Role of *rpjF* in Virulence and Exoenzyme Production of *Xanthomonas axonopodis* pv. *glycines*, the Causal Agent of Bacterial Pustule of Soybean

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Accepted for publication 1 July 2008.

ABSTRACT

Thowthampitak, J., Shaffer, B. T., Prathuangwong, S., and Loper, J. E. 2008. Role of *rpjF* in virulence and exoenzyme production of *Xanthomonas axonopodis* pv. *glycines*, the causal agent of bacterial pustule of soybean. *Phytopathology* 98:1252-1260.

Ten strains of *Xanthomonas axonopodis* pv. *glycines*, the causal agent of bacterial pustule of soybean, which were isolated from various soybean growing regions of Thailand, produced an extracellular diffusible factor (DSF) related to a well-characterized quorum sensing molecule produced by other *Xanthomonas* spp. Genomic DNA of the 10 strains of *X. axonopodis* pv. *glycines* contained *rpjF*, a gene encoding for the biosynthesis of the DSF in *X. campestris* pv. *campestris*. The *rpjF* gene from one strain of *X. axonopodis* pv. *glycines* was fully sequenced, and the 289 aa product is closely related to RpfF of other *Xanthomonas* spp. (95 to 98%

identical). Three independently generated *rpjF* mutants of *X. axonopodis* pv. *glycines* strain No12-2 were defective in the production of a DSF, as expected if *rpjF* encodes for DSF biosynthesis in *X. axonopodis* pv. *glycines*. The *rpjF* mutants of *X. axonopodis* pv. *glycines* exhibited reduced virulence on soybean and produced less than wild-type levels of extracellular polysaccharide and the extracellular enzymes carboxymethylcellulase, protease, endo- β -1,4-mannanase, and pectate lyase. Transcripts for three genes that encode for the extracellular enzymes protease, endoglucanase, and pectate lyase were at lower abundance in an *rpjF* mutant than in the parental strain of *X. axonopodis* pv. *glycines*. These results indicate that *X. axonopodis* pv. *glycines* produces a diffusible signal related to the DSF of *X. campestris* pv. *campestris*, which contributes to virulence and exoenzyme production by this phytopathogenic bacterium.

Xanthomonas axonopodis pv. *glycines* is the causal agent of bacterial pustule of soybean (*Glycine max*), a serious disease in several major soybean-producing countries (50). Symptoms of the disease are small pale green spots with raised centers, which develop on either or both leaf surfaces. When disease is severe, lesions expand, coalesce, and become necrotic, which leads to premature defoliation (36). In Thailand, the disease can cause yield losses of up to 20 to 35% in sensitive cultivars (36). Severe disease is observed in Central Thailand during the rainy season, when temperatures are warm, rainfall is frequent, and humidity is high.

Xanthomonas is an important genus of plant-pathogenic bacteria that cause diseases with diverse symptoms on at least 124 monocotyledonous and 268 dicotyledonous plants (21). Among the best characterized members of the genus is *X. campestris* pv. *campestris*, which produces several virulence factors including extracellular enzymes, which degrade plant cell components, and an extracellular polysaccharide (EPS) (18,44). These virulence factors are expressed in a cell density-dependent fashion upon the accumulation of a diffusible signal factor (DSF) (3,23,43). The biosynthesis of DSF by *X. campestris* pv. *campestris*, which has been identified as *cis*-11-methyl-2-dodecanoic acid (48), requires RpfF, a protein similar to enoyl-CoA hydratases (3). Other genes in the *rpf* gene cluster of *X. campestris* pv. *campestris* encode for proteins that sense the DSF and initiate a regulatory cascade con-

trolling genes required for virulence factors, such as exopolysaccharide and exoenzyme biosynthesis (17,43,44). A diffusible signal related to the DSF of *X. campestris* pv. *campestris* is also produced by *X. axonopodis* pv. *citri* (41), *X. oryzae* pv. *oryzae* (7), and *Xylella fastidiosa* (6,34), and *rpjF* mutants of these bacteria exhibit altered virulence towards their plant hosts.

Many bacteria coordinate gene expression in a cell density-dependent fashion by producing small, diffusible signaling molecules. Such phenomena, in which bacterial traits are expressed only when a population exceeds a minimum threshold density, are often referred to as quorum sensing. Density-dependent signaling molecules have diverse structures, as *N*-acyl homoserine lactones (AHL), small peptides, butyrolactone derivatives, or fatty acids such as DSF (4,48), but they share the characteristic of enhanced production with increasing cell density. Genes expressed under the control of these signaling molecules have diverse functions including biofilm formation, antibiotic and extracellular enzyme production, bioluminescence, and virulence (6,11,23,47,49). While these functions are beneficial to bacteria living in groups, they are of little value to solitary cells, and their expression could be a futile waste of metabolic resources for individual bacterial cells. For pathogens, quorum sensing may ensure that virulence traits are expressed only when cells are in sufficient numbers to infect a host, thereby avoiding premature triggering of host defense responses before cells are of adequate density to evade them. Indeed, the virulence of many pathogens is reduced when the ability to produce signaling compounds is disrupted by mutation (4,47,49). This is the case for several plant pathogenic members of *Xanthomonas* spp., in which *rpjF* mutants deficient in DSF production exhibit reduced virulence (3,7,16,18,41). Despite these commonalities, there are also differences in DSF

