Intermolecular interactions between HD-GYP and GGDEF domain proteins mediate virulence-related signal transduction in *Xanthomonas campestris*

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In the plant pathogen *Xanthomonas campestris* pv. *campestris* (Xcc) a two component system comprising RpfG and the complex sensor kinase RpfC is implicated in sensing and responding to the cell-cell signaling molecule DSF to positively regulate the synthesis of virulence factors such as extracellular enzymes, biofilm structure and motility. RpfG is a two-component regulator with a CheY-like receiver domain attached to an HD-GYP cyclic di-GMP phosphodiesterase domain. In a recent paper we showed that the physical interaction of RpfG with two proteins with a diguanylate cyclase (GGDEF) domain, acts to control a sub-set of RpfG-regulated virulence functions. These protein-protein interactions required the conserved GYP motif in the HD-GYP domain of RpfG and were dependent on DSF signaling. Here we discuss these findings, considering in particular different scenarios for the role of RpfG in multiple signaling pathways involving cyclic di-GMP that impinge on virulence.

**Introduction**

Bacteria belonging to the related genera *Xanthomonas* and *Xylella* are plant pathogens that cause diseases in many economically important plants in many geographical locations throughout the world. The virulence of these bacteria depends in part on cell-to-cell signaling mediated by the signal molecule DSF (for Diffusible Signaling Factor). DSF from the crucifer pathogen *Xanthomonas campestris* pv. *campestris* (Xcc) has been shown to be the unsaturated fatty acid cis-11-methyl-dodecenoic acid. The synthesis of DSF is dependent on RpfF, which has some amino acid sequence similarity to enoyl CoA hydratases, whereas the two-component system comprising the sensor kinase RpfC and regulator RpfG is implicated in DSF perception and signal transduction. RpfC is a complex sensor kinase with a predicted membrane-associated sensory input domain. RpfG is a regulator with a CheY-like receiver (REC) domain attached to an HD-GYP domain, which acts to degrade the second messenger bis (3',5')-cyclic diguanosine monophosphate (cyclic di-GMP). The function of this Rpf/DSF signaling system has been most extensively studied in Xcc. In this organism, mutation of *rpfF*, *rpfG* and *rpfC* leads to a coordinate reduction in the synthesis of virulence factors, including extracellular protease, endoglucanase and endomannanase enzymes and the extracellular polysaccharide xanthan; reduced pili-dependent motility; alterations in biofilm formation; and a reduction in virulence. Addition of synthetic DSF can restore virulence factor synthesis to *rpfF* mutants but not to Xcc strains with mutations in *rpfG* or *rpfC*. These observations together are consistent with the involvement of RpfC/RpfG in perception and transduction of the DSF signal in Xcc.

The role of RpfG (and the HD-GYP domain) in DSF signal transduction is still relatively poorly understood. A consensus sequence of the HD-GYP domain from alignment of over 200 proteins indicated that the signature HD and GYP...
motifs might be more usefully considered as part of the larger motifs HDxGK and HHExxDGxGYP which are present in sub-domains separated by a region of high sequence diversity (Fig. 1). In addition there are a number of other well-conserved charged and hydrophobic residues. We have shown previously that the HD diad of the HD-GYP domain is required for both the enzymatic activity against cyclic di-GMP and for the regulation of synthesis of virulence factors. It has been proposed that other conserved H and D residues (i.e., those not in the HD diad) have a role in metal binding in these metal-dependent enzymes and that the GYP motif has a role in determining substrate specificity but currently there is no experimental evidence.

In addition to the HD-GYP domain, two other protein domains are implicated in the synthesis or degradation of cyclic di-GMP; the GGDEF domain is a diguanylate cyclase (DGC) involved in cyclic di-GMP synthesis whereas the EAL domain is a second cyclic di-GMP phosphodiesterase (PDE). In common with many bacteria, the genome of Xcc encodes multiple proteins with GGDEF, EAL or HD-GYP domains. Many of these proteins contain additional signaling domains, suggesting that their role is to transduce perception of environmental signals or cues into alterations in cyclic di-GMP levels. This raises the issue of the functional organization of these signaling systems; are they part of interactive regulatory networks or are they dedicated to specific cellular tasks? A related question is whether cyclic di-GMP exists as a general pool, as a set of discrete localized pools or as both.

