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Dual Signaling Functions of the Hybrid Sensor Kinase RpfC of *Xanthomonas campestris* Involve Either Phosphorelay or Receiver Domain-Protein Interaction*[S]

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The hybrid sensor kinase RpfC positively regulates the expression of a range of virulent genes and negatively modulates the synthesis of the quorum sensing signal diffusible signal factor (DSF) in *Xanthomonas campestris.* Three conserved amino acid residues of RpfC implicated in phosphorelay (His^{198} in the histidine kinase domain, Asp^{512} in the receiver domain, and His^{657} in the histidine phosphotransfer domain) were essential for activation of the production of extracellular enzymes and extracellular polysaccharide (EPS) virulence factors but were not essential for repression of DSF biosynthesis. Domain deletion and subsequent in *trans* expression analysis revealed that the receiver domain of RpfC alone was sufficient to repress DSF overproduction in an *rpfC* deletion mutant. Further deletion and alanine scanning mutagenesis analyses identified a peptide of 107 amino acids and three amino acid residues (Gln^{496}, Glu^{504}, and Ile^{552}) involved in modulating DSF production. Co-immunoprecipitation and far Western blot analyses suggested an interaction between the receiver domain and RpfF, the enzyme involved in DSF biosynthesis. These data support a model in which RpfC modulates two different functions (virulence factor synthesis and DSF synthesis) by utilization of a conserved phosphorelay system and a novel domain-specific protein-protein interaction mechanism, respectively. This latter mechanism represents an added dimension to conventional two-component signaling paradigms.

Two-component regulation is the predominant form of signal recognition and response coupling mechanism used by bacteria to sense and respond to diverse environmental stresses and cues ranging from common environmental stimuli to host signals recognized by pathogens and bacterial cell-cell communication signals (1–3). Bacteria chromosomes encode numerous two-component systems, implying diversified roles in signal modulation of microbial physiology and ecology. For example, the human pathogen *Pseudomonas aeruginosa* and plant pathogen *Xanthomonas campestris* pv. *campestris* carry, respectively, >60 and >100 pairs of genes encoding two-component systems (4, 5). Typically, recognition of a signal by the sensor component results in autophosphorylation at a histidine residue; the phosphoryl group is subsequently transferred to an aspartate residue in the CheY-like receiver (REC)[2] domain of the cognate response regulator (2, 6).

The structures of both sensors and regulators are modular, and numerous variations in domain architecture and composition have evolved to tailor to specific needs in signal perception and signal transduction (2, 7). Among the extremely diversified family of histidine kinase sensors, the simplest (also known as orthodox kinases) consists of only sensing and kinase domains. The more complex hybrid sensors contain, in addition to sensing and kinase domains, a REC domain typical of two-component regulators and in some cases a C-terminal histidine phosphotransferase (HPT) domain (2, 6). The family of sensor kinases with this latter domain organization includes ArcB of *Escherichia coli*, BvgS of *Bordetella* sp., GacS of *Pseudomonas* sp., and RpfC of *X. campestris* pv. *campestris* (8–13). In the case of such hybrid sensor-regulator kinases, the phosphoryl group from the autophosphorylated histidine (His_1) residue is transferred to an aspartate (Asp_1) residue of the REC domain and is further relayed to a histidine residue (His_2) in the HPT domain. Subsequently, the His_2 transfers the phosphoryl group to an aspartate (Asp_2) residue in the REC domain of the cognate response regulator (12–14). Although the REC domain of hybrid sensor kinases is implicated in phosphorelay, it is by no means clear that this is its sole function (15).

In *X. campestris* pv. *campestris*, the hybrid sensor kinase RpfC and cognate regulator RpfG are implicated in the positive regulation of biofilm dispersal and the production of virulence factors (16, 17). This two-component system is believed to respond to the cell-cell communication signal DSF (16–19), which has been characterized as *cis*-11-methyl-2-dodecenoic acid (20). Synthesis of DSF requires an enzyme encoded by the *rpfF* gene (18). Recent microarray and genetic analyses have revealed that DSF also modulates additional functions associated with stress resistance and adaptation (21). A number of lines of evidence support a role for RpfC/RpfG in the perception and transduction of the DSF signal. The addition of DSF can restore virulence factor production and induce biofilm dispersal in *rpfF* mutants but not in *rpfC* and *rpfG* mutants, respec-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1 and S2.

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[2] The abbreviations used are: REC, receiver; HPT, histidine phosphotransferase; rpm, revolutions/min; EPS, extracellular polysaccharide; RT, reverse transcription; DSF, diffusible signal factor; HK, histidine protein kinase.

tively (16, 17). Furthermore, the RpfC/RpfG two-component system has been reconstructed in *P. aeruginosa* and shown to confer responsiveness to exogenously added DSF, as seen through its effects on swarming motility (19). Importantly, mutation of *rpfC* (but not of *rpfG*) leads to overproduction of DSF. These findings suggest that the RpfC sensor kinase may control two signaling pathways in *X. campestris* pv. *campestris*, with one activating virulence factor production and the other inhibiting DSF biosynthesis. The former pathway is dependent on the RpfG response regulator, but the latter apparently is not. The work in this paper had the aim of establishing the molecular mechanisms underlying this dual signaling action of RpfC.