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doi:10.1094/PHYTO-98-12-1252

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signaling among *Xanthomonas* spp.: DSF is involved in regulation of virulence factors such as extracellular enzymes and extracellular polysaccharide (EPS) in *X. campestris* pv. *campestris* (3,16,18,43) and *X. axonopodis* pv. *citri* (41), whereas it is involved in iron uptake in *X. oryzae* pv. *oryzae* (7). The goal of this study was to determine if a DSF is produced by *X. axonopodis* pv. *glycines*, and if *rpjF* has a role in the virulence and exoenzyme production by this pathogen.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are described in Table 1. *X. axonopodis* pv. *glycines* was grown routinely at 27°C on NYGB (46), which is composed of (per liter): 5 g of bacto-peptone (Difco, Becton, Dickinson Co., Sparks, MA); 3 g of yeast extract (Difco), and 20 g of glycerol. NYGA is NYGB containing 15 g/liter of agar. *Escherichia coli* was grown on Luria-Bertani (LB) agar or broth at 37°C.

DSF Bioassay. DSF production by *X. axonopodis* pv. *glycines* was detected using the DSF biosensor *X. campestris* pv. *campestris* strain 8523 (pKLN55), which has a green fluorescent protein reporter gene (*gfp*) fused to the DSF-inducible promoter region of *engXCA*, which encodes for endoglucanase (34). Test strains were streaked in a line on NYGA plates, and plates were incubated for 24 h at 27°C. The biosensor strain was then streaked perpendicular to the test strain, without allowing the indicator and test strain to touch. Following 24 h incubation at 27°C, DSF production by the test strains could be detected by fluorescence of the biosensor. Fluorescence was viewed on a Zeiss SV11 stereoscope with Kramer epifluorescence/Optronix Color DEI450. The experiment was done three times with identical results.

Nucleotide sequence of *rpjF* of *X. axonopodis* pv. *glycines*. The *rpjF* genes were amplified from the genomic DNA isolated from the 10 strains of *X. axonopodis* pv. *glycines* listed in Table 1 using primers complementary to conserved regions of *rpjF* from other *Xanthomonas* spp. The primer sets (5'-CTGCAGTTCA-ACCCTTCA-3' and 5'-ACCCAGATTTCGGTGATG-3') amplified a 772-bp fragment from each strain. The polymerase chain reaction (PCR) amplification products from each strain were sequenced using the same primer set and sequences were compared to those of published *rpjF* sequences from other *Xantho-*

monas spp. The entire *rpjF* gene from one DSF-producing strain, No12-2, was amplified from primer sets designed from conserved regions both internal and flanking *rpjF* of other *Xanthomonas* spp. Sequences from these PCR amplification products were assembled to derive the *rpjF* sequence of strain No12-2 (Accession No. EU862234).

Derivation of *rpjF* mutants of strain No12-2. A 385-bp fragment internal to *rpjF* was obtained following the PCR with primers *rpjF*.F3 (5'-CACCCCTCGCCGTCACCC-3') and *rpjF*.R2 (5'-GCACCTCGGGCAATCCCA-3'). The PCR product was cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA) and integrated into the destination vector pLVC-D (31) using the clonase protocol described by Invitrogen. The resultant plasmid (pLVC-D containing 385 bp of *rpjF*) was introduced into the mobilizing strain *E. coli* S17-1 (42) by transformation, and transferred from S17-1 to No12-2 via conjugation, selecting for transconjugants on 925 medium (28) amended with sucrose (15 g/liter) as a sole carbon source and tetracycline at 10 µg/ml. Because pLVC-D is a suicide plasmid in *Xanthomonas* spp., tetracycline-resistant colonies of No12-2 were expected to have undergone a single-crossover event between the DNA cloned in pLVC-D and the corresponding sequence in the No12-2 chromosome, resulting in two nonfunctional copies of *rpjF* in the genome, one with a 3' and the other with a 5' truncation. Gene disruption and plasmid insertion were confirmed using PCR with primers specific to the pLVC-D vector (primers *LattB2* [5'-CTTTGTACAAGAAAGCTGGGT-3'] and *UattB1* [5'-AGTT-TGTACAAAAAAGCAGGCT-3']) and genomic DNA sequences up- and down-stream from the targeted genes (forward primer 5'-CTGCAGTTCAACCCTTCA-3', and reverse primer 5'-ACC-CAGATTTCGGTGATGCG-3'). Three independently derived mutants of No12-2 (T3A, T3B, and T3C), each having the expected insertion in *rpjF*, were selected for further analysis. No differences in the growth rates of the *rpjF* mutants versus the parental strain were observed in routine cultures. Growth rates of mutant T3A and No12-2 were compared in shake cultures incubated at 27°C in yeast extract broth, which was composed of (per liter): 5 g of yeast extract, 10 g of tryptone, 5 g of NaCl, 5 g of sucrose, 0.5 g of MgSO₄·7H₂O, pH 7.0 (51), and shown to be similar (data not shown).