The work discussed here addressed aspects of both the structure-function of the HD-GYP domain and the functional organization of different cyclic di-GMP signaling systems within the same organism. The background to the experiments was a yeast two-hybrid analysis that showed that the HD-GYP domain of RpfG of the citrus canker pathogen X. axonopodis pv. citri interacts with a subset of GGDEF domain-containing proteins. This posed the question of the relevance of RpfG interaction with GGDEF domain-containing proteins for DSF/Rpf regulated virulence functions. Specifically, these interactions influence pilus-dependent motility, but have no effect on other virulence-associated processes such as synthesis of extracellular enzymes or biofilm formation. The interaction of RpfG with these GGDEF domain proteins requires the GYP motif within the HD-GYP domain. RpfG regulation of the synthesis of extracellular enzymes and biofilm formation is by contrast not dependent on the GYP motif. Consistent with these observations, double mutation of XC_0249 and XC_0420 (but not single mutation) leads to a reduction in motility, but has no effect on synthesis of extracellular enzymes or biofilm formation.

Intermolecular interactions of RpfG with XC_0249 and XC_0420 increase during DSF signaling (as measured by FRET), and correlate with the re-localization of RpfG to the cell poles, where the DSF sensor kinase RpfC is located. The available data suggest that this cellular localization of RpfG is dependent on the REC domain, since the HD-GYP domain alone is not localized within the bacterial cell. On the basis of these findings, we proposed that the localization of RpfG in response to DSF is associated with the sensing of the signal by RpfC, which triggers autophosphorylation of the sensor and subsequent phosphotransfer to the REC domain of RpfG. For many two-component regulators, interactions between the unphosphorylated receiver domain and the effector domain prevent effector domain activity by restricting it in an unfavorable conformation; phosphorylation of the REC domain relievers this inhibition. Accordingly, we proposed that in RpfG, the unphosphorylated REC domain negatively influences the ability of the HD-GYP domain to interact with GGDEF domain proteins and the encoding the ‘target’ GGDEF domain proteins. In parallel, protein-protein interactions in Xcc were directly examined by fluorescence resonance energy transfer (FRET) analysis and protein localization was examined using GFP fusions. Our findings indicated that physical interactions between RpfG and two GGDEF domain proteins, XC_0249 and XC_0420 occur within the Xcc cell where they act to regulate a subset of DSF/Rpf regulated virulence functions. Specifically, these interactions influence pilus-dependent motility, but have no effect on other virulence-associated processes such as synthesis of extracellular enzymes or biofilm formation. The interaction of RpfG with these GGDEF domain proteins requires the GYP motif within the HD-GYP domain. RpfG regulation of the synthesis of extracellular enzymes and biofilm formation is by contrast not dependent on the GYP motif. Consistent with these observations, double mutation of XC_0249 and XC_0420 (but not single mutation) leads to a reduction in motility, but has no effect on synthesis of extracellular enzymes or biofilm formation.

See Figure 1 for an illustration of conservation of amino acid residues and motifs within the HD-GYP domain.
enzymatic activity against cyclic di-GMP. In this model, sensing of DSF would lead to phosphorylation of the REC domain, thus relieving this inhibition and promoting the physical interaction with GGDEF domain proteins. Phosphorylation of the REC domain would also lead to enhanced PDE activity of RpfG.

**Protein-protein Interactions and the Regulation of Specific Phenotypes**

A theme from the body of work on cyclic di-GMP signaling in a number of bacteria is that higher levels of the nucleotide promote biofilm formation and sessility whereas lower levels promote motility. In contrast to this generality, XC_0249 and XC_0420 are active DGCs but act to positively regulate motility. Such divergences from the general correlation between enzymatic activities of specific cyclic di-GMP signaling proteins and their regulatory functions taken together with the identification of dedicated tasks for particular signaling proteins have led to the concept of the existence of discrete pools of cyclic di-GMP that may be generated and act in a highly localized fashion. The interaction of RpfG with XC_0249 and XC_0420 may lead to alteration in the level of such a localized pool of cyclic di-GMP with consequences for the regulation of motility. These effects may occur through the juxtaposition of PDE and DGC enzymes with opposite activities in cyclic di-GMP turnover, although the possibility that the physical interaction serves to inhibit the DGC activity of the two GGDEF proteins and/or to stimulate the PDE activity of RpfG should not be excluded. The effect of RpfG on extracellular enzyme synthesis and biofilm formation may be exerted through influences on a different pool(s) of the nucleotide (Fig. 2). Intriguingly, recent findings have implicated the cyclic-AMP receptor-like protein Clp as an element responsible for linking Rpf/DSF signaling (and alteration in cyclic di-GMP) to the expression genes encoding extracellular enzymes. DSF signaling has been shown to activate transcription of the clp gene, although the mechanism remains obscure. Furthermore, Clp has been shown to bind cyclic di-GMP at physiologically relevant levels to negatively influence binding of the transcriptional activator to the promoter of the engXCA gene, which encodes the major endoglucanase (Fig. 2). In Xcc, the Rpf/DSF system influences biofilm formation as well as the synthesis of extracellular enzymes and motility. Clp regulates many functions in the DSF ‘regulon’ in addition to the expression of genes encoding extracellular enzymes but is not apparently involved in regulation of biofilm dynamics. Similarly XC_0249 and XC_0420 influence motility but have no apparent effect on biofilm formation. These findings may indicate the existence of yet further cyclic di-GMP-mediated pathways that influence multicellular behavior (Fig. 2).

