AN ANALYSIS OF C-DI-GMP SIGNALLING IN *XYLELLA FASTIDIOSA* VIRULENCE

Project Leaders:
David Appel
Dept. of Plant Pathology & Microbiology
Texas A&M University
appel@ag.tamu.edu

Paul de Figueiredo
Dept. of Plant Pathology & Microbiol.
Texas A&M University
pjdefigueiredo@tamu.edu

Researchers:
Shiping Wei
Dept. of Plant Pathology & Microbiol.
Texas A&M University

Tom Kurdyla
Dept. of Plant Pathology & Microbiol.
Texas A&M University

Reporting Period: The results reported here are from work conducted April 24, 2007 to October 9, 2007.

ABSTRACT

Pierce's disease (PD) poses a grave threat to many commercially important plants, including grapevine, and has placed the wine industries of Texas, California and other states at risk. Although *Xylella fastidiosa* (*Xf*) is recognized as the causal agent of the disease, the mechanism by which this xylem-limited, insect-transmitted bacterium induces sickness in plants remains almost completely unknown. Here, we present results from experiments that explore the role that cyclic diguanylate (c-di-GMP), a small regulatory molecule produced by many human and plant bacterial pathogens, plays in regulating *Xf* virulence. Specifically, we describe our efforts: (1) To generate *Xf* strains harboring deletions in genes encoding putative c-di-GMP signaling proteins; (2) To examine the effects of c-di-GMP on *Xf* gene expression; (3) To show that *Xf* biofilm formation *in vitro* is inhibited by c-di-GMP treatment. Taken together, these findings enhance our understanding of the molecular mechanisms mediating *Xf* virulence, and thereby, provide new insights into controlling PD.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a Gram-negative non-flagellated bacterium that causes a number of economically important plant diseases, including PD of grapevine, citrus variegated chlorosis, pear leaf scorch, and almond leaf scorch (Purcell and Hopkins 1996). Disease symptoms occur as a result of water stress and nutritional deficiencies caused by blockage of xylem vessels by bacterial biofilms. The bacteria are transmitted to plants by xylem-feeding insect vectors, such as glassy-winged and blue-green sharpshooters. To date, the molecular virulence mechanisms of *Xf*, as well as how it interacts with plant hosts, remain obscure. Our project focuses on exploring the role that the putative c-di-GMP signaling system in *Xf* genes plays in mediating these events.

Cyclic dinucleotide bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger that regulates cell-cell signaling, biofilm formation, motility, differentiation, and virulence (Tamayo et al 2007). High concentrations of c-di-GMP are associated with biofilm formation, exopolysaccharide production, attachment to surfaces, and attenuation of motility and virulence. c-di-GMP is produced from two molecules of GTP by diguanylate cyclase enzymes (DGCs). DGC activity resides in the GGDEF (Gly-Gly-Asp-Glu-Phe) domain of these proteins. c-di–GMP is degraded to GMP, via the linear pGpG by phosphodiesterases (PDEs). PDE activity has been show to reside proteins containing EAL (Glu-Ala-Leu) or HD-GYP (His-Asp, Gly-Tyr-Pro) domains. The interplay of DGC and PDE activities controls intracellular c-di-GMP concentration and hence c-di-GMP signaling. GGDEF, EAL, and HD-GYP domain containing proteins have been described in several bacterial species, and have generated significant interest as targets for the possible control of bacterial pathogenesis. In addition, a PilZ domain, which is thought to possess c-di-GMP binding activity, has recently been described (Amikam and Galperin 2006, Ryjenkov et al. 2006). Upon c-di-GMP binding, PilZ containing proteins are believed to regulate downstream events, including biofilm formation, motility and virulence (Cotter1 and Stibitz 2007). We are exploring the possibility that proteins participating in the c-di-GMP signaling system may play a role in regulating *Xf* virulence, and thereby, enable a strategy for Pierce's disease control.