As outlined above, RpfC is a hybrid sensor kinase in which the sensory input and kinase domains are fused to a receiver domain and a C-terminal HPT domain. By domain deletion and site-directed mutagenesis approaches, we have shown here that RpfC transduces signals for the regulation of virulence factor production through a phosphorelay system but modulates DSF biosynthesis through a domain-specific protein-protein interaction mechanism involving the REC domain. This latter finding, which offers an insight into possible additional roles of the REC domain of hybrid sensor kinases, presents a new dimension to the conventional two-component signaling paradigms.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The wild-type *X. campestris* pv. *campestris* strain Xc1 has been described previously (20). The Xc1 derivatives carrying various mutations or constructs were described in Figs. 1–5. *X. campestris* pv. *campestris* strains were grown at 30 °C in YEB medium (22), unless otherwise stated. *E. coli* strains were grown at 37 °C in LB medium. Antibiotics were added at the following concentrations when required: 100 μg/ml kanamycin, 50 μg/ml rifampicin, and 15 μg/ml tetracycline; X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucopyranoside) was included in the medium at 60 μg/ml for the detection of GUS (β-glucuronidase) activity. DSF signal was added to the medium in a final concentration of 3 μM when necessary.

Chromosomal Deletions in RpfC and Preparation of Constructs for in Trans Expression—RpfC was analyzed using the Simple Modular Architecture Research Tool (SMART). The open reading frame and the coding sequences for various domains of RpfC were deleted using the allelic exchange vector *pK18mobsacB* following the methods described previously (17). Briefly, for generation of the *rpfC* deletion mutant Xc1ΔC, two *rpfC* DNA fragments rpfC-1 (the 5′ region of ~531 bp) and rpfC-2 (the 3′ region of ~649 bp) were amplified using two primer pairs, rpfC-1-FOR and rpfC-1-REV and rpfC-2-FOR and rpfC-2-REV (supplemental Table S1). The resultant DNA fragments were cleaved with BamHI and ligated by T4 DNA ligase. The fusion fragment rpfC-12 was then amplified using the ligation mixture as the template with primer pair rpfC-1-FOR and rpfC-2-REV. The fusion fragment was cloned into the SmaI site of the vector pK18*mobsacB*. After sequence verification, the recombinant plasmid was mobilized into strain Xc1 by triparental mating. Transconjugants were selected on LB medium supplemented with rifampicin and kanamycin. A second selection was done on LB medium containing 5% (w/v) sucrose and rifampicin to select for resolution of the vector by a second crossover event. The in-frame deletion of *rpfC* was confirmed by PCR and sequencing. Similar methods were applied to generate the HPT domain deletion mutant RpfCΔ1, the REC-HPT domain deletion mutant RpfCΔ2, the HK-REC-HPT domain deletion mutant RpfCΔ3, and the transmembrane-HK-REC domain deletion mutant RpfCΔ4, using the primers listed (supplemental Table S1).

For the preparation of in *trans* expression constructs, the coding sequence of the REC domain of RpfC or its truncated versions was obtained by PCR amplification using the primers listed in supplemental Table S1. The PCR fragments were cleaved with BamHI and HindIII and cloned under the control of the *lac* promoter in the broad host range vector pLAFR3. The recombinant constructs were sequence-verified and mobilized into the RpfC null mutant Xc1ΔC by triparental mating. The resultant transformants were selected on LB medium supplemented with rifampicin and tetracycline.

Quantification of DSF and Virulence Factor Production—DSF synthesis, bioassay, and quantification were performed as described previously (20). DSF signals were extracted from the supernatants of bacterial cultures 40 h after inoculation, unless otherwise indicated. For determination of biofilm formation, 1 ml of bacterial cell culture at A_{600} = 1.6 was centrifuged at 10,000 rpm for 2 min, and the existence of a gum-like substance on the top of the bacterial pellet was checked as described previously (21). Quantification of EPS production and the activities of extracellular enzymes were performed as described previously (21, 23, 24).

RNA Extraction and Reverse Transcription (RT)-PCR Analysis—The detailed methods for RNA extraction and oligomicroarray analysis have been described previously (21). Briefly, bacterial cells at A_{600} = 1.6 were harvested by centrifugation at 4 °C for 4 min at 10,000 rpm. RNA was purified by using an RNeasy midicolumn (Qiagen) following the protocol provided by the manufacturer. RT-PCR analysis was done using the Qiagen® OneStep RT-PCR kit following the manufacturer's instructions. The primers used for RT-PCR analysis were listed in supplemental Table S1, and a total of 250 ng of total RNAs were used for each reaction.

In Situ Site-directed and Alanine Scanning Mutagenesis—Three conserved amino acid residues of RpfC predicted to be involved in phosphorelay (His_1^{198} in the HK domain, Asp_1^{512} in the REC domain, and His_2^{657} in the HPT domain) were identified via the sequence alignment with the following homologues: RpfA (NCBI accession number(s) U62023), LemA (M80477), BvgS (M25401), GacS (AB219364), ArcB (X53315), and CheY (M13463). The three conserved residues were changed to alanine or valine by using substituted PCR primers (supplemental Table S1). The resultant PCR fragments were cloned into the SmaI site of pK18*mobsacB*. The recombinant constructs were verified by DNA sequencing and mobilized into strain Xc1 by triparental mating. Transconjugants were selected on LB medium supplemented with rifampicin and kanamycin. The second selection was done on YEB medium containing 5% (w/v) sucrose and rifampicin. The potential mutants were selected based on DSF production and biofilm formation phenotypes.

The point mutation was verified by PCR amplification of the corresponding DNA fragment and DNA sequencing.

For alanine scanning mutagenesis of the REC domain, the coding region was amplified using the primers rpfC-F3 and rpfC-R3 listed in supplemental Table S1 and cloned into the vector pGEMT-easy. Point mutation was conducted using the QuikChange® site-directed mutagenesis kit following the manufacturer's instructions. After DNA sequencing verification, the mutated REC fragments were cut by BamHI and HindIII and cloned under the control of the *lac* promoter in expression vector pLAFR3. These constructs were then separately mobilized into strain Xc1ΔC by triparental mating.

