CELL-CELL COMMUNICATION IN *XANTHOMONAS CAMPESTRIS PV. CAMPESTRIS*

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SUMMARY

Xanthomonas campestris pv. *campestris* (*Xcc*) is a major bacterial pathogen of cruciferous plants worldwide. The pathogen produces polysaccharide and extracellular enzymes (including proteases, pectinases and endoglucanase), which are key virulence factors. The production of these factors is regulated by a diffusible signal factor (DSF). However, little is known about the structure and regulatory mechanism of DSF. In this study, genetic, chemical and biochemical, genomic and bioinformatic approaches were used to address these intriguing questions.

DSF bioassay system was established and two DSF overproduction mutants, Xc1853 and Xc4199, were identified from about 7000 *Xcc* strain Xc1 mutants generated by Tn*5* random insertion. DSF was purified and chemically characterized as *cis*-11-methyl-2-dodecenoic acid, which is a novel α,β unsaturated fatty acid. The DSF-like activity was detected in at least 25 strains of 11 bacterial species, suggesting that DSF might be a widely conserved signal.

For investigation of the genomic profiles of DSF regulation, *Xcc* oligomicroarray was designed and analysis was conducted by comparison of the gene expression patterns of wild-type strain Xc1 and its DSF-deficient mutant ΔrpfF, as well as those of the mutant ΔrpfF in the presence or absence of DSF signals. The analyses led to identification of 165 genes, whose expression was significantly influenced by DSF signals. These genes encode proteins and enzymes belonging to at least 12 functional groups. In addition to those previously known DSF-dependent activities such as production of extracellular enzymes and extracellular polysaccharides, microarray

analyses also revealed new functions mediated by DSF, such as flagellum synthesis, resistance to toxins and oxidative stress, and aerobic respiration. Phenotype analyses confirmed that DSF signaling contributed to resistance to toxin acriflavin and hydrogen peroxide, and to the survival of bacterial cells at different temperatures.

The hybrid sensor kinase RpfC positively regulates the expression of a range of virulence genes and negatively modulates the synthesis of quorum sensing signal DSF in *Xcc*. Site-directed mutagenesis showed that the three conserved amino acid residues of RpfC implicated in phosphorelay (H198 in the histidine kinase domain, D512 in the receiver domain and H657 in the histidine phosphotransfer domain) were essential for activation of the production of extracellular enzymes and extracellular polysaccharide (EPS) virulence factors, but not 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-aa and three amino acid residues (Q496, E504 and I552) involved in modulating DSF production. Co-immunoprecipitation and far western blot analyses suggest 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.

RpfG is essential for DSF-dependent virulence factor production. Domain deletion analysis also showed that the HD-GYP domain of RpfG, which is the cognate

response regulator of RpfC, is sufficient for virulence factors production and biofilm dispersion. The finding has facilitated the subsequent demonstration that the HD-GYP domain is a novel cyclic di-GMP phosphodiesterase, which degrades the nucleotide second messenger cyclic di-GMP to generate GMP.

Clp, a conserved global regulator showing a strong homology to the cAMP nucleotide receptor protein Crp of *E. coli*, is essential for DSF regulation of virulence factor production but not for biofilm dispersal. Deletion of *clp* in *Xcc* changed the transcriptional expression of 299 genes including a few encoding transcription factors. Further genetic and microarray analysis led to identification of a homologue of the transcriptional regulator Zur, and a novel TetR-type transcription factor FhrR. These two regulatory factors regulated different sets of genes within Clp-regulon. These results outline a hierarchical signaling network by which DSF modulates different biological functions, and may also provide a clue on how the novel nucleotide signal coupled to its downstream regulatory system.