CHARACTERIZATION OF REGULATORY PATHWAYS CONTROLLING VIRULENCE IN *XYLELLA FASTIDIOSA*

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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. Here we report a specific example of this approach that has helped to define genes involved in aggregation, biofilm formation, and virulence of *Xf*. In previous work with *X. fastidiosa*, we mutated the global regulatory gene *rsmA*, and found a number of genes that were over-expressed in this mutant when grown in vitro, implying that these genes are normally repressed by the post-transcriptional regulator RsmA in the wild-type. In addition, the *rsmA*$^-$ mutant formed much more biofilm than wild type. Among the genes repressed by *rsmA* was another regulatory gene, *algU*, which regulates important virulence factors in *Pseudomonas*. In this study, an *algU::nptII* mutant had reduced cell-cell aggregation, attachment, biofilm formation, and lower virulence in grapevines. DNA microarray analysis showed that 42 genes had significantly lower expression in *algU::nptII* than wild type, including several genes which could contribute to cell aggregation and biofilm formation, as well as other physiological processes that could contribute to virulence and survival. Thus, *rsmA* appears to control biofilm formation and other traits partly through its repression of the positive regulator, *algU*.

INTRODUCTION
Many virulence genes in bacterial pathogens are coordinately regulated by "global" regulatory genes. The gene *rsmA*, for example, is known to regulate pathogenicity and secondary metabolism in a wide group of bacteria (Blumer and Haas, 2000; Mukherjee et al., 1996). Conducting DNA microarray analysis with mutants for such genes, compared with wild-type, can help to refine the list of genes that may contribute to virulence. We have reported on such an analysis with an *rsmA* mutant of *Xylella fastidiosa (Xf)*, and identified a number of genes that were overexpressed in the mutant (Cooksey, 2004). Among those were *pil* genes that have been subsequently confirmed to be important in twitching motility and long-distance spread of *Xf* in grapevines (Hoch and Burr, 2005; Meng et al., 2005), as well as enzymes or other structural proteins. In addition, a few genes controlled by *rsmA* were "secondary" regulatory genes, such as *algU*, which controls exopolysaccharide production in certain human and plant pathogens and contributes to virulence (Schnider-Keel, et al., 2001; Yu et al., 1995; Yu et al., 1999).

AlgU is a an alternative sigma factor whose role in regulation of biosynthesis of the exopolysaccharide (EPS) alginate has been extensively studied in *Pseudomonas aeruginosa* and *P. syringae*. Alginate functions as a virulence factor in *P. aeruginosa* during infection of cystic fibrosis patients (May and Chakrabarty, 1994), and also contributes to both virulence and epiphytic survival of the plant pathogen *P. syringae* (Yu et al., 1999). In *P. aeruginosa,* AlgU activates AlgU-dependent promoters of *algD* and *algR*. AlgR regulates *algC* and *algD* in cooperation with AlgU (Martin et al., 1994). *mucD* is a negative regulator of *algU* activity in *P. aeruginosa*. Homologs of *algU* (PD1284), *algZ* (PD1154), *algS* (PD0347), *algR* (PD1153), *algC* (PD0120), *algH* (PD1276) and *mucD* (PD1286) were detected in the *Xf* genome (Simpson et al., 2000; Van Sluys et al., 2003), but there are no homologs of the alginate biosynthesis genes *algA, algD, algG, algF, algI* and *algJ*. The alginate homolog genes in *Xf* are therefore probably not involved in alginate biosynthesis, but may be involved in synthesis of other EPS or of lipopolysaccharide (LPS), which could play a role in biofilm formation and cell attachment. In *P. aeruginosa*, the *algC* gene encodes a bifunctional enzyme that is involved in alginate production (phosphomannomutase activity) and lipopolysaccharide (LPS) production (phosphoglucomutase activity) (Coyne et al., 1994). We have constructed an insertional mutation in *algU* in *X. fastidiosa*, which reduced cell-cell aggregation, attachment, biofilm formation, and virulence. DNA microarray analysis of the *algU* mutant was then conducted to determine which genes it regulates.

OBJECTIVES

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

RESULTS

Phenotype of an *algU* mutant

The *algU* gene in *X. fastidiosa* strain A05 was amplified by PCR, cloned into pUC129 and randomly mutagenized *in vitro* with the EZ::TN^{TM} system. A mutant was selected with Tn5 inserted 79bp from the ATG code of the *algU* ORF and used to replace the wild-type *algU* gene in strain A05. Analysis by RT-PCR showed that there was no expression of *algU* within the *algU::nptII* mutant cells but strong expression was detected within wild-type cells. The *algU::nptII* strain exhibited a more random distribution of cells on agar surfaces, whereas the wild-type grew in clumps. The wild-type formed large aggregates in liquid culture, whereas the *algU::nptII* strain was impaired in its ability to aggregate in a quantitative assay (Fig.1, Left). The exponential and stationary phases of growth of the mutant were similar to those of the wild-type parent A05 in PD3 medium, but the ability of the mutant to adhere to glass surfaces was reduced (Figure1, Right). Biofilm analysis revealed that the *algU::nptII* strain had a greatly reduced ability to form biofilm (Figure 2, Left).

