathway. The yeast two-hybrid assay revealed that RpfC interacts with RimM; XAC3455, which is 2-isopropylmalate synthase; XAC1136, which is a propionate catabolism regulatory protein; Xac1282, which is a two-component sensor histidine kinase; AvrBs2; and CMF (XAC0330), which is a conditioned medium factor (Andrade et al. 2006). RpfC interaction with one or more of these proteins could lead to a pathway independent of QS, such as an interaction with CMF. CMF was first found as a glycoprotein that was secreted under conditions of nutritional stress and served as a QS signal in *Dictyostelium discoideum* (Deery and Gomer 1999). A CMF homolog in *X. citri* subsp. *citri* contains a putative signal sequence and could be secreted to the periplasm or be bound to the inner membrane, implying that CMF could interact with RpfC to alter gene regulation (Andrade et al. 2006).

In conclusion, we have significantly advanced the understanding of DSF-mediated QS regulation by characterizing the RpfC and RpfG regulons in addition to the RpfF regulon. The comparison of these three regulons led to the identification of a core group of genes controlled by all three QS components, suggesting that the RpfC-RpfG two-component system is a

Fig. 3. Mutations in *rpfF*, *rpfC*, or *rpfG* reduced the attachment of *Xanthomonas citri* subsp. *citri* to abiotic and biotic surfaces. **A**, Attachment of strains grown in XVM2 media to a polypropylene surface. Bacteria grown in nutrient broth (NB) showed similar results (*data not shown*). **B**, Quantification of bacterial attachment to polypropylene surfaces. Columns represent the mean of crystal violet staining measured spectrophotometrically (A590 nm) and error bars are standard deviations; ■ represents the strains grown in NB and □ represents the strains grown in XVM2. Means were calculated using 10 tubes for each strain. The assay was performed three times independently, and the representative results from one experiment are shown. **C**, Attachment to abaxial surfaces of Duncan grapefruit leaves of strains grown in NB or XVM2 media. The assay was performed three times with six leaves each time, and similar results were obtained. Phosphate buffer (pH 7.0) was used as the negative control in all assays.

major and conserved signal perception and transduction system for DSF family signal-mediated QS in *X. citri* subsp. *citri*. The unique genes controlled by RpfF alone indicate the complexity of the QS pathway and the involvement of additional sensory mechanisms in *X. citri* subsp. *citri*. The unique genes controlled by RpfC and RpfG support the possibility that RpfC and RpfG play broader roles in gene regulation beyond transduction of QS signals.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

All of the strains used in this study are listed in Supplementary Table S5. *X. citri* subsp. *citri* mutant strains were generated in this study as described below. The wild-type strain 306 (rifampicin resistant) (da Silva et al. 2002) and the mutant strains were routinely grown in NB, on nutrient agar (NA), or in nutrient yeast glycerol (NYG) medium (Daniels et al. 1984) at 28°C. The composition of XVM2 medium is described elsewhere (Wengelnik et al. 1996). *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C. Antibiotics were used at

the following concentrations: rifampicin (Rif), 50 µg/ml; kanamycin (Kn), 50 µg/ml; ampicillin (Ap), 50 µg/ml; spectinomycin (Sp), 50 µg/ml; gentamicin (Gm), 5 µg/ml; and chloramphenicol (Cm), 35 µg/ml.

Construction of strains with mutations in genes involved in DSF-mediated QS signaling.

To construct the *rpfF* deletion mutant, the 1,610-bp fragment containing the entire *rpfF* gene was amplified using genomic DNA from *X. citri* subsp. *citri* 306 as a template and the primers rpfFF and rpfFR (Supplementary Table S6). The fragment was cloned into a pGEM-T easy vector, resulting in the construct pGEM-rpfF. After digestion with *AgeI* and *KpnI*, the construct pGEM-rpfF was blunt ended using T4 DNA polymerase and self-ligated and denoted pGEM-ΔrpfF. From pGEM-ΔrpfF, an *ApaI*-*SpeI* fragment containing the *rpfF* gene with the internal deletion was transferred into *ApaI*-*XbaI*-digested pOK1, resulting in pOK-ΔrpfF. The construct pOK-ΔrpfF was sequenced to confirm the deletion, before conjugation. *E. coli* DH5αλPIR (Huguet et al. 1998) were transformed with pOK-ΔrpfF, which was subsequently transferred to *X. citri*