Virulence assays on soybean. Virulence of No12-2 and *rpjF* mutants was assessed on soybean cultivar SJ4, using previously

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic ^z	Reference or source
<i>X. axonopodis</i> pv. <i>glycines</i>		
No12-2	Field isolate, pathogenic, DSF ⁺	Nakornratchasima, Thailand
CM60-1	Field isolate, pathogenic, DSF ⁺	Nakornratchasima, Thailand (27)
NKR13	Field isolate, pathogenic, DSF ⁺	Nakornratchasima, Thailand (27)
NKR17	Field isolate, pathogenic, DSF ⁺	Nakornratchasima, Thailand (27)
NKR21	Field isolate, pathogenic, DSF ⁺	Nakornratchasima, Thailand (27)
NO15	Field isolate, pathogenic, DSF ⁺	Nakornsawon, Thailand (27)
NO21	Field isolate, pathogenic, DSF ⁺	Chiangmai, Thailand (27)
RE07	Field isolate, pathogenic, DSF ⁺	Khonkhan, Thailand (27)
RE08	Field isolate, pathogenic, DSF ⁺	Loei, Thailand (27)
ST91-051	Field isolate, pathogenic, DSF ⁺	Sukhothai, Thailand (27)
T3A	<i>rpjF</i> ::pJEL6351 mutant of No12-2, DSF ⁻ , Tc ^r (also called JL2553)	This study
T3B	<i>rpjF</i> ::pJEL6351 mutant of No12-2, DSF ⁻ , Tc ^r (JL2554)	This study
T3C	<i>rpjF</i> ::pJEL6351 mutant of No12-2, DSF ⁻ , Tc ^r (JL2555)	This study
<i>X. campestris</i> pv. <i>campestris</i>		
8004	Rifampicin-resistant derivative of wild-type strain NCPPB 1145	(46)
8523	<i>rpjF</i> ::Tn5lac mutant of 8004	(3)
8523 (pKLN55)	DSF biosensor	(34)
Plasmid		
pKLN55	DSF-inducible promoter of <i>engXCA</i> in pPROBE-KT ^r , resulting in DSF-inducible expression of GFP	(34)
pLVC-D	pBR322 containing <i>att</i> sites and mobilization region from pRK2, Tc ^R	(31)
pJEL6351	pLVC-D with 360-bp internal to <i>rpjF</i> from <i>X. axonopodis</i> pv. <i>glycines</i> No12-2, Tc ^R	This study

^z Tc = tetracycline.

described methods (25). Briefly, aqueous cell suspensions of *X. axonopodis* pv. *glycines* ($OD_{600} = 0.2$, ca. 10^8 colony forming units (CFU)/ml), supplemented with 5 g/liter 600 mesh carborundum, were sprayed on leaves of plants maintained in a greenhouse. At 7 to 10 days after inoculation, disease severity was scored on three leaves per plant, one each from the apical, middle, or basal portions. A scoring card having nine holes (Fig. 1) was placed over each leaflet, and the presence or absence of pustules visible through each hole was recorded. Disease severity was expressed as the percentage of holes through which pustules were visible. No significant differences in this percentage were observed among leaves at three different positions (top, middle, and low) on the plant ($P > 0.98$), nor were significant interactions between treatment and leaf position observed. Therefore, values from leaves in all positions on the plant were pooled in analyses comparing the virulence of No12-2 and *rpfF* mutants. The experiment was done three times, once with 10 replicate plants and twice with four replicate plants.

Potato tuber maceration. The capacity of No12-2 and the *rpfF* mutants to cause soft rot symptoms was assessed on slices of potato tubers (*Solanum tuberosum*) that were inoculated by stabbing with a toothpick dipped in a bacterial culture grown for 24 h on NYGA. Potato slices were incubated on moist filter paper in a petri dish at 28°C for 24 h before observation for symptoms of soft rot. The experiment was done twice.

Exoenzyme plate assays. Relative levels of exoenzyme production were assessed by radial diffusion assays. No12-2 and the *rpfF* mutants were grown in NYGB at 27°C overnight, unless a different time is specified later. Cells were pelleted by centrifugation, and the supernatants were collected and sterilized by filtration (0.2 µm). Thirty microliters of the filter-sterilized supernatant was placed in a 0.5-cm well, made using a cork borer, in an agar medium in a petri plate. The medium composition, incubation conditions for assay plates, and detection conditions for each assay are specified later. Enzyme activity was estimated from the diameter of zones surrounding the culture supernatants of each strain. All experiments were done twice, once with four replicate plates and once with three replicate plates.

Carboxymethylcellulase. Inoculated plates containing an assay medium (0.1% carboxymethyl cellulose, 25 mM sodium phosphate, pH 7.0, and 0.8% agarose) (2) were incubated at room

temperature overnight, stained with 0.1% Congo red for 20 min, and washed twice with 1 M NaCl. Carboxymethyl cellulase (CMCase) activity was visualized as white halos surrounding the wells.

α-Amylase. Inoculated plates containing alpha-amylase assay medium (0.5% yeast extract, 1.0% tryptone, 0.25% NaCl, 0.2% soluble starch, and 0.8% agarose) were incubated at room temperature overnight and stained with potassium iodine for 10 min as described by Ray et al. (39) and modified by Lory (30). α-Amylase was detected as clear halos surrounding the wells.