Anti-FLAG Co-immunoprecipitation—The REC coding sequence was fused in-frame by PCR with that of FLAG using two primers listed in supplemental Table S1. After digestion with BamHI and HindIII, the PCR fragment was cloned in vector pLAFR3. The construct was transferred to the *rpfC* deletion mutant Xc1ΔC by conjugation. The expression of FLAG-REC fusion protein was confirmed by Western blot analysis and DSF bioassay. A total soluble protein sample was prepared when bacterial cell density reached 1.0 at A_{600} and then applied onto EZview™Red Anti-FLAG® M2 affinity gel (Sigma) following the manufacturer's instructions. In brief, the gel was washed with TBS buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4), and the FLAG-tagged protein and its binding proteins were eluted with 0.1 M glycine HCl, pH 3.5. After condensation with Microcon YM-10 (Amicon), the eluted proteins were resolved by SDS-PAGE and stained with Coomassie Blue. Visible protein bands were excised from the gel, and the peptide sequences were deciphered by mass spectrometry (quadrupole time-of-flight).

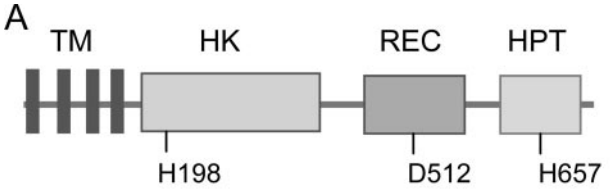
Protein Purification and Anti-serum Preparation—The REC coding sequence was fused in-frame to the coding sequence of the His_6 tag in expression vector pET-14b (Novagen) and transformed into *E. coli* strain BL21 (DE3). Cells were grown at 28 °C with shaking at 250 rpm to 0.7 at A_{600}, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.25 mM, and growth was continued overnight at 18 °C with a gentle shaking at 200 rpm. The cells were harvested by centrifugation at 4000 rpm for 30 min and resuspended in the lysis buffer (pH 8.0) containing 50 mM NaH_2PO_4, 0.3 M NaCl, 10 mM imidazole, and 0.1 mM protease inhibitor mixture (Sigma). The cells were sonicated on ice with 5 × 15-s bursts and 90-s cooling intervals. The cell debris was removed by centrifugation at 14,000 rpm for 30 min. The supernatant was then filtered using a 0.45-μl filter before adding to an affinity column containing Ni^{2+}-chelating Sepharose fast flow resin (Amersham Biosciences) for affinity binding. The column was washed with a buffer solution of the same pH containing 50 mM NaH_2PO_4, 0.3 M NaCl, and 20 mM imidazole. The bound His_6-REC protein was eluted from the column with a 250 mM imidazole gradient and used as an antigen to obtain polyclonal antisera by immunizing rabbits through subcutaneous injections at two-week intervals. RpfF and green fluorescent protein were also purified in the same way for far Western blot analysis. Preparation of recombinant AlbD protein was described previously (25).

Western Blot and Far Western Protein-Protein Interaction Assay—Western blotting was performed as described previously (26). *In vitro* far Western blot assay was performed following the method of Hall (27). Briefly, purified REC (0.1 μg), RpfF (10 μg), AlbD (10 μg), and green fluorescent (10 μg) proteins were resolved by SDS-PAGE and transferred to an Immun-Blot™ polyvinylidene difluoride membrane (Bio-Rad), which was then blocked with phosphate-buffered saline containing 0.05% Tween 20 and 3% nonfat powdered milk overnight at room temperature. The blocked membrane was overlaid with REC domain protein (30 μg/ml in blocking buffer) for 4 h at room temperature. After washing with phosphate-buffered saline with 0.05% Tween 20 four times, the blots were incubated with primary polyclonal anti-REC serum followed by washing and incubation with secondary goat anti-rabbit IgG(H+L)-horseradish peroxidase conjugate (Bio-Rad). The hybridization signal was detected using SuperSignal® West Pico chemiluminescent substrate (Pierce).

RESULTS

Conserved Phosphorelay Mechanism of RpfC Is Essential for Induction of Virulence Gene Expression but Not for Down-regulation of DSF Biosynthesis—RpfC is a hybrid sensor regulator, consisting of a transmembrane, an HK, a REC, and a HPT domain (Fig. 1*A*). Multiple sequence alignment analysis of RpfC and homologues identified several potential residues implicated in phosphotransfer, including the autophosphorylatable His_1^{198} in the HK domain, the phosphor-accepting Asp residue (Asp_1^{512}) in the REC domain, and the phosphor-accepting His_2^{657} in the HPT domain of RpfC (Fig. 1*A* and supplemental Fig. S1). Similarly, we also identified the potential phosphor-accepting aspartate (Asp_2^{80}) residue in the REC domain of response regulator RpfG (data not shown). The conserved structure implies that the RpfC/RpfG two-component system may sense and transduce signals (including DSF) through the $His_1 \rightarrow Asp_1 \rightarrow His_2 \rightarrow Asp_2$ multiple phosphorelay system.