OBJECTIVES

1. To examine whether c-di-GMP suppresses *Xf* biofilm formation in *vitro*, and disease progression *in planta*.
2. To examine whether putative *Xf* c-di-GMP biosynthesis and catabolic genes regulate bacterial biofilm formation in culture and disease *in planta*.

RESULTS

Cyclic di-GMP signaling proteins in *Xf.* Several GGDEF, EAL and HD-GYP domain containing proteins, which are implicated in c-di-GMP signaling, were identified in the genome of *Xf* Temecula strain. BLAST analysis revealed six proteins containing GGDEF domains, EAL domains and/or HD-GYP domains. Of the six proteins, two contained only a single GGDEF domain, one contained only a single EAL domain, two contained both a GGDEF and EAL domain, and one contained a single HD-GYP domain (Figure 1). The BLAST analysis also revealed that the proteins with GGDEF, EAL and HD-GYP domains contained additional signaling domains, including REC, conserved transmembrane regions (e.g., DUF21, 7TMR-DISMED2 and 7TMR-DISM_7TM), and a response regulator containing CheY-like receiver domain (COG3437), which senses and responds to environmental cues. Multiple proteins with PilZ domains, which are candidate receptors for c-

di-GMP, have been identified in several bacteria (Amikam and Galperin 2006, Ryjenkov et al 2006). BLAST analysis revealed two proteins with PilZ domains (PD1497 and PD0726) in the *Xf* Temecula genome.

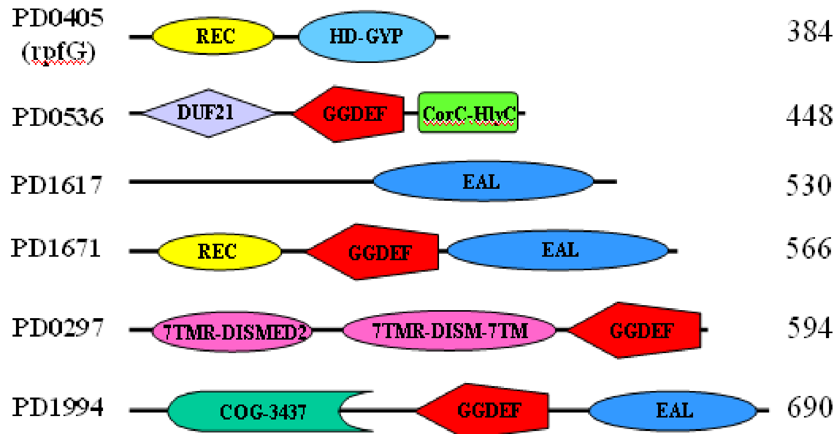

Figure 1. Architectures of GGDEF, EAL and HD-GYP domain proteins coded by the *Xylella fastidiosa* Temecula genome. The protein codes are shown to the left, and the number of amino acids in each protein number is shown to the right.

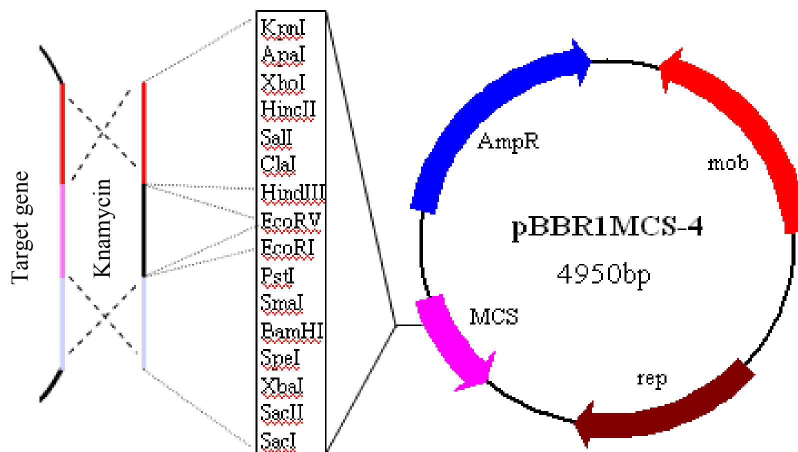

Figure 2. Construction of gene deletion vectors and schematic of double cross over strategy for the generation of gene deletions.