Protease. Inoculated plates containing NYGA supplemented with 0.5% skimmed milk were incubated at room temp for 48 h, as described by Barber et al. (3). Extracellular protease production was detected visually as clear halos surrounding the wells.

Endo-β-mannanase. Inoculated plates containing a locust bean gum medium (5) were incubated at 32°C for 18 to 20 h. The agar surface was then washed in a 1.0:4.3 mixture of 0.1 M citric acid and 0.2 M Na_2HPO_4 (pH 7.0) for 30 min, stained for 30 min in 0.5% (wt/vol) Congo red, washed for 2 min in water, fixed for 10 min in 80% ethanol, washed three times in McIlvaine buffer (pH 7) (32) for 20 min each, and washed several times in 1 M NaCl until color development was complete. Endo-β-mannanase was visualized as clear halos surrounding the wells.

Pectolytic enzyme activity. A medium for assessing pectolytic enzyme activity was composed of 1.0% agarose and 0.1% polygalacturonic acid (PGA), which was dissolved by boiling in 100 mM potassium phosphate (pH 6.5), as described by Kaewnum (26). Culture filtrates were prepared as previously described except the NYGB cultures were grown for 48 h. Following inoculation of the medium, plates were incubated at room temperature overnight, and the surface of medium was flooded with 0.05% (wt/vol) ruthenium red for 20 min and rinsed in water. Pectolytic enzyme activity was observed as clear halos surrounding the wells.

Pectate lyase activity was assessed as described by Dow et al. (18). Briefly, strains were grown overnight in MMX minimal medium (13) supplemented with casamino acids (1.5 g/liter) and 0.25% (wt/vol) polygalacturonic acid, and cells were harvested by centrifugation. Cells were suspended in a solution containing 0.05M Tris-HCl buffer, pH 8.5, 1 mM $CaCl_2$, and 0.25% (wt/vol) polygalacturonic acid, and absorbance at 235 nm was assessed hourly (33). A unit of pectate lyase activity was defined as a change in optical density of 1 unit min^{-1} corresponding to the production of 0.5 µmol unsaturated product. The assay was done three times, with four replicate cultures in each assay.

Siderophore production. Siderophore production was detected using the universal siderophore detection method of Schwyn and Neilands (40). The siderophore indicator medium described by Chatterjee and Sonti (7) was spotted with 10 µl of an aqueous bacterial suspension ($OD_{600} = 0.1$), and incubated for 24 h at 27°C. Siderophore production was observed as yellow halos surrounding the bacterial growth on the agar surface. The test was done three times with identical results.

EPS production. Cultures were grown in NYGB medium for 48 h, and the supernatants were collected following centrifugation of cultures at 14,000 rpm for 10 min. Two volumes of ethanol were added into the supernatants, and the resulting solution was held at -20°C for 30 min. The precipitated EPS was collected by centrifugation, dried overnight at 55°C, and dry weights were recorded as an estimate of EPS (44). Four replicate cultures were assessed in each assay, and three independent assays were done.

RNA purification. Four replicate cultures of *X. axonopodis* pv. *glycines* strain No12-2 and three replicate cultures of the *rpfF* mutant T3A were grown at 27°C with shaking in yeast extract broth (51). Because *rpfF* is known to affect gene expression by stationary-phase cells of *X. campestris* pv. *campestris* grown in this medium (22), cultures of *X. axonopodis* pv. *glycines* were

Fig. 1. Diagram of scoring card used to estimate disease severity of soybean pustule. To estimate disease severity, a scoring card (4.0 cm × 7.0 cm) having nine circular holes (0.5-cm diameter) was placed over soybean leaflets. The presence or absence of pustules visible through each hole was recorded. Three leaves, one each from the apical, middle, or basal portion, were assessed, for a total of 81 observations per plant (nine leaflets and nine observations per leaflet). This estimated method has been described by Prathuangwong et al. (37).

grown to early stationary phase ($OD_{600} \approx 3.5$). Cells were pelleted by centrifugation and washed with 0.02 mM NaOH and 0.3 mM NaCl. Washed cell pellets were resuspended in 5 ml of RNA Protect (Qiagen, Valencia, CA), and then vortexed immediately. Cells were pelleted again and stored at -80°C . RNA was extracted by using the Qiagen RNA/DNA Midi Kit, and DNA was removed using an on-column DNase treatment (RNeasy Mini Kit with DNase I; Qiagen). PCR was performed on 0.5 μg of the RNA to determine that detectable DNA had been removed, and RNA samples were analyzed for quality using a BioAnalyzer 2100 (Agilent, Palo Alto, CA). cDNA was generated from 5 μg of RNA using SuperScript II (Invitrogen) and random hexamers. To confirm that DNA was removed, samples processed in parallel without reverse transcriptase served as negative controls in quantitative PCR experiments (Q-PCR) described in the following paragraph. Following reverse transcription, RNA was hydrolyzed with 2.5 M NaOH, and samples were neutralized with 2 M HEPES-free acid.