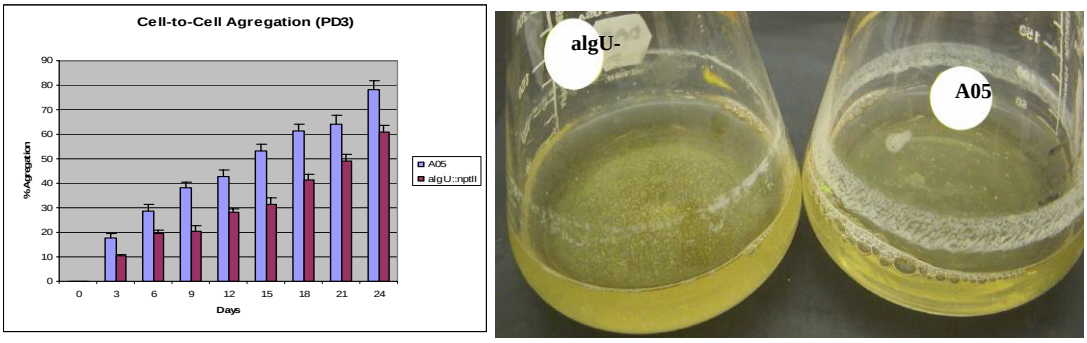

Figure 1. Cell-to-cell aggregation and attachment analysis of *Xf algU::nptII* mutant and wild-type. **Left.** *Xf algU::nptII* mutant was impaired in the ability to form cell-to-cell aggregates in liquid culture. **Right**. Reduced adherence of the *algU::nptII* mutant to a glass surface.

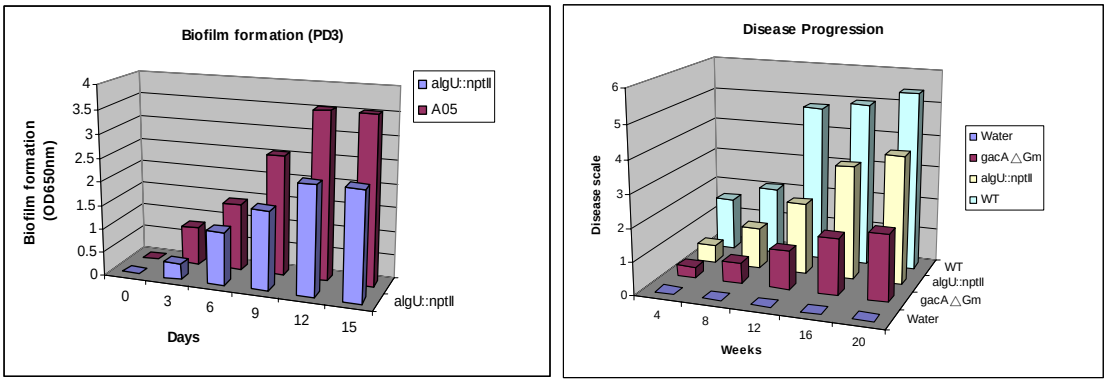

Figure 2. Left: Biofilm formation by *Xf* wild-type and *algU::nptII* mutant **Right**: Disease progression of grapevines inoculated with *Xf* wild-type and *algU::nptII* mutant. Disease severity was based on a visual disease scale of 0 to 5 and was assessed 4, 8, 12, 16, and 20weeks after inoculation. The data are an average of 10 independent replications. The water-inoculated control did not show any symptoms during 20 weeks.

Virulence assay

Grapevines inoculated with the *Xf algU::nptII* mutant developed less severe disease than did those inoculated with the wild type strain 20 weeks after inoculation (Figure 2, Right). Grapevines inoculated with the *algU::nptII* mutant 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 600 nm with that of positive control *Xf* concentrations. The cell populations of the *algU::nptII* mutant were less than that of the wild-type, indicating that *algU* affects the growth and possibly the movement of *Xf* inside the xylem resulting in reduced pathogenicity.

