CHARACTERIZATION OF REGULATORY PATHWAYS IN *XYLELLA FASTIDIOSA*: GENES AND PHENOTYPES CONTROLLED BY GacA

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Reporting Period: The results reported here are from work conducted October 2006 to September 2007.

ABSTRACT
We are pursuing a strategy to identify traits important in virulence of *Xylella fastidiosa* (*Xf*) through the mutagenesis of "global" regulatory genes, which are known to broadly regulate virulence functions in other microbes. In addition to phenotypic characterization of such mutants, we are using whole-genome microarrays to identify which genes are regulated by these global regulators and examine these genes as putative virulence factors. One such global regulatory gene, *gacA*, controls various physiological processes and pathogenicity factors in many gram negative bacteria, including biofilm formation in *P. syringae* pv. *tomato* (*Pst*) DC3000. Cloned *gacA* of *Xf* was found to restore the hypersensitive response and pathogenicity in *gacA* mutants of *Pst* and *E. amylovora* (*Ea*). An *Xf gacA* mutant (DAC1984) had significantly reduced abilities to adhere to a glass surface, form biofilm, and incite disease symptoms on grapevines compared to the parent A05. cDNA microarray analysis identified seven genes positively regulated by GacA, including genes encoding putative outer membrane proteins XadA and Hsf, and 20 negatively-regulated genes including *gumC* and *cvaC*, predicted to encode an antibacterial toxin. These results suggest that GacA of *Xf* regulates many factors, which contribute to attachment and biofilm formation, as well as some physiological processes that may enhance the adaptation and tolerance of *Xf* to environmental stresses and the competition within the host xylem.

INTRODUCTION
The xylem-limited, insect-transmitted bacterium *Xylella fastidiosa* (*Xf*) causes Pierce's disease in grapes through cell aggregation and vascular clogging. Pathogenic bacteria use gene regulatory mechanisms to rapidly respond to and survive in changing environments (Storz and Hengge-Aronis, 2000). Inside the plant's xylem, *Xf* is exposed to a range of variable stress factors, such as changes in osmolarity, availability of nutrients, and agents generating reactive oxygen intermediates (Alves et al., 2004). To ensure survival, *Xf* may respond to these stress situations via specific regulatory mechanisms involving specific regulatory genes. We have previously reported the role in virulence of the regulatory gene, *algU*, and identified a number of genes regulated by AlgU through microarray analysis of an *algU* mutant (Shi et al, 2007). Among other regulators identified in pathogenic and environmental bacteria, the GacS and GacA regulators are involved in sensing environmental cues and signals (Heeb and Haas, 2001). In this system, hypothetically, GacS is a putative sensor kinase that perceives a signal or environmental cues, and GacA is a response regulator, which functions as the transcriptional activator of one or more genes. Genes controlled by GacA include those regulating pathogenicity factors, quorum sensing, and toxins, and also genes involved in motility, biofilm formation, extracellular polysaccharides (EPS) in a wide range of pathogenic bacteria including *P. syringae, E. carotovora* and *P. aeruginosa* (Chatterjee, 2003; Cui, 2001; Parkins, 2001). The high similarity between *gacA* of *Xf* (designed as $gacA_{Xf}$) and *gacA* of *P. syringae* (designed as $gacA_{DC3000}$) suggests that, like $gacA_{DC3000}$, $gacA_{Xf}$ may regulate pathogenicity of *Xf* by acting as a global regulator during infection and the process of disease development. Interestingly, while a *gacA* homolog was identified in *Xf*, a *gacS* homolog was not found, which suggests that there may be specific regulatory roles for *gacA* in *Xf* (Simpson et al., 2000). However, the role of *gacA* and its regulation in *Xf* is unknown. In this study, we cloned and characterized $gacA_{Xf}$ and analyzed the effect of a *gacA* deletion of *Xf* (DAC1984). We also performed whole-genome microarray analysis of gene expression in the mutant in comparison with the parent and identified genes whose expression *in vitro* is controlled by GacA. Functional studies of global regulatory genes in *Xf* should identify the specific regulatory pathways and roles of specific virulence factors in disease development, which in turn, could reveal the pathogenicity mechanisms of the bacteria in responding to, adapting, and surviving in the stress environment of xylem.

OBJECTIVES
1. Conduct DNA microarray analysis of gene expression patterns in regulatory mutants of *Xf.*
2. Characterize mutants in regulatory genes and genes that they regulate for changes in virulence and other phenotypes.