Quantitative PCR. The expression of genes encoding for protease (*pro*), endoglucanase (*engXCA*), and pectate lyase (*pelB*) was determined by quantitative PCR (Q-PCR) of cDNAs isolated from *X. axonopodis* pv. *glycines* strain No12-2 and the *rpff* mutant T3A. Because the nucleotide sequences of *pro*, *engXCA*, and *pelB* in *X. axonopodis* pv. *glycines* are unknown, primers were designed from conserved regions of these genes in other *Xanthomonas* spp. PCR amplification products were sequenced to confirm that the expected gene was amplified in each case (Table 2).

Q-PCR was performed on 1 μg of the cDNA using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche, Indianapolis, IN) on a Roche Lightcycler II (Roche) following manufacturer's specifications. An external standard curve, generated using a purified *ihfA* (integration host factor A) (9) PCR product over a dilution range of known concentrations, was used to estimate template concentrations (in pg). Primers for *ihfA* were 5'-TGACGAAAGCGGAGATGGCCG-3' and 5'-TGCCCGGATC-CAGCATAAGCC-3'. Melting curve analysis was used to verify amplification of a specific product. The concentration of amplification products from negative controls (RNA samples to which no superscript was added) was nondetectable (*pro*) or at concentrations of 10^{-4} (*engXCA*) or 10^{-2} (*pelB*) of that of the corresponding cDNA samples, indicating lack of interference from contaminating DNA.

RESULTS

Strains of *X. axonopodis* pv. *glycines* produce a DSF. Ten strains of *X. axonopodis* pv. *glycines*, isolated from soybean in

different regions of Thailand (Table 1), produced a diffusible signal that was recognized by the DSF biosensor *X. campestris* pv. *campestris* strain 8523 (pKLN55) (Fig. 2). Furthermore, *rpff* was present in the genomes of each of the 10 strains, as determined from nucleotide sequences of PCR amplification products of genomic DNA using primers complementary to *rpff* of other *Xanthomonas* spp. (data not shown). The partial *rpff* sequences obtained from the 10 strains were identical to one another. The entire *rpff* gene from one strain, No12-2, was sequenced. The deduced sequence of the 289 aa product is most closely related to RpfF of *X. campestris* pv. *vesicatoria* strain 85-10 (CAJ23597) (98% identical), *X. campestris* pv. *campestris* strain ATCC33913 (AAM41146) (97% identical), *X. oryzae* pv. *oryzicola* BLS256 (ZP_02243158), and *X. oryzae* pv. *oryzae* strains (AAL06345, YP_201508, and YP_451752) (95% identical). Therefore, both a diffusible signal recognized by a DSF biosensor and a homolog of *rpff*, which is essential for the biosynthesis of DSF in other *Xanthomonas* spp. (3,7,41), were detected in *X. axonopodis* pv. *glycines*.

***X. axonopodis* pv. *glycines* requires *rpff* to produce a DSF.** To establish the role of *rpff* in DSF production by *X. axonopodis* pv. *glycines*, we derived three *rpff* mutants of strain No12-2 through allelic exchange mutagenesis. DSF production by No12-2 was detected by the green fluorescence of the DSF biosensor (Fig. 2A), whereas the three *rpff* mutants (called T3A, T3B, and T3C) did not produce DSF detectable by the biosensor (Fig. 2B).

***rpff* mutants of *X. axonopodis* pv. *glycines* exhibit reduced virulence.** Lesions characteristic of bacterial pustules were observed on 61 to 67% of soybean leaf sections (0.5-cm diameter) evaluated at 7 days following spray inoculation with strain No12-2 in three replicated experiments (Table 3). The appearance of lesions caused by No12-2 and the *rpff* mutants was identical, but lesions were observed on only 19 to 26% of the leaf sections on soybean plants sprayed with *rpff* mutants of No12-2. Therefore, the *rpff* mutants were less virulent than the parental strain of *X. axonopodis* pv. *glycines*. In two of the three experiments, mutant T3B caused slightly higher levels of disease than did the other two *rpff* mutants. The variation in disease severity caused by the different mutant strains ($\approx 4\%$) was small in contrast to the 34 to 44% reduction in disease severity caused by the wild-type strain relative to the mutants. Therefore, we concluded that all mutants exhibited reduced virulence and did not further investigate the small differences among them that were seen in some experiments.

Extracellular products influenced by *rpff* in *X. axonopodis* pv. *glycines*. Due to the influence of *rpff* on exoenzyme and EPS production by other *Xanthomonas* spp. (3,18,41,43,44), and the important role of the exoproducts in virulence of *Xanthomonas*

TABLE 2. Quantitative polymerase chain reaction (Q-PCR) primers and sequence analysis of amplification products

				Most closely related sequences ^z					
Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Accession number	% ID	Gene name or locus tag	Annotated function	Organism	Reference
<i>pro</i>	CGTCAAATACAAAGACGG	GGTACATGATCTGGTTCG	254	AAM35817	98	XAC0929	Extracellular protease	<i>X. axonopodis</i> pv. <i>citri</i> strain 306	(12)
<i>engXCA</i>	CCTCTCCAATAAGGAACGA	GTCTTGAGCACTTCCG	327	AAM35501	100	XAC0612	Endoglucanase	<i>X. axonopodis</i> pv. <i>citri</i> strain 306	(12)
				AAY47719	92	XC0639	Endoglucanase	<i>X. campestris</i> pv. <i>campestris</i> strain 8004	(38)
				AAA27612	92	<i>engXCA</i>	Endoglucanase	<i>X. campestris</i> pv. <i>campestris</i>	(20)
<i>pelB</i>	GTGACATGGTCCACCC	CGAGTTGAAAGCTGCATT	360	AAM38405	93	XAC3562	Pectate lyase	<i>X. axonopodis</i> pv. <i>citri</i> strain 306	(12)
				AAC41522	85	<i>pelB</i>	Pectate lyase	<i>X. campestris</i> pv. <i>malvacearum</i>	(29)