To determine whether the conserved phosphorelay mechanism in RpfC is involved in regulation of the dual functions, *i.e.* induction of virulence factor production and down-regulation of DSF production, we substituted His_1^{198} and His_2^{657} of RpfC with alanine and Asp_1^{512} with valine by site-directed mutagenesis. These altered *rpfC* alleles were transferred to the chromosome to replace the wild type. RT-PCR analysis showed that these point mutations did not affect the expression of *rpfC* (supplemental Fig. S2) but resulted in decreased production of EPS and reduced activity of cellulase and protease, which was similar to the RpfC null mutant Xc1ΔC (Fig. 1*B*). Surprisingly, however, strains expressing the RpfC phosphotransfer-deficient variants H198A, D512V, and H657A produced a low level of DSF similar to the parental wild-type strain Xc1 (Fig. 1*B*) and unlike the *rpfC* deletion mutant Xc1ΔC, which produced elevated levels of DSF. These findings suggested that the conserved His^{198}-Asp^{512}-His^{657} phosphorelay mechanism is required for induction of extracellular enzyme and EPS virulence factors but not for the RpfC-dependent inhibition of DSF synthesis.



B

Strains	EPS (% WT)	Protease (% WT)	Cellulase (% WT)	DSF (units)
WT	100 (± 5)	100 (± 9)	100 (± 6)	8.7 (±1.0)
Xc1ΔC	69 (± 7)	60 (± 8)	71 (± 6)	84.3 (±14.2)
H198A	64 (± 2)	60 (± 5)	67 (± 4)	6.2 (±0.8)
D512V	72 (± 5)	55 (± 6)	62 (± 4)	5.7 (±0.9)
H657A	61 (± 3)	50 (± 8)	70 (± 5)	4.5 (±0.8)

FIGURE 1. **The conserved phosphorelay residues are essential for RpfC signaling modulation of EPS and extracellular enzyme production.** *A*, RpfC domain structure and relative position of conserved residues. *B*, effect of site-directed mutagenesis of conserved residues on enzyme activity and production of EPS and DSF signals. All of the strains used were derived from wild-type (*WT*) strain Xc1 (31). Xc1ΔC, an internal fragment of a 2157-bp coding sequence of rpfC from position 10 to 2166, was deleted in-frame; H198A, D512V, and H657A were generated by substituting the histidine residues at positions 198 and 657 and aspartic acid residue at position 512 of RpfC by alanine and valine, respectively. *TM*, transmembrane.

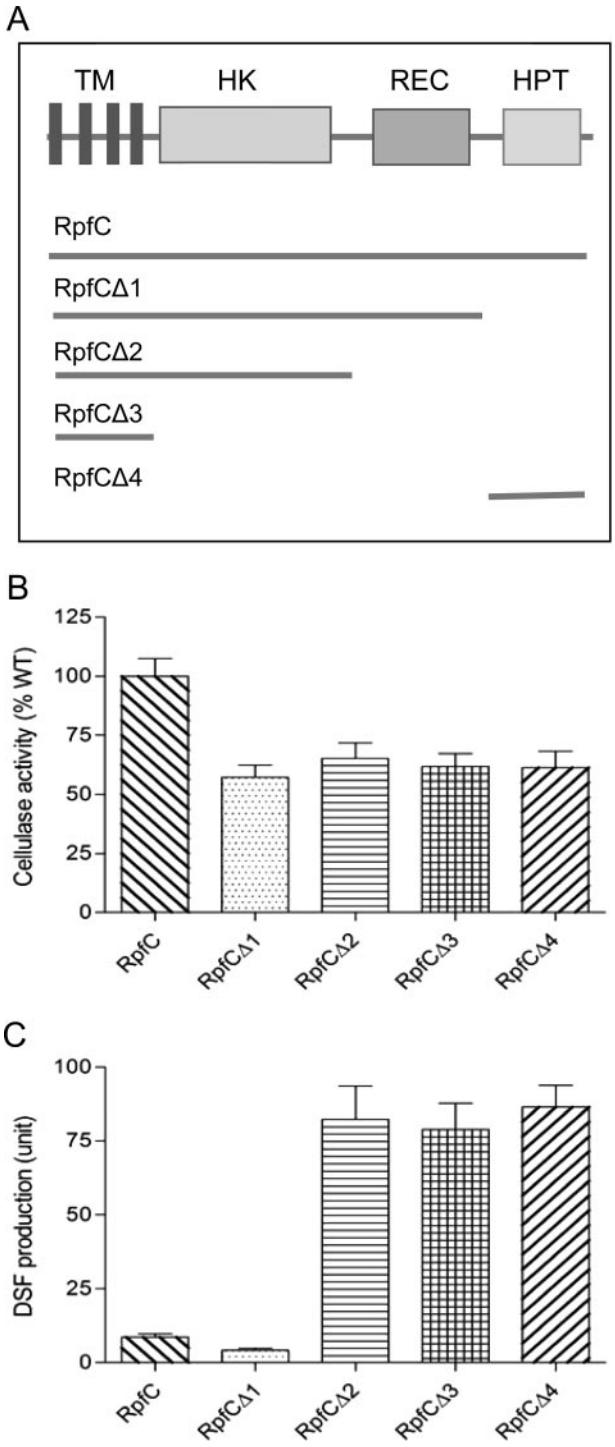


FIGURE 2. **Identification of essential domains for the regulation of extracellular enzyme activity and DSF production.** *A*, illustration of RpfC deletion derivatives. *B*, effect of domain deletion on Xc1 cellulase activity. *WT*, wild type. *C*, effect of domain deletion on DSF production. *TM*, transmembrane.