RESULTS
Effects of $gacA_{xf}$ on the hypersensitive response (HR) and pathogenicity
An alignment of the predicted amino acid sequences of GacA protein from *Xf* and *Pst* DC3000 showed that the sequences are 211aa in length and are 43% identical and 69% similar overall (data now shown). In the HR and pathogenicity test experiments, *Pst*AC81 and *Pst*AC812 elicited typical HR in tobacco (*Nicotiana tabacum* L. cv. Samsun), whereas water control, *Pst*AC811 and *Pst*AC813 did not (Figure 1, left, A), confirming that it is $gacA_{xf}$ that restores the elicitation of HR in

tobacco. *Ea*EC19 and *Ea*EC192 produced disease symptoms in African violet leaves. In contrast, *Ea*EC191 and*Ea*EC193 failed to produce disease symptom (Figure 1 Left, B), suggesting that $gacA_{xf}$ restores the ability of *Ea*EC192 to cause disease in African violet leaves. This demonstrated that $gacA_{xf}$ can complement *gacA* deficiencies of *P. syringae* and *E. amylovora* in the hypersensitive response and pathogenicity.

Figure 1. Left. Complementation of *gacA* function by $gacA_{xf}$ in *gacA*-deficient mutants of *P. syringae* pv. *tomato* DC3000 (*Pst*) and *E. amylovora* (*Ea*). **A.** Effect of $gacA_{xf}$ in *Pst*AC811 on the elicitation of the hypersensitive response (HR) in tobacco leaf (*Nicotiana tabacum* L. cv. Samsun). Leaf panels were infiltrated with bacterial cells. Site 1, *P. syringae* pv. tomato DC3000 (*Pst*AC81) (1×10^7 CFU/ml); site 2, DC3000 $gacA_{DC3000}^-$ (*Pst*AC811) (1×10^7); site 3, $gacA_{DC3000}^-$ carrying $gacA_{xf}$ (*Pst*AC812) (5×10^7); site 4, $gacA_{DC3000}^-$ carrying pCPP47 (*Pst*AC813) (5×10^7); and site 5, water. **B.** Disease symptoms caused by *E. amylovora* and its $gacA^-$ mutant in Africa violet. Site 1, *E. amylovora* (*Ea*EC19); site 2, *E. amylovora* $GacA^-$ mutant (*Ea*EC191); site 3, *E. amylovora* $gacA^-$ mutant carrying $gacA_{xf}$ (*Ea*EC192) (5×10^7); site 4, *E. amylovora* $GacA^-$ mutant carrying pCPP47 (*Ea*EC193) (5×10^7). **Right**. The ability of DAC1984 to adhere to a glass surface was reduced.

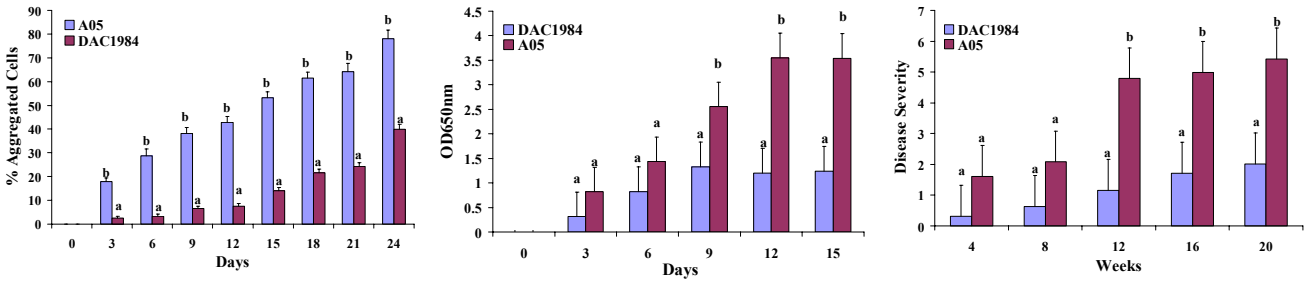

Figure 2. Cell-to-cell aggregation, biofilm formation and virulence of *Xf* DAC1984 and A05. **Left.** Quantitative assessment of *Xf* A05 or DAC1984 cell-to-cell aggregation. Student's *t* test, $p < 0.05$. **Middle**. DAC1984 had reduced ability to form biofilm. Student's *t* test, $p < 0.05$. **Right**: Pierce's disease progression of grapevines inoculated with *Xf* A05 and its *gacA*-deficent derivative DAC1984. Disease severity was based on a visual disease scale of 0 to 5 and was assessed 4, 8, 12, 16, and 20 weeks after inoculation. Student's *t* test, $p < 0.05$. The data are an average of 10 independent replications.