Fig. 4. Microscopic analysis of bacterial attachment on abaxial surfaces of Duncan grapefruit leaves. **A**, **B**, and **C**, Green fluorescent protein (GFP)-labeled strains grown in XVM2 were used in confocal laser scanning microscopy analysis. **A**, Bacterial attachment to leaves after 1 h of incubation. **B**, Bacterial attachment to leaves after 6 h of incubation. **C**, Zoomed-in image of GFP-labeled wild-type strain attached to leaves after 6 h of incubation. **D**, Scanning electron microscopy analysis of bacterial attachment to a leaf surface after 6 h of incubation. Three independent experiments were performed for each strain, and similar results were obtained. Scale bars in all images represent 20 µm.

subsp. *citri* 306 by triparental mating with a helper *E. coli* strain containing pRK2013 (Swarup et al. 1991). Transconjugants were selected on NYG medium supplemented with Rif and Sp. Positive colonies were replicated on NA plates supplemented with 5% (wt/vol) sucrose, Sp, and Rif, as well as NA plates containing only Rif. The sucrose-sensitive colonies were selected from NA plates containing Rif and then dilution plated onto NA containing Rif and 5% sucrose to select for the resolution of the construct by a second cross-over event. The resultant markerless deletion mutant of *rpjF* was confirmed by PCR and sequencing.

To construct the *rpjC* deletion mutant of *X. citri* subsp. *citri*, the 3,017-bp fragment containing the entire *rpjC* gene was amplified using genomic DNA from *X. citri* subsp. *citri* 306 as a template and the primers *rpjCF* and *rpjCR*, and then cloned into a pGEM-T easy vector, resulting in pGEM-*rpjC*. The construct pGEM-*rpjC* was digested by *Bst*EII to remove 1,116-bp from the interior of *rpjC* and then self-ligated and denoted pGEM- Δ *rpjC*. An *Apal*-*Spe*I fragment containing *rpjC* with the internal deletion was transferred from pGEM- Δ *rpjC* into *Apal*-*Xba*I-digested pOK1, generating pOK- Δ *rpjC*. The deletion construct pOK- Δ *rpjC* was transferred into *X. citri* subsp. *citri* 306 by triparental mating, and a deletion mutant of *rpjC* was selected using the method described above.

The in-frame deletion mutant of *rpjG* was constructed as follows. The 2,037-bp fragment containing the entire *rpjG* gene was amplified using genomic DNA from *X. citri* subsp. *citri* 306 as a template and the primers *rpjGF* and *rpjGR*, and then cloned into a pGEM-T easy vector, resulting in pGEM-*rpjG*. The pGEM-*rpjG* was digested by *Bss*HII to remove 648 bp from the interior of *rpjG* and then self ligated to form pGEM- Δ *rpjG*. An *Apal*-*Spe*I fragment containing *rpjG* with the in-frame deletion was transferred from pGEM- Δ *rpjG* into an *Apal*-*Xba*I-digested pOK1 to generate pOK- Δ *rpjG*. The deletion construct pOK- Δ *rpjG* was transferred into *X. citri* subsp. *citri* 306 by triparental mating, and the *rpjG* deletion mutant was selected using the method described above.

Complementation of the *rpjF*, *rpjC*, and *rpjG* mutants.

To generate the complementation plasmid for the *rpjF* mutant, the fragment containing the entire *rpjF* gene and its promoter was amplified using genomic DNA from *X. citri* subsp. *citri* 306 as a template and the primers *rpjFF* and *rpjFR*. The fragment was cloned into a PCR 2.1-TOPO vector, resulting in the construct PCR-*rpjF*. The *Hind*III-*Xba*I fragment containing the *rpjF* gene from PCR-*rpjF* was blunt ended by T4 DNA polymerase and then cloned into pUFR053 that had been digested by *Hind*III, blunt ended, and treated with calf intestinal alkaline phosphatase. The derivative construct, p53-*rpjF*, was transferred into the *rpjF* mutant by triparental mating. The transconjugants were selected on NA with Rif and Gm.