An alternative scenario that is also consistent with the data does not invoke the existence of discrete pools of cyclic di-GMP influenced by RpfG but instead envisages that the same cellular pool of cyclic di-GMP controls both extracellular enzyme synthesis and motility but that the different systems have different threshold levels for activation. In this view, the protein-protein interaction modulates the PDE activity of RpfG such that the cellular level of cyclic di-GMP is low enough to allow both motility and extracellular enzyme synthesis. In the absence of interaction, RpfG is less active and the cellular level of cyclic di-GMP is higher, sufficient to inhibit motility but still allow extracellular enzyme synthesis. It is noteworthy that alanine substitution of the HD diad does not allow either motility or extracellular enzyme synthesis to occur but the physical interaction of the AA-GYP variant of RpfG with either of the GGDEF domain protein ‘targets’ is unaltered. In other words, the physical interaction per se is not sufficient to drive motility, which also requires low cellular levels of the nucleotide. In summary, interactions between RpfG and specific GGDEF domain proteins could set the level of a cellular pool of cyclic di-GMP so that...
E. coli regulates bacterial swimming velocity in elegant work showing that cyclic di-GMP.

Three very recent reports have described the regulation of swimming motility in Escherichia coli of the flagellar motor slow the rotation of the flagellar motor, leading to reduced swimming velocity. A network of five DGCs is responsible for the generation of the cyclic di-GMP that exerts this effect during bacterial starvation, whereas the PDE YhjH prevents binding of this transcriptional activator to the promoters of regulated genes. Differences in the affinity for cyclic di-GMP of regulators influencing motility and extracellular enzyme synthesis (to include Clp) could conceivably account for different threshold levels of the nucleotide required to influence these traits.

**Broad Relevance of the Findings**

The findings expand our knowledge of the HD-GYP domain and the potential multiple roles of this class of regulator in signal transduction. The occurrence of a second PDE unrelated to the EAL domain raises the question of why such an alternative activity arose. In the context of the experiments described here, it is of interest to note that in yeast two-hybrid experiments, the HD-GYP domain of RpfG interacts with a subset of GGDEF domain proteins and not with those proteins with both a GGDEF and EAL domain. Furthermore, no interactions were detected when "non-target" GGDEF domains were used as prey in yeast two-hybrid experiments or probed by Far-western analysis. This raises the possibility that the HD-GYP domain evolved to regulate the action of specific GGDEF domain proteins through dynamic interactions driven by environmental cues or signals. Along the same lines, proteins with an EAL domain may also interact with a sub-set of GGDEF domain proteins, although there is as yet no experimental evidence for this. It is tempting to speculate that in some cases, transient interactions between certain GGDEF and EAL domain proteins evolved to produce a permanent attachment of the two domains in the GGDEF-EAL domain proteins. Notably, a small number of proteins that contain both GGDEF and HD-GYP domains have been described.

**Future Prospective**

It is evident that a real understanding of the molecular mechanism of signal transduction via RpfG in Xcc and further insight into the role of the HD-GYP domain will require answers to a number of questions. What effects do protein-protein interaction between RpfG and GGDEF domain proteins have on the enzymatic activities of all protein partners involved? What residues within the GGDEF domain are responsible for interaction with the HD-GYP domain? Do other residues of the HD-GYP domain such as those within the more extended motif HHExxDGxGYP have a role in interaction? How do RpfG-GGDEF domain interactions influence motility? Determination of the structure of RpfG and of the HD-GYP domain both alone and particularly in combination with ‘target’ GGDEF domains may offer information on the interacting surfaces and insight into the molecular basis of any alteration of enzyme activity that may occur upon interaction. Further yeast two-hybrid analysis using the GGDEF domain proteins as ‘bait’ in conjunction with FRET analysis may identify proteins that link or transduce the effects of RpfG-GGDEF interaction to the apparatus for pilus-dependent motility. The results of such efforts should contribute to our understanding of the functional organization of cyclic di-GMP signaling systems in bacteria as well as identifying key steps for interference in expression of virulence determinants or biofilm formation, with consequences for strategies for disease control.

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