Characterization of a Δ*gacA::Gm* mutant

The replacement of the *gacA* ORF by a gentamycin cassette (Gm) in the genome of the Δ*gacA::Gm* mutant (DAC1984) was confirmed by electrophoresis (data not shown). Sequence analysis indicated that Gm physically replaced most of the *gacA* ORF, from 25 bp downstream from the ATG start codon to 35 bp upstream of the TGA terminal codon of the *gacA* ORF. After streaking five to eight times on PD3 Gelrite medium with 10 µg/ml gentamycin, DAC1984 still grew well and was indistinguishable from the parent, indicating that the mutant is genetically stable. RT-PCR analysis showed that there was no expression of *gacA* within DAC1984 cells, but strong expression was detected in wild-type A05 cells (data not shown). *In vitro* growth curves of *Xf* A05 and DAC1984 over 21 days were similar (data not shown), indicating that mutation of the *gacA* gene did not affect the growth pattern of *Xf* in culture. As is typical of *Xf*, cells of A05 attached to the surface of the flasks and formed wide rings, but while DAC1984 cells attached to the surface formed rather light rings (Figure 1, Right). An optical density assay was used to quantify the effect of DAC1984 on cell-to-cell aggregation and showed that the percentage of aggregated cells of DAC1984 was significantly lower than that of A05 (Figure 2, Left). The ability of

DAC1984 to form biofilm was investigated further by a crystal violet staining method. *Xf* A05 formed more biofilm in PD3 broth than did DAC1984 (Figure 2, Middle).

Virulence assay

Grapevines inoculated with the *Xf* DAC1984 mutant developed less severe disease than did those inoculated with the wild type strain 12-20 weeks after inoculation (Figure 2). Grapevines inoculated with DAC1984 showed i) later symptom development, ii) slower disease progression over a period of 20 weeks, and iii) late appearance of leaf scorching, in comparison with the wild type. Bacterial populations at 25cm and 50cm above inoculation points were estimated from ELISA assays by comparing the OD at 600nm with that of positive control *Xf* concentrations (data not shown). The cell populations of the DAC1984 were less than that of the wild-type, indicating that *gacA* affects the growth and possibly the movement of *Xf* inside the xylem resulting in reduced pathogenicity.

Table 1. Genes differentially expressed in *Xf* Δ*gacA::Gm* mutant (DAC1984) *in vitro*

ORF	Gene[c]	Description	Mutant /Wild type	Signfig.[b]	The expression in mutant
PD0264	*oprO*	porin O precursor.	0.24	*	lower
PD1926		pilus assembly fimbrial protein	0.12	*	lower
PD1294	*actP*	acetate permea-cotranscribed with the acs gene	0.337	*	lower
PD2039		Oxidoreductase-Putative multicopper oxidases	2.75	*	higher
PD1688	*bioI*	cytochrome P450-like enzyme	2.45	*	higher
PD1703		Conserved Domains: Lysophospholipase	6.31	*	higher
PD1702		Conserved Domains: LIP, Secretory lipase	5.29	*	higher
PD0215	*cvaC*	colicin V precursor-antibacterial polypeptides toxin	3.63	*	higher
PD0216	*cvaC*[d]	colicin V precursor-antibacterial polypeptides toxin	0.51	*	lower
PD0731	*XadA*	outer membrane protein-Autotransporter adhesin	8.93	*	lower
PD0744	*hsf*	surface protein-Autotransporter adhesin	3.24	*	lower
PD1395	*gumC*	protein involved in exopolysaccharide biosynthesis	3.17	*	higher
PD0243		Conserved Domains-Membrane-fusion protein	3.01	*	higher
PD0244		Conserved Domains-acriflavin resistance protein	2.7	*	higher
PD0956		V8-like Glu-specific endopeptidase	4.77	*	higher
PD1299		Conserved Domains-polyvinylalcohol dehydrogenase	6.18	*	higher
PD1295		putative membrane protein-unknown function	0.20	*	lower
PD0521		unknown	3.24	*	higher
PD0657		unknown	3.28	*	higher
PD0743		unknown	6.62	*	higher
PD0955		unknown	2.67	*	higher
PD0911		phage-related proteins, unknown function	6.04	*	higher
PD0912		phage-related proteins, unknown function	8.41	*	higher
PD0917		phage-related proteins, unknown function	3.94	*	higher
PD0924		phage-related proteins, unknown function	6.39	*	higher
PD0925		phage-related proteins, unknown function	9.12	*	higher
PD0930		phage-related proteins, unknown function	3.96	*	higher