^z Amplification products from PCR were sequenced, and submitted as blastx searches to identify the most closely related sequences in the NCBI database. The sequence with highest percent identity (% ID) is shown. For *engXCA* and *pelB*, additional sequences are shown for genes that were functionally characterized and shown to encode endoglucanase (20,38) and pectate lyase (29), respectively.

spp., we compared No12-2 and *rpjF* derivatives for exoenzyme and EPS production. *rpjF* mutants exhibited reduced production of protease, CMCase, endo- β -mannanase, and endo-glucanase, and less pectolytic activity compared to the parental strain No12-2 (Fig. 3, Table 4). In contrast, α -amylase production did not differ detectably between No12-2 and *rpjF* derivatives. The *rpjF* mutants also produced less EPS (Table 4) and less pectate lyase (Fig. 4) than was produced by the parental strain.

No12-2 produced a siderophore, which was detected by an orange halo surrounding bacterial growth on siderophore indicator medium (Fig. 3). The *rpjF* mutants produced larger zones than the wild type on the medium, which typically indicates siderophore overproduction (7,40).

Influence of *rpjF* on gene expression. Reverse transcriptase-PCR was done to determine if the effect of the *rpjF* mutation on exoenzyme production was associated with an effect on gene expression. Transcript abundance for genes encoding endoglucanase (*engXCA*), protease (*pro*), and pectate lyase (*pelB*) were lower in the *rpjF* mutant T3A than in the wild-type strain No12-2 (Fig. 5). Therefore, the influence of *rpjF* on the production of these exoenzymes was associated with a reduction in the rate of transcription or transcript stability in *X. axonopodis* pv. *glycines*.

DISCUSSION

The results of this study demonstrate that 10 strains of *X. axonopodis* pv. *glycines* obtained from various soybean growing regions of Thailand produce an extracellular DSF that plays an important role in virulence and exoenzyme and EPS production by *X. axonopodis* pv. *glycines*. These 10 strains possess *rpjF*, as demonstrated by PCR analysis, and the gene from one strain was sequenced and shown to be nearly identical to *rpjF* from other *Xanthomonas* spp. Three independently generated *rpjF* mutants of *X. axonopodis* pv. *glycines* strain No12-2 were defective in the

production of a DSF, as expected if *rpjF* is required for DSF biosynthesis in *X. axonopodis* pv. *glycines*, as in other *Xanthomonas* spp. As for *rpjF* mutants of *X. oryzae* pv. *oryzae* (7), *X. campestris* pv. *campestris* (3,16,43,45), and *X. axonopodis* pv. *citri* (41), the *rpjF* mutants of *X. axonopodis* pv. *glycines* exhibited reduced virulence on a compatible plant host. Despite the common role of DSF in virulence of *Xanthomonas* spp., the mechanisms by which DSF acts are quite distinct among these pathogens. *rpjF* mutants of *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* produce less than wild-type levels of EPS and the extracellular enzymes CMCase and protease (3,41), which was also observed for *rpjF* mutants of *X. axonopodis* pv. *glycines* in this study. *rpjF* mutants of *X. campestris* pv. *campestris* also produce less than wild-type levels of endo- β -1,4-mannanase (16) and polygalacturonate lyase (3), characteristics that were exhibited by *rpjF* mutants of *X. axonopodis* pv. *glycines*. Pectolytic activity, which has been associated with the hypersensitive response in *X. axonopodis* pv. *glycines* (26), was also diminished

TABLE 3. Virulence of *Xanthomonas axonopodis* pv. *glycines* strain No12-2 and *rpjF* mutants on soybean

Strain	% Leaf segments exhibiting symptoms ^z		
	Experiment 1	Experiment 2	Experiment 3
No12-2	63 a	67 a	61 a
T3A	22 c	26 b	21 c
T3B	26 b	26 b	25 b
T3C	19 c	25 b	23 bc

^z Virulence was assessed in three experiments evaluating three leaves on each of four replicate plants (experiments 1 and 2) or 10 replicate plants (experiment 3). The percentage of leaf segments exhibiting lesions typical of bacterial pustule was calculated for each replicate plant. Values followed by different letters differed significantly (*P* = 0.05) according to the Duncan's multiple range test.