The REC (but Not the HPT) Domain Is Required for Repression of DSF Biosynthesis—To address the mechanistic basis of RpfC modulation of DSF biosynthesis, we first generated a panel of mutants with in-frame deletions in the chromosomal copy of the *rpfC* gene, which expressed variants of RpfC lacking one or more domains (Fig. 2*A*). RT-PCR analysis confirmed that these deletions did not affect the expression of the altered *rpfC* protein (supplemental Fig. S2). As expected, deletion of any of the three domains (HK, REC, or HPT) containing the conserved phosphorelay residues resulted in decreased activity of cellulase (Fig. 2*B*) and reduced EPS production and protease activity (data not shown). However, although deletion of both REC and HPT domains led to overproduction of DSF, the truncated RpfC lacking only the HPT domain remained active in the repression of DSF biosynthesis (Fig. 2*C*). Furthermore, all of the deletion mutants (RpfCΔ2, RpfCΔ3, and RpfCΔ4) lacking the REC domain overproduced the signal (Fig. 2, *A* and *C*). These data, which seem to exclude the involvement of HPT in modulation of DSF signal generation, suggest a role for the REC domain in this function.

The Isolated REC Domain Can Repress DSF Biosynthesis—To further test the hypothesis that the REC domain is involved in RpfC-dependent repression of DSF biosynthesis, we cloned the coding region of this domain (the amino acid residues 450–599 of RpfC) under the control of the *lac* promoter in the expression vector pLAFR3 for in *trans* expression in the *rpfC* deletion mutant Xc1ΔC and the wild-type strain Xc1. Fig. 3 shows that Xc1ΔC produces an elevated level of DSF, whereas overexpression of the REC domain in Xc1ΔC reduced DSF production to a

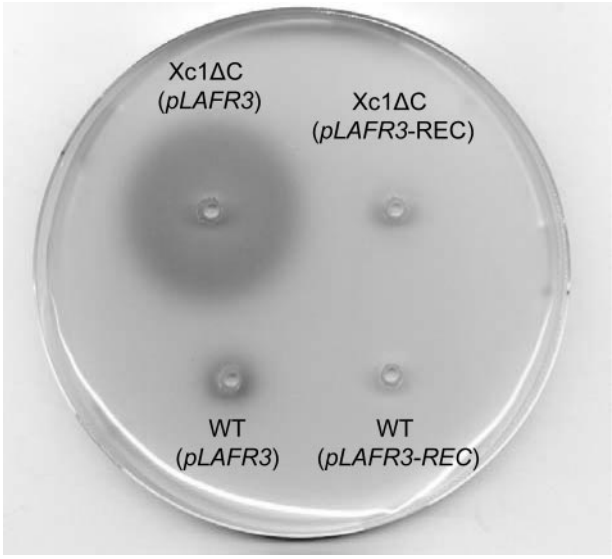


FIGURE 3. **The REC domain of RpfC inhibits DSF biosynthesis.** The coding sequence of the REC domain was cloned under the control of the *lac* promoter in the cloning vector pLAFR3, and strains carrying the REC construct or vector control were added to wells in the bioassay plate. The *black* zone indicates DSF activity.

level less than the wild-type control. Consistent with these findings, expression of REC in wild-type strain Xc1 decreased DSF production to an undetectable level (Fig. 3).

The REC domain contains 150 amino acids. To locate the minimum active region, we generated seven further deletion variants of the REC domain (Fig. 4*A*). Variants RΔ1–RΔ4 lack the first 15, 29, 45, 60 amino acids, and RΔ5–RΔ7 lack the last 15, 29, and 45 amino acids of the REC domain, respectively. The constructs encoding these truncated REC variants were introduced into Xc1ΔC, and the effect of deletion on the repression of DSF biosynthesis (Fig. 4*A*) and the level of expression of each of the truncated proteins (Fig. 4*B*) were determined by Western analysis. These combined analyses suggested that the N-terminal amino acids 1–29 and the 15 amino acids at the C-terminal of REC are not essential for the repression of DSF biosynthesis. Western analysis failed to detect any REC derivatives with further deletion at either the N or C terminus. The findings were extended by in *trans* expression of the truncated REC domain without the first 29 N-terminal amino acids and the last 15 C-terminal amino acids (Fig. 4*A*, RΔ8). When expressed in Xc1ΔC, this peptide of 107 amino acids could repress DSF biosynthesis to a level lower than that in the wild-type strain Xc1, throughout growth (Fig. 4*C*).

Identification of Key Amino Acid Residues Implicated in REC Down-regulation of DSF Biosynthesis—The minimal REC region for repression of DSF biosynthesis contains 107 amino acids, including 47 hydrophobic amino acids (Fig. 5*A*). For identification of the key amino acid residues involved in down-regulation of DSF biosynthesis, we employed alanine-scanning mutagenesis to alter each of the 90 amino acids (with the exception of the 17 alanines). These modified peptides were expressed in *trans* in mutant Xc1ΔC, and DSF production was determined. The analysis led to the identification of three amino acids (Gln^{496}, Glu^{504}, and Ile^{552}) for which alteration to an alanine residue reduced but did not totally abolish the REC