Generation of targeted gene deletion mutants of *Xf* Temecula. A replicative plasmid in *Xf*, pBBR1MCS-4, was used to construct several gene deletion vectors. The gene deletion vectors, p118LKR, p384LKR, p448LKR, p530LKR, p566LKR, p594LKR and p690LKR, have been constructed for deleting PD1497, PD0405, PD0536, PD1617, PD1671, PD0297 and PD1994 respectively. These vectors have been transformed into *Xf* (Figure 2). The transformation efficiency is about 30 transformants per microgram of plasmid DNA. The identification of knock-out mutants by PCR and Southern analysis is underway.

c-di-GMP suppression of biofilm formation in *vitro*. Bacterial biofilm formation is thought to play an important role in *Xf* pathogenesis and Pierce's Disease. To investigate the effect of c-di-GMP on biofilm formation, we treated bacterial cells with concentrations of c-di-GMP that have been shown to affect biofilm formation and virulence in other bacterial pathogens, including *Vibrio cholerae* and *Pseudomonas aeruginosa* (Tischler and Camilli 2005, Kulasakara et al 2006). We then quantified biofilm formation using the crystal violet method (Karaolis et al 2005). When cells were grown in PD3 medium for 10 days in the absence of c-di-GMP, significant amounts of biofilm formation was observed (our unpublished data). However, when cells were similarly grown in the presence of c-di-GMP, a significant decrease in biofilm formation was seen (our unpublished data). Our preliminary data therefore indicate that bacterial c-di-GMP synthesis and signaling may contribute to *Xf* biofilm formation.