Fig. 2. Detection of diffusible signal factor (DSF) production by *Xanthomonas axonopodis* pv. *glycines* No12-2. The DSF biosensor *X. campestris* pv. *campestris* 8523 (pKLN55), which has a green fluorescent protein reporter gene (*gfp*) fused to a DSF-inducible promoter (34), is grown to the right of test strains **A**, *X. axonopodis* pv. *glycines* No12-2, **B**, the *rpjF* mutant T3A of *X. axonopodis* pv. *glycines*, **C**, the known DSF-producing strain 8004 of *X. campestris* pv. *campestris*, which served as a positive control, and **D**, the *rpjF* mutant *X. campestris* pv. *campestris* strain 8523, which served as a negative control. Green fluorescence indicates the detection of DSF, which has diffused from the test strain to the biosensor. Fluorescence was viewed on a Zeiss SV11 stereoscope with Kramer epifluorescence/Optronix Color DEI450.

by an *rpfF* mutation in this pathogen. To our knowledge, *rpfF* was not known previously to influence pectolytic activity of *Xanthomonas* spp. Although *rpfF* mutants of *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri*, and *X. axonopodis* pv. *glycines* share the phenotypes of diminished extracellular enzyme and EPS production, *rpfF* mutants of *X. oryzae* pv. *oryzae* produce these compounds at wild-type levels. In *X. oryzae* pv. *oryzae*, a DSF appears to be a key regulator of iron metabolism, and *rpfF* mutants overproduce siderophores, a physiological response to severe iron limitation that was also observed for the *rpfF* mutants of *X. axonopodis* pv. *glycines* in this study. Therefore, the DSF signaling pathway in *X. axonopodis* pv. *glycines* shares components with *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri*, and *X. oryzae* pv. *oryzae*, including the effects on extracellular products and iron acquisition found in these other pathogens.

The influence of *rpfF* on extracellular enzyme production by *X. axonopodis* pv. *glycines* was evaluated at the transcriptional level for three genes conferring exoenzyme production: a protease, endoglucanase, and pectate lyase. Transcript levels for all three genes were lower in the *rpfF* mutant than in the parental strain. These data correspond well to results from *X. campestris* pv.

campestris (22), *X. axonopodis* pv. *citri* (1), and *Xylella fastidiosa* (8), in which a DSF or *rpfF* influence transcript abundance of genes encoding DSF-regulated products. In *X. campestris* pv. *campestris*, the DSF regulon includes transcripts of 165 genes, including those involved in extracellular enzyme production (22). As in *X. campestris* pv. *campestris*, genes encoding for extracellular endoglucanase (*engXBC*), protease (*pro*), and pectate lyase (*pelB*) were positively regulated by *rpfF* in *X. axonopodis* pv. *glycines*. According to a current model (17), DSF may serve as a primary signal in a signal transduction pathway that links the sensing of a specific environmental cue, cell density, to the physiology of the bacterial cell via the secondary intracellular signal cyclic di-GMP (bis-[3'-5']-cyclic di-guanosine monophosphate). As the primary signal, DSF is thought to trigger a phosphorelay cascade mediated by a two-component regulatory system composed of the hybrid sensor RpfC and the response regulator RpfG. By analogy to other two component systems, signal transduction may involve autophosphorylation of RpfC in response to DSF binding, followed by phosphotransfer to RpfG. Upon phosphorylation, RpfG functions as a cyclic di-GMP phosphodiesterase, which alters the level of cyclic di-GMP in the cell (17). Mechanisms of cyclic-di-GMP signaling in bacteria are

Fig. 3. Exoenzyme activity and siderophore production of *Xanthomonas axonopodis* pv. *glycines* No12-2 and *rpfF* mutants (T3A, T3B, and T3C). Counter-clockwise from upper left: No12-2 (wt), T3A, T3C, and T3B. Filtrates from bacterial cultures were placed in wells in assay media to assess **A**, CMCase, **B**, protease, **C**, endo- β -mannanase, and **D**, pectolytic enzyme activity. **E**, Soft rot symptoms on potato tuber slices inoculated with bacterial cells. **F**, Siderophore production exhibited as yellow zones surrounding bacterial colonies on siderophore indicator medium (40). The *rpfF* mutant T3B was not tested for siderophore production. Assays were done twice, once with four replicates and once with three replicates, and a representative plate for each assay is shown.

TABLE 4. Exoenzyme and extracellular polysaccharide (EPS) production by *Xanthomonas axonopodis* pv. *glycines* No12-2 and *rpfF* mutants

Strain	Diameter of clear zone (cm) ^y						EPS ^z (mg/ml)
	Protease	CMCase	Pectolytic activity	Mannanase	Alpha-amylase		
No12-2	2.1	2.8	2.6	1.7	2.8		1.8
T3A	0.5	1.3	1.5	0.7	2.7		1.3
T3B	0.5	1.4	1.4	0.7	2.7		1.3
T3C	0.5	1.3	1.4	0.8	2.7		1.3

^y Filtrates of overnight cultures grown in NYGB were placed in wells (0.5-cm diameter) in agar media specific for detection of each exoenzyme. Enzyme activity was estimated from the diameter of zones surrounding each well. A value of 0.5 cm, the diameter of the well, indicates that no enzyme activity was detected. All experiments were done twice, once with four replicate plates and once with three replicate plates. The standard deviation for each assay was ≤ 0.1 cm. CMCase, carboxymethyl cellulase.

^z EPS was extracted from four replicate cultures grown in NYGB medium for 48 h and dried, and mean dry weights are presented. The standard deviation for each treatment (strain) was ≤ 0.1 mg/ml. Three independent experiments were done, and results from a representative experiment are presented.

not fully understood, but they are known to include regulation of downstream products at the transcriptional, translational, and posttranslational levels (24), which is consistent with the effects of *rpjF* on transcript abundance that was observed in this study.