[a] Hybridization signal intensity obtained with mutant was divided by that from wild-type in order to obtain the M/W ratio. [b] Based on standard deviation calculations, genes having $\geq$ 1.5 or $\leq$ 0.66 final M/W ratios were selected as statistically significant up-regulated or down-regulated genes, respectively. Student's *t*-test, $p < 0.001$. [c] Genes were detected based on *Xf* Temecula genomic sequences at the NCBI site. [d] Currently annotated as colicin V precursor proteins.

DNA microarray analysis of gene expression *in vitro*

RNA was prepared from DAC1984 and wild type A05, and cDNA was synthesized to hybridize to a genomic DNA microarray from NimbleGen Systems. This oligo-based, high-density microarray contains multiple probes for every gene found in the Pierce's disease strain sequence as well as those unique to the CVC strain sequence. Expression levels of 2188 genes between wild type and were analyzed. Twenty seven genes were differentially expressed in DAC1984 compared to A05 (Table 1). Differential expression of *hsf, gumC, cvaC* (PD0216), fimbrial protein (PD1926), and PD1295 were validated by RT-PCR (data not shown). Genes involved in surface structures and attachment components, such as fimbrial proteins, PD1926, *Hsf,* and *XadA*, were positively regulated, and *GumC* was negatively regulated by GacA in *Xf*. *hsf* (PD0744) has a high similarity to the *hsf* adhesin gene of *Haemophilus influenza* (St Geme et al., 1996), and *XadA* encodes a putative afimbrial outer membrane protein adhesion (Simpson et al., 2000). The expression of *hsf* and *XadA* were decreased in DAC1984, likely contributing to a reduced ability to adhere to xylem cell walls. GumC may be responsible for gum

polymerization or secretion through the membrane of *Xf* (Vojnov et al., 1998; de Pieri et al., 2004). *gumC* was negatively regulated by GacA *in vitro*, but is likely to be expressed *in planta*. Genes involved in cell transport and physiological metabolism, such as *oprO* and *actP*, were positively regulated, and an oxidoreductase and *bioI* were negatively regulated by GacA in *Xf*. Culture-independent analysis of bacterial populations inside plants in relation to *Xf* suggest that bacterial endophytic population are much more diverse than previously realized (Araujo et al., 2002; Cooksey and Borneman, 2005). Colicin V is an antibacterial polypeptide toxin produced by *E. coli*, which acts against closely related sensitive bacteria (Havarstein et al., 1994). *cva*C may play a role for *Xf* in competing with indigenous microbes to survive in the poor nutriment xylem environment.

CONCLUSIONS

Attachment and biofilm formation should be key mechanisms by which *Xf* persists in plants. It is hypothesized that there are different steps for attachment to xylem vessels and biofilm formation (de Souza et al., 2004). Previously, we showed that mutation of the regulatory gene *algU* resulted in decreased attachment, decreased biofilm formation, and decreased virulence (Shi et al., 2007). Similar results were obtained here with deletion of *gacA*. However, of the many genes found to be regulated by these two regulatory genes, only four were regulated by both. PD0216, encoding a putative antibacterial toxin, and PD1295, encoding a putative membrane protein, were positively regulated by both AlgU and GacA. However, PD1926, encoding a putative pilus assembly protein, was negatively regulated by AlgU but positively regulated by GacA. Also, PD0521, of unknown function, was positively regulated by AlgU but negatively regulated by GacA. Several select candidate pathogenicity genes that were regulated by AlgU and by GacA are being mutated, and the effects of the mutations on phenotype and virulence are being assessed. The intent of this research is to identify essential virulence factors that may serve as targets for novel control approaches.

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FUNDING AGENCIES
Funding for this project was provided by the California Department of Food and Agriculture, and the University of California Agriculture Experiment Station.