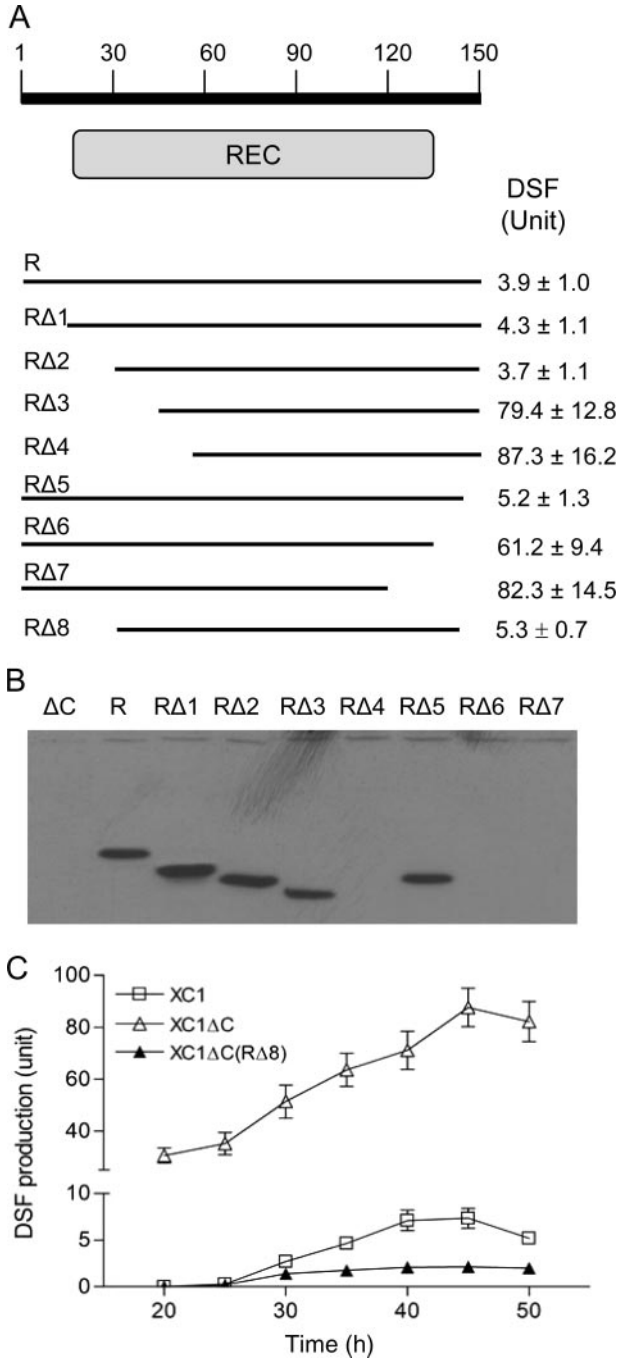


FIGURE 4. **Deletion analysis of REC domain.** *A*, effect of deletion on DSF biosynthesis. The corresponding deletion derivatives of REC domain (*R*) were cloned separately in vector pLAFR3 (as described in the legend to Fig. 3), and DSF was bioassayed when bacterial cell density reached ~A_{600} = 2.4. *B*, effect of deletion on protein expression. The total soluble protein extracts from the above strains were separated by electrophoresis and hybridized using REC-specific antiserum. *C*, DSF production profiles of wild-type strain Xc1, the *rpfC* deletion mutant Xc1ΔC, and Xc1ΔC expressing the minimal active region of REC (shown as RΔ8 in *A*).

repressor activity (Fig. 5*B*). To test for any synergistic effect among these three amino acid residue alterations, two constructs for in *trans* expression of the REC domain with double and triple alterations (Q496A,I552A and Q496A,E504A,I552A, respectively) were generated. However, no significant difference in DSF production was noticed when comparing the

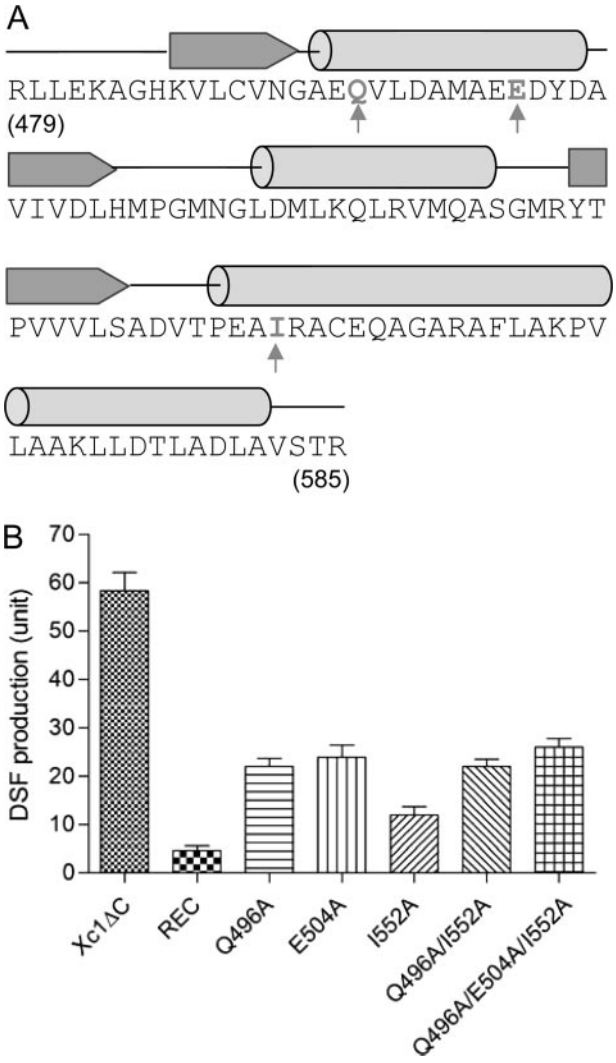


FIGURE 5. **Key amino acid residues implicated in REC-mediated repression of DSF biosynthesis.** *A*, REC secondary structure prediction (GOR4 program) and the three key residues (indicated by *vertical arrow*) influencing REC activity. *Arrows* represent β-sheets, whereas *cylinders* represent α-helices. *B*, effect of key amino acid residue substitutions on DSF production. The coding sequence of minimum REC region RΔ8 (Fig. 4) and its alanine-substituted derivatives was cloned in pLAFR3 and expressed in *rpfC* deletion mutant Xc1ΔC.