To construct the complementation plasmid for the *rpjC* mutant, the entire *rpjC* gene was amplified from genomic DNA of *X. citri* subsp. *citri* 306 using PCR with the primers *rpjC*-*Kpn*F and *rpjC*-*Kpn*R, which contain a *Kpn*I restriction site. The fragment was cloned into a PCR 2.1-TOPO vector, resulting in PCR-*rpjC*. The *Kpn*I-digested fragment containing the *rpjC* gene was transferred from PCR-*rpjC* into the *Kpn*I site of pUFR053. The derivative constructs were sequenced to confirm the orientation of *rpjC* insertion. The construct containing the *rpjC* gene in the same orientation as the vector *lacZ* and driven by *lac* promoter was selected as p53-*rpjC*. The p53-*rpjC* was transferred into the *rpjC* mutant by triparental mating and selected on NA with Rif and Gm.

To construct the complementation plasmid for the *rpjG* mutant, an *Eco*RI fragment containing *rpjG* and its promoter was transferred from pGEM-*rpjG* and ligated into the *Eco*RI site of

pUFR034, resulting in p34-*rpjG*. The construct p34-*rpjG* was transferred into the *rpjG* mutant by triparental mating and selected on NA with Kn.

RNA extraction.

Individual bacterial colonies were selected and grown in 5 ml of NB at 28°C for 24 h with shaking and then transferred into 50 ml of NB for overnight incubation. The bacterial cultures in the exponential phase were centrifuged and washed with XVM2 medium once, and inoculated in the XVM2 medium with an initial concentration of optical density at 600 nm (OD_{600}) = 0.03. Bacteria were grown in XVM2 medium with constant shaking at 200 rpm and 28°C, and culture samples were collected at 11 and 25 h, according to the growth curve in XVM2. Four biological replicates were used for each strain per time point. RNA was stabilized immediately by mixing bacterial culture with two volumes of RNeasy Protect Bacteria reagent (Qiagen, Valencia, CA, U.S.A.) and incubation at room temperature for 5 min. Bacterial cells were centrifuged at $5,000 \times g$ for 10 min, and cell pellets were used for RNA extraction.

Cell pellets were treated with lysozyme and proteinase K for 15 min, and RNA extractions were performed using an RNeasy Mini kit (Qiagen). Contaminating genomic DNA was removed from the RNA samples by treatment with a TURBO DNA-free kit (Ambion, Austin, TX, U.S.A.). The RNA quantity was initially determined on an ND-8000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.), and the RNA quality was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, U.S.A.).

Microarray hybridization.

Microarray analysis using the Agilent microarray platform was performed at the Interdisciplinary Center for Biotechnology Research Microarray Core Facility, the University of Florida. Labeled cDNA was generated using a Fairplay III microarray labeling kit (Agilent Technologies). Total RNA input (5 μ g) was used to generate labeled cDNA according to the manufacturer's protocol. Briefly, cDNA was synthesized from 5 μ g of total RNA with AffinityScript HC and random primers, and then modified cDNA was labeled with either Cy3 or Cy5; labeled cDNA was purified following the manufacturer's instructions. The microarray analysis was performed using the Agilent 8 $\times$ 15K *X. citri* subsp. *citri* genome array (Guo et al. 2011). Four independent biological replicates were performed for two time-point comparisons with a dye-swap design. A total of 300 ng of labeled cDNA per sample was used for the hybridization. A dye swap was performed to remove any bias from the labeling dyes. Hybridization was performed using the Gene Expression Hybridization Kit (Agilent Technologies) according to the user's manual and was done in a hybridization oven for 17.5 h at 65°C. The arrays were washed according to the manufacturer's recommended protocols. Briefly, arrays were washed with Gene Expression Wash Buffer 1 containing 0.005% Triton X-102 for 1 min at room temperature, washed with 37°C Gene Expression Wash Buffer 2 containing 0.005% Triton X-102 for 1 min, and dried with Agilent Stabilization and Drying Solution. The arrays were scanned using a dual-laser DNA microarray scanner (Model G2505C) (Agilent Technologies). Data were extracted from scanned images using Feature Extraction 10.1.1.1 software (Agilent Technologies).

Microarray data analysis and statistical methods.