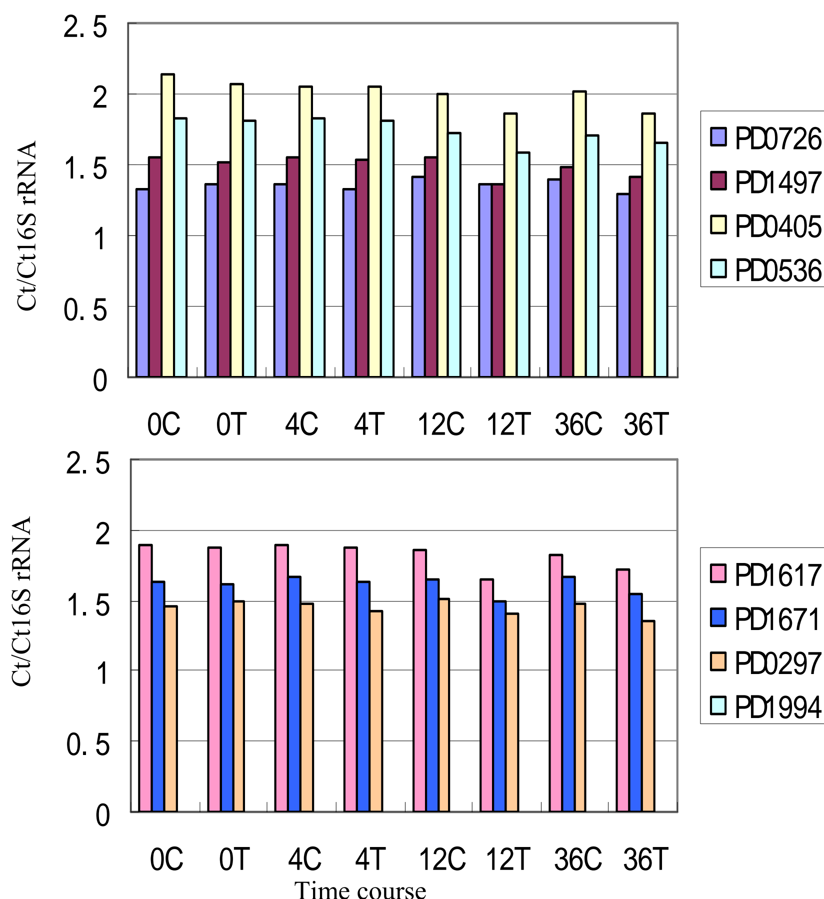

Figure 3. Expression analysis of the genes coding GGEDF, EAL, HD-GYP, PliZ domain proteins in response to c-di-GMP treatment.

Expression analysis of c-di-GMP pathway genes. To examine whether *Xf* gene expression changes in response to c-di-GMP treatment, we treated bacterial cells with c-di-GMP and then analyzed gene expression levels at different times post-treatment using real-time PCR methods. Eight genes (PD0726, PD1497, PD0405, PD0536, PD1617, PD1671, PD0297 and PD1994) that participate in the putative *Xf* c-di-GMP signaling pathway were analyzed, and revealed that the expression of these genes is not significantly altered by the tested concentrations of c-di-GMP (Figure 3).

CONCLUSIONS

We have initiated an effort to generate targeted deletions in genes contained in the putative *Xf* c-di-GMP pathway. In addition, we have shown that c-di-GMP addition to *Xf* liquid cultures inhibits bacterial biofilm formation. Finally, we have demonstrated that c-di-GMP treatment of *Xf* cultures does not alter the expression of genes contained in the c-di-GMP pathway. Taken together, these experiments provide a solid foundation for research into the role that the *Xf* c-di-GMP signaling system plays in mediating Pierce's disease.

REFERENCES

Purcell, A. H. and D. L. Hopkins 1996. Fastidious xylem-limited bacterial plant pathogens. Annu. Rev. Phytopathol. 34:131-151.

Tischler, A. D. and A, Camilli 2005. Cyclic diguanylate regulates *Vibrio cholerae* virulence gene expression. Infect. Immun. 73:5873–5882.

Karaolis, D. K. R., M. H. Rashid, R. Chythanya, W. Luo, M. Hyodo, and Y. Hayakawa 2005. c-di-GMP (3'-5'-Cyclic Diguanylic Acid) inhibits *Staphylococcus aureus* cell-cell interactions and biofilm formation. Antimicrobial Agents and Chemotherapy 45:1029–1038.

Ryjenkov, D. A., R. Slmm, U. Romling and M. Gomelsky 2006. The PilZ domain is a receptor for the second messenger c-di-GMP. Journal of Biological Chemistry. 281:30310-30314.

Dorit Amikam and Michael Y. Galperin 2006. PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinformatics 22:3-6.

Kulasakara, H., V. Lee, A. Brencic, N. Liberati, J. Urbach, S. Miyata, D. G. Lee, A. N. Neely, M. Hyodo, Y. Hayakawa, F. M. Ausubel, and S. Lory 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. Proc. Natl. Acad. Sci. USA 103:2839–2844.
Cotter1, P. A. and S. Stibitz 2007. c-di-GMP-mediated regulation of virulence and biofilm formation. Current Opinion in Microbiology 10:17–23.
Tamayo, R., J. T. Pratt, and A. Camilli 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu. Rev. Microbiol. 61:131–48.

FUNDING AGENCIES

Funding for this project was provided by a Mexican American and U.S. Latino Research (MALRC) Fellowship Grant and a Ralph E. Powe Junior Faculty Enhancement Award to P.dF, and a USDA APHIS award to D.A. and P.dF. The Texas A&M Experiment Station and Texas A&M University provided P.dF. with additional funding and resources in support of this research.

Additional note: Dr. Carlos Gonzalez provided critical assistance in culturing *Xf* (Temecula) and Ms. Veronica Ancona provided valuable assistance with the real-time PCR experiments.