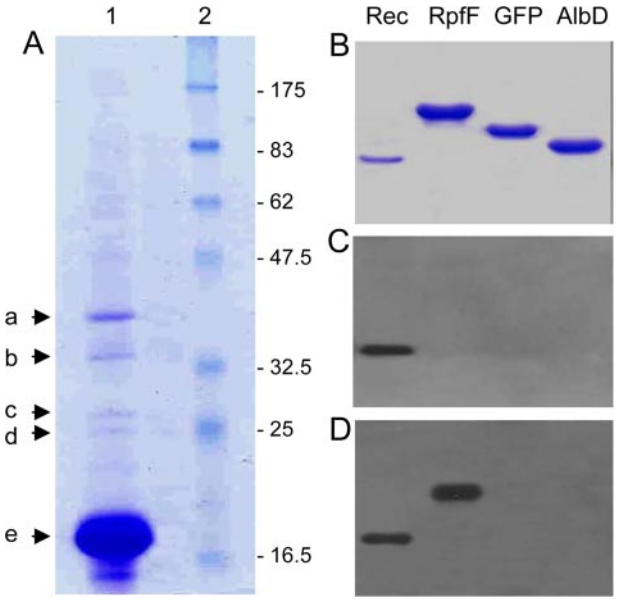


FIGURE 6. **REC-RpfF interaction to form a stable complex.** *A*, electrophoresis separation of the proteins co-eluted with FLAG-REC fusion protein (*e*) from the affinity column. The other proteins were characterized by peptide sequencing as elongation factor Tu-B (*a*), RpfF (*b*), 2-hydroxyhepta-2,4-diene-1,7-dioateisomerase/5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioatedecarboxylase (*c*), and 30 S ribosomal protein S3 (*d*), respectively. *B*, electrophoresis separation of REC, RpfF, and two control proteins, AlbD and green fluorescent protein. *C*, Western blot analysis using REC-specific antiserum. *D*, far Western blot detection of the proteins capable of formatting stable complex with REC using REC-specific antiserum.

effects of the expression of these multiply altered proteins with the corresponding single alterations (Fig. 5*B*).

Identification of RpfF as a REC-interacting Protein by Co-immunoprecipitation and Far Western Analysis—On the basis of the above findings, we posited that the effect of RpfC on DSF synthesis was due to binding of a (regulatory) protein by the REC domain and that such binding may be influenced by the RpfC conformation. To test the first part of this hypothesis, we searched for REC-interacting proteins using co-immunoprecipitation. A construct expressing the REC domain tagged with a 9-amino-acid FLAG peptide (see "Experimental Procedures") was introduced into the *rpfC* deletion mutant. The total proteins isolated from the cell culture at $A_{600} = 1.0$ were passed through the EZview™Red Anti-FLAG® M2 affinity gel (Sigma) for affinity binding. Electrophoretic separation of the proteins eluted from the affinity column and subsequent analysis by mass spectrometry identified four proteins in addition to the bait FLAG-REC as elongation factor Tu-B, RpfF, 2-hydroxyhepta-2,4-diene-1, 7-dioateisomerase/5-carboxymethyl-2-oxo-hex-3-ene-1, 7-dioatedecarboxylase, and 30 S ribosomal protein S3 (Fig. 6). The finding that RpfF, an enzyme essential for DSF biosynthesis, was able to bind to the REC domain was intriguing. To test whether any of the other three interacting proteins were also required for DSF biosynthesis, the cognate genes were deleted in both wild-type and *rpfC* deletion mutant backgrounds. Bioassay of these mutant strains did not reveal any effect on DSF biosynthesis (data not shown).

To verify specific binding of REC to RpfF, we conducted far Western blot analysis using two unrelated proteins, albicidin hydrolase (AlbD) (25) and the green fluorescent protein as negative controls (Fig. 6*B*). Western analysis showed that the anti-REC antiserum only recognized REC but not RpfF or the other two proteins (Fig. 6*C*). However, after soaking the membrane containing the separated proteins with a REC protein solution and subsequent stringent washes, probing with the anti-REC antiserum revealed a cross-reacting band at the position of the RpfF protein band in addition to the expected REC hybridization signal (Fig. 6*D*). The results thus demonstrated a specific REC-RpfF protein-protein interaction that is maintained throughout the stringent washing regime.

DISCUSSION

The hybrid sensor kinase RpfC of *X. campestris* pv. *campestris* acts to positively regulate the synthesis of extracellular enzymes and EPS virulence factors and negatively regulates the synthesis of the cell-cell signal DSF. The findings presented here are consistent with a model by which RpfC modulates

these diverse biological functions through two distinct molecular mechanisms; activation of virulence factor synthesis requires phosphorelay, whereas regulation of DSF production involves domain-specific protein-protein interaction with RpfF, the enzyme directing DSF synthesis.

Many microbial two-component systems with hybrid sensors, including AcrB/ArcA and TorS/TorR of *E. coli* (10, 28), BvgS/BvgA of *Bordetella pertussis* (29), the LuxN(Q)-LuxU-LuxO of *Vibrio harveyi* and *Vibrio cholerae* (30), and the Kin/Spo of *Saccharomyces cerevisiae* (31), adopt a conserved four-step phosphorelay mechanism in signal transduction following signal perception. In most cases, His_1-Asp_1-His_2 is in the sensor and Asp_2 in the response regulator, although in *Vibrio* sp., His_2 is carried by the separate protein LuxO. RpfC contains several functional domains, *i.e.* transmembrane, HK, REC, and HPT with the three essential phosphorelay residues His_1^{198}, Asp_1^{512}, and His_2^{657} located in the HK, REC, and HPT domains, respectively. Substitution of these three key residues with other amino acids or deletion of the HPT domain that contains the critical His_2^{657} residue abrogated the RpfC activity in induction of EPS and virulence factor production. Given these results, we have concluded that RpfC uses the conserved His_1-Asp_1-His_2 phosphorelay mechanism to perceive and transduce environmental signals, which include DSF, with consequent activation of the synthesis of virulence factors.