The raw data were imported into the R environment, and statistical tests were performed using BioConductor statistical software, which is an open-source and open-development software project for the analysis of microarray and other high-

throughput data based primarily on the R programming language (Gentleman et al. 2004). Data preprocessing and normalization were performed using the Linear Models for Microarray Data (LIMMA) package (Smyth 2004). Raw mean signal intensities from all microarray spots were background corrected and normalized using a within-array lowess approach. Log₂-transformed values were used for statistical analysis. Histograms, box plots, and pairwise scatter plots were generated to examine the data quality and comparability. A linear modeling approach and the empirical Bayes statistics as implemented in the LIMMA package (Smyth 2004) were then employed for differential expression analysis. The *P* values were adjusted using the Benjamini and Hochberg method, designated as the FDR (Benjamini and Hochberg 1995). Differentially expressed genes were ranked based on FDR, and genes with an FDR less than 0.01 and a minimum absolute value of a log₂-fold change greater than 1 (equivalent to twofold) were considered as significantly differentially expressed. If a gene had three probes and only one was filtered, the gene was removed from further analysis. The log₂-fold change values of the differentially expressed genes were averaged from the values of the two or three probes of the corresponding genes. Annotation for the differentially expressed genes was extracted from the Integrated Microbial Genome database and the JCVI database and manually verified.

Details of our microarray experiments and the MIAME-compliant microarray data have been deposited in the Gene Expression Omnibus database, the National Center of Biotechnology Information (accession number GSE29877).

Real-time two-step QRT-PCR.

To verify the microarray result, QRT-PCR assays were conducted using the same set of RNA as for microarray analysis. The aliquoted RNA sample (1 µg) used for microarray was reverse transcribed using a QuantiTect Reverse Transcription kit with random hexamer primers (Qiagen) for two-step QRT-PCR. Gene-specific primers were designed to generate products 100 to 250 bp in length from sequences on the *X. citri* subsp. *citri* genome, using DNASTAR software (DNASTAR, Madison, WI, U.S.A.). QRT-PCR was performed for all four biological replicates of the *rpfG* mutant and the wild-type strain on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) using a QuantiTect SYBR Green PCR kit (Qiagen) following the manufacturer's instructions. 16S rRNA was used as an endogenous control. The relative fold change of gene expression was calculated by using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). The values of fold change were log₂ transformed to compare with the values generated from microarray analysis.

Motility assays.

The medium for motility assays was NB or XVM2 containing 0.7% agar. Bacteria were grown in NB overnight with shaking at 200 rpm, and then centrifuged, washed, and diluted to OD₆₀₀ = 0.3 in sterile water. A 1-µl suspension was spotted on the center of plate, which was incubated at room temperature. The assay was repeated three times, independently, in quadruplicate.

Protease activity test.

To measure the activity of extracellular proteases produced by bacteria, bacterial cells were grown in NB at 28°C overnight with shaking at 200 rpm, then centrifuged, washed, and diluted to OD₆₀₀ = 0.3 in sterile water. A 1-µl suspension was spotted on a 5% skim-milk-containing NA plate, which was incubated at room temperature. The protease activity was detected as zones of hydrolysis around the colonies. The assay was repeated three times, independently, in quadruplicate.

Pathogenicity assay.

Pathogenicity assays were conducted in a quarantine greenhouse facility at the Citrus Research and Education Center, Lake Alfred, FL, U.S.A. Assays were performed using fully expanded, immature leaves of Duncan grapefruit (*Citrus paradisi* Macfadyen). The *X. citri* subsp. *citri* wild-type and mutant strains used in this assay were grown with shaking overnight at 28°C in NB, centrifuged, and suspended in sterile tap water, and the concentrations were adjusted to 10⁸ CFU/ml. For the pathogenicity assays, bacterial solutions of 10⁸, 10⁵, or 10⁴ CFU/ml were infiltrated into leaves with needleless syringes (Rybak et al. 2009; Vilorio et al. 2004). The test was repeated three times, and similar results were obtained. Disease symptoms were photographed at 6, 12, and 18 DPI. For the lesion test, inoculated areas were marked on leaves and photographed on 18 DPI. Canker lesions from 10 inoculated leaves were quantified, and the inoculated areas were calculated using the program ImageJ version 1.44p (Abramoff et al. 2004). The PROC GLM procedure of SAS 9.2 (SAS Institute, Cary, NC, U.S.A.) was used to analyze the significance of difference in virulence among those inoculated strains.

To mimic the natural infection process of *X. citri* subsp. *citri*, Duncan grapefruit leaves were inoculated with bacterial suspensions of 10⁸ CFU/ml by spraying on the abaxial surface. The inoculated plants were covered with plastic bags for 24 h to maintain 100% relative humidity and then kept in a greenhouse (approximately 50% relative humidity) for the symptoms to develop.

Attachment assays.