The fatty acid signaling compounds produced by *Xanthomonas* spp. remain largely uncharacterized, with cis-11-methyl-2-dodecanoic acid (the DSF produced by *X. campestris* pv. *campestris*) being the only compound whose structure is known (48). A similar compound, tentatively identified as 12-methyl-tetradecanoic acid (10), functions as a signaling compound in *X. fastidiosa*. The chemical nature of the extracellular signal produced by *X. axonopodis* pv. *glycines* remains to be determined, but the high degree of similarity between the *rpjF* genes of *X. axonopodis* pv. *glycines* and *X. campestris* pv. *campestris*, which is known to encode for DSF biosynthesis (48), along with the recognition of the factor by the DSF biosensor suggests that the extracellular factor is closely related to the DSF of *X. campestris* pv. *campestris*. DSF biosensors provide sensitive and specific tools for the detection of fatty acid signaling molecules, which are present in minute quantities in cultures of *Xanthomonas* spp. (48). Biosensors based upon the DSF-inducible *engXCA* promoter have been used successfully to identify and characterize *rpjF* mutants of *X. campestris* pv. *campestris* (43,48) and *X. fastidiosa* (34) and

were instrumental in the purification of DSF from *X. campestris* pv. *campestris* (48). Structural features important for *engXCA* promoter induction have been identified as the length, double bond at the α , β position, cis conformation, and methyl group at the C-11 position of the fatty acid (48). Accordingly, recognition by the DSF biosensor suggests that the extracellular signaling molecule produced by *X. axonopodis* pv. *glycines* is related to the DSF of *X. campestris* pv. *campestris*.

The discovery of a DSF-based quorum sensing system in *X. axonopodis* pv. *glycines* opens up the possibility of new biologically-based disease management strategies targeting this system. Degradation of a density-dependent signal can interfere with a quorum sensing system, leading to reduced virulence of a pathogen, a phenomenon termed quorum quenching (14,15,19). Through quorum quenching, traits such as virulence or exoenzyme production, which would normally be expressed when cell densities are high, are suppressed by altering the levels of cell density-dependent signaling compounds. Recently, bacterial strains that degrade DSF signals produced by *X. campestris* pv. *campestris* and *Xylella fastidiosa* have been identified (35). Several strains of naturally occurring leaf-inhabiting bacteria representing diverse genera (*Bacillus*, *Paenibacillus*, *Microbacterium*, *Staphylococcus*, and *Pseudomonas*) were found to degrade these DSFs. Coinoculation of susceptible plants with *X. campestris* pv. *campestris* and DSF-degrading bacteria reduced disease severity up to twofold compared with plants inoculated only with the pathogen. Similarly, disease severity caused by *X. fastidiosa* on grapevine was reduced significantly when the pathogen was coinoculated with DSF-degrading bacterial strains (35). The DSF-based quorum sensing system of *X. axonopodis* pv. *glycines* could also be disrupted through a quorum quenching approach, which could provide new directions for management of soybean pustule disease through biological control.

ACKNOWLEDGMENTS

We thank S. Lindow for providing the DSF biosensor, K. Hockett for constructing the *rpjF* mutants of *X. axonopodis* pv. *glycines*, V. Stockwell for assistance in statistical analysis of the virulence data, M. Henkels and C. Baker for preparing the figures, and the Center for Genomic Research and Biocomputing Core Laboratories, Oregon State University for sequencing and bioanalyzer services. This research was supported by a fellowship to J. Thowthampitak from the Royal Golden Jubilee program of the Thailand Research Fund.

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Fig. 4. Pectate lyase activity exhibited by *Xanthomonas axonopodis* pv. *glycines* No12-2 and *rpjF* mutants (T3A, T3B, and T3C). Bacterial cells were suspended in a buffer solution containing polygalacturonic acid, and pectate lyase was assessed hourly by measuring changes in absorbance at 235 nm (33). A unit of pectate lyase activity was defined as a change in optical density of 1 unit min^{-1} corresponding to the production of 0.5 μmol unsaturated product. The assay was done three times, with four replicate cultures in each assay, and a representative experiment is shown.

Fig. 5. Influence of *rpjF* on abundance of transcripts for genes encoding exoenzymes. Levels of mRNA for **A**, *pelB* (encoding for pectate lyase), **B**, *engXCA* (encoding for endoglucanase), or **C**, *pro* (encoding for extracellular protease) were estimated by quantitative polymerase chain reaction (Q-PCR) from cultures of *Xanthomonas axonopodis* pv. *glycines* strain No12-2 (gray bars) and the *rpjF* mutant T3A (black bars). RNA was isolated from three (mutant) or four (parental strain) replicate cultures grown in YEB medium (5 g of beef extract, 1 g of yeast extract, 5 g of peptone, and 5 g of sucrose) to early stationary phase ($\text{OD}_{600} = 3.5$). Error bars denote standard errors of the mean. The Q-PCR was done twice with similar results, and results from one experiment are shown.

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Erratum

Corrections were made to pages 1253, 1256, and 1257 of this article on November 21, 2008.