Our findings also indicate that RpfC modulates DSF biosynthesis by a novel mechanism that is independent of the HPT domain and phosphorelay but involves interaction of the REC domain with RpfF, the key enzyme responsible for DSF synthesis (18). These conclusions depend upon site-directed mutagenesis and deletion analysis of the chromosomal copy of the *rpfC* gene, examination of the effects of in *trans* expression of the REC domain and its truncated variants on DSF synthesis in the RpfC null mutant, and direct evidence of protein-protein interaction by co-immunoprecipitation and far Western analysis. Deletion analysis narrowed down the minimal region required for repression of DSF biosynthesis to a peptide of 107 amino acids. The alanine scanning mutagenesis peptide revealed that three amino acid replacements, Q496A, E504A, and I552A, partially decreased the repressor activity, although most alterations had no effect. Multiple mutations did not further reduce the repressor activity. Q496, E504, and I552 may either be directly involved in the interaction with the RpfF protein or in maintenance of a conformation of the REC domain that promotes that interaction.

Previous quantitative analysis has shown that DSF production in wild-type *X. campestris* pv. *campestris* is growth phase-dependent and is maximal in the late stationary phase (20). In the well characterized quorum sensing systems involving acyl homoserine lactones, signal production is autoregulated; genes within the *luxI* family, which encode for acyl homoserine lactone synthases, are inducible by acyl homoserine lactone signals (32, 33). However, in the DSF quorum sensing system, the transcription of *rpfF* occurs throughout growth and is not influenced by DSF (18, 21). Furthermore, although *rpfC* mutants produce highly elevated levels of DSF, this is accompanied by only modest (up to 2-fold) changes in the level of *rpfF* transcript (17). The elevated DSF production in RpfC null mutants is seen throughout growth, even in the early growth phase where the wild-type *X. campestris* pv. *campestris* signal is low or undetectable (20). These findings, together with the data from this study, suggest a model for the control of virulence factor synthesis and DSF auto-induction by RpfC (Fig. 7). At low cell density or in an unconfined environment, the extracellular concentration of DSF is below a threshold, and autophosphorylation of RpfC is not initiated. Unphosphorylated RpfC adopts a structure that allows binding of RpfF to the REC domain, thus inhibiting DSF synthesis, which remains at a basal level. When the cell density is high or when bacteria enter a confined environment, the level of extracellular DSF increases. Upon reaching a threshold level, DSF binding causes RpfC to autophosphorylate, which results in a conformational change allowing release of RpfF, thus increasing DSF biosynthesis and facilitating the four-step phosphorelay that activates RpfG and the down-stream DSF regulon (Fig. 6*B*). In this manner, auto-induction of DSF could be achieved without substantial elevation in *rpfF* gene transcription. We cannot exclude the possibility that DSF production may also be regulated by substrate availability, which in turn may be negatively influenced by RpfC. However, this putative role of RpfC would also have to be independent of the phosphorelay, because H198A, D512V, and H657A variants all support wild-type levels of DSF.

Although phosphorylation-induced conformational changes in sensor kinases remain to be investigated, such phenomena have been well documented among response regulators (2). The distinct mechanisms of RpfC in modulating different functions have thus presented a new dimension to the conventional

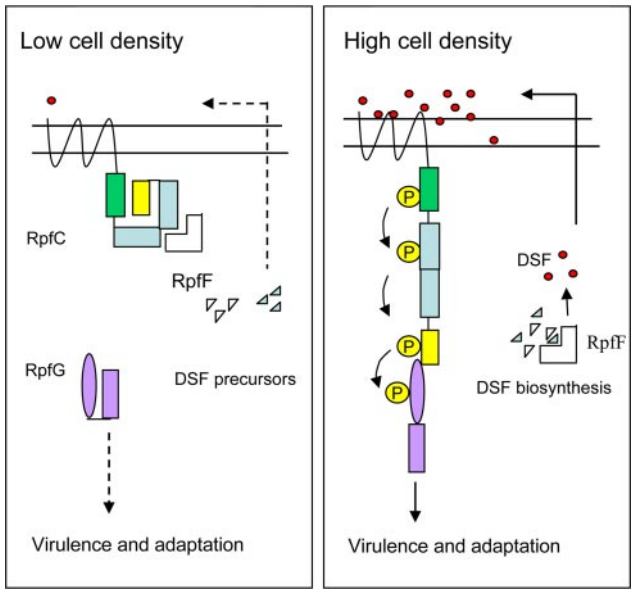


FIGURE 7. **Schematic representation of a model of RpfC in DSF signal perception and signal transduction.** At a low cell density or in an unconfined environment, RpfC maintains a conformation that forms a complex with the DSF synthase RpfF. No phosphor transfer is initiated. *Dashed arrows* indicate no signal flow and basal signal generation. At a high cell density or when bacteria are confined, elevated extracellular DSF levels induce conformational changes in RpfC, which initiate autophosphorylation and phosphorelay to RpfG and release RpfF. The *solid arrows* indicate strong signal flow or signal generation.

two-component signaling paradigms, although it remains to be seen how widespread such mechanisms are.

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