To measure the level of cells adhered to an abiotic surface, overnight cultures of the wild-type, *rpfF*, *rpfC*, and *rpfG* mutant strains of *X. citri* subsp. *citri* in NB or XVM2 medium were centrifuged to recover cell pellets, washed, and resuspended in 10 mM phosphate buffer (pH 7.0) to OD₆₀₀ = 1.0 (10⁹ CFU/ml). Each bacterial suspension (200 µl) was aliquoted into 1.5-ml plastic microcentrifuge tubes and incubated for 6 h at 28°C. The adhesion was monitored by staining the attached bacteria with CV. Bacterial adhesion was measured after repetitive washing of the tubes to remove nonadherent cells and then staining with 0.1% CV for 45 min at room temperature. Excess stain was removed by washing under running tap water, and the CV stain was solubilized by the addition of 250 µl of 99% ethanol to each tube. The amount of retained CV was quantified with an Agilent 8453 UV-visible spectrophotometer at 590 nm. The assay was repeated three times with 10 replicates each time.

To observe the bacterial adhesion to biotic surfaces, bacterial suspensions (OD₆₀₀ = 1.0) were prepared as described above. Bacterial suspension (20 µl) was plated on the abaxial surface of detached Duncan grapefruit leaves, which were then incubated for 6 h in a moist chamber at 28°C. Inoculated leaves were washed twice with sterile tap water and stained with CV as for the abiotic surfaces (Gottig et al. 2009).

To directly observe the bacterial adhesion to the leaf surface using a confocal laser-scanning microscope, bacteria were transformed with a constitutively GFP-expressing pUFZ75 plasmid (Zhang et al. 2009) by electroporation. No difference in growth or adhesion to leaf and polypropylene surfaces could be detected between GFP-labeled bacteria and those lacking the GFP-expressing plasmid. The bacterial suspension was prepared and dropped onto detached leaves as described above. The inoculated leaves were incubated in a moist chamber at 28°C and observed at 1 and 6 h postinoculation. After washing twice with sterile tap water, inoculated areas were dissected from the leaf for microscopic observation. Bacterial adhesion was visualized using a Leica TCS SL confocal laser-scanning micro-

scope (Leica Microsystems Inc., Buffalo Grove, IL, U.S.A) with a 63× water objective in the microscope lab at the Citrus Research and Education Center, Lake Alfred, FL, U.S.A. Three excitation and emission lines were used in the observation, as follows: green (excitation 488 nm and emission 500 to 545 nm) for the detection of GFP-labeled strains, red (excitation 543 nm and emission 600 to 630 nm) for the detection of the cuticle, and blue (excitation 633 nm and emission 680 to 750 nm) for the detection of chlorophyll. The assay was performed three times independently in quadruplicate.

To further compare the location of bacterial cells relative to leaf tissue, we used a scanning electron microscope (SEM) in the microscope lab at the Citrus Research and Education Center. The leaves, after inoculation with bacteria for 6 h, were washed twice with sterile tap water, and inoculated areas were dissected from the leaf (approximately 0.5 cm²) and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight. The fixed leaf discs were washed three times with 0.1 M phosphate buffer (pH 7.2), postfixed in 2% OsO₄ in 0.1 M phosphate buffer (pH 7.2) for 4 h, and rinsed twice in 0.1 M phosphate buffer (pH 7.2). After rinsing, the leaf discs were subjected to dehydration in a sequence of ethanol solutions (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%) for 10 min each. The final wash in 100% ethanol was repeated three times. Leaf discs were critical-point dried in a LADD critical point dryer (LADD Research, Williston, VT, U.S.A.) and then fixed on stubs and coated with gold-palladium using a LADD sputter coater (LADD Research). SEM micrographs were taken using an SEM S-530 (Hitachi, Tokyo) at 20 kV. SEM analysis was performed twice, with six replicates for each sample.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- BioConductor website: www.bioconductor.org
- Gene Expression Omnibus (GEO) website: www.ncbi.nlm.nih.gov/geo
- The Integrated Microbial Genome (IMG) *Xanthomonas axonopodis* pv. *citri* 306 genome database: img.jgi.doe.gov/cgi-bin/w/main.cgi?section=TaxonDetail&page=taxonDetail&taxon_oid=637000342
- The J. Craig Venter Institute (JCVI) *Xanthomonas axonopodis* pv. *citri* 306 genome database: cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=ntxa01