Table 1. Genes differentially expressed in the *Xf algU::nptII* mutant

ORF	Gene[c]	Description	Mutant /Wild type	Signfig.[b]	The expression in mutant
PD0347	*algS*	sugar ABC transporter ATP-binding protein	0.41	*	down
PD0120	*algC*	Phosphomannomutase	0.3	*	down
PD1153	*algR*	two-component system, regulatory protein	0.617	*	down
PD1276	*algH*	transcriptional regulator	0.652	*	down
PD1286	*mucD*	periplasmic protease	0.616	*	down
PD0664	*clpS*	ATP-dependent Clp protease adaptor protein	0.448	*	down
PD0665	*clpA*	ATP-dependent Clp protease subunit	0.485	*	down
PD1685	*clpB*	ATP-dependent Clp protease subunit	0.421	*	down
PD1371	*grpE*	heat shock protein GrpE	0.40	*	down
PD1370	*dnaK*	heat shock protein-Hsp70	0.378	*	down
PD1280	*hspA*	heat shock protein(Hsps)	0.469	*	down
PD2123	*rpmH*	50S ribosomal protein L34, unknown function	0.449	*	down
PD0439	*rplW*	50S ribosomal protein L23, unknown function	0.499	*	down
PD0444	*rplP*	50S ribosomal protein L16, unknown function	0.426	*	down
PD0445	*rpmC*	50S ribosomal protein L29, unknown function	0.479	*	down
PD0447	*rplN*	50S ribosomal protein L14, unknown function	0.477	*	down
PD0450	*rpsN*	30S ribosomal protein S14, unknown function	0.244	*	down
PD0451	*rpsH*	30S ribosomal protein S8, unknown function	0.256	*	down
PD0452	*rplF*	50S ribosomal protein L6, unknown function	0.323	*	down
PD0454	*rpsE*	30S ribosomal protein S5, unknown function	0.437	*	down
PD0455	*rpmD*	50S ribosomal protein L30, unknown function	0.348	*	down
PD0458	*rpsM*	30S ribosomal protein S13, unknown function	0.394	*	down
PD0488	*rpmB*	50S ribosomal protein L28, unknown function	0.436	*	down
PD0489	*rpmG*	50S ribosomal protein L33, unknown function	0.353	*	down
PD0749	*rpmE*	50S ribosomal protein L31, unknown function	0.350	*	down
PD0750	*gltA*	citrate synthase-Energy production and conversion	0.496	*	down
PD1926	none	Type II secretion system-pilus assembly fimbrial protein	2.478	*	up
PD1709	*mopB*	outer membrane protein	0.479	*	down
PD1807	*ompW*	outer membrane protein	0.391	*	down
PD1065	*SecB*	Type II secretion system-Preprotein translocase	0.409	*	down
PD1672	*bfr*	Bacterioferritin-ferritin-like diiron-carboxylate proteins	0.178	*	down
PD0095	*rsmA*	RsmA homologue-regulate virulence determinants	0.403	*	down
PD0066	*hfq*	host factor-I protein, ubiquitous RNA-bing protein hfq	0.32	*	down
PD0216	cvaC[d]	colicin V precursor-antibacterial polypeptides toxin	0.40	*	down
PD0159		unknown	0.479	*	down
PD0521		unknown	0.439	*	down
PD1354		unknown	0.392	*	down
PD0968		unknown (Helix-turn-Helix motif)	0.495	*	down
PD1028		unknown	0.425	*	down
PD1058		putative transcriptional regulatory protein	0.484	*	down
PD1295		putative integral membrane protein	0.469	*	down
PD1668		putative integral membrane protein	0.413	*	down
PD1667		HesB-like protein-unknown function	0.462	*	down

[a] Hybridization signal intensity obtained with the mutant was divided by that from wild-type 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. Significant T-test, $t < 0.001$.
[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.
RNA was prepared from the *algU::nptII* mutant and wild type AO5, 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. Many of the differentially expressed genes were validated by RT-PCR. Forty three genes were differentially expressed in *algU::nptII* compared with the wild type (Table 1). One gene, predicated to encode a fimbrial protein (PD1926), had increased expression in the mutant, but the other 42 genes had decreased expression and are therefore considered to be positively regulated by *algU* in wild-type *Xf* (Table 1). These included homologs of the alginate genes *algS*, *algC*, *algR*, *algH*, and *mucD*, which may have a role in LPS or EPS biosynthesis. Several genes involved in cell structural components and secretion (*mopB, ompW,* and *secB*) are also positively regulated by *algU*,. MopB was shown to bind specifically to xylem tissue (Bruening et al., 2005), and its decreased expression in the *algU::nptII* may have contributed to its decreased attachment and aggregation. Genes involved in physiological metabolism under stress, such as heat shock protein genes *cplS, clpA, clpB*, *dnaK, grpE* and *hspA,*, and the iron storage and detoxification gene, *bfr*, are positively regulated by *algU,* as well as a colicin V precursor (PD0216) that may function in competition with other microbes (Pashalidis et al., 2005). Interestingly, *rsmA* and *hfq*, involved in posttranslational regulation, are also positively regulated by *algU* in *Xf*, while it was shown previously that RsmA negatively regulates *algU*.

CONCLUSIONS
Investigating expressed gene profiles of the *algU::nptII* mutant compared with wild-type via microarray analysis revealed that *algU* regulate various factors which contribute to attachment and biofilm formation, as well as physiological processes that may enhance tolerance to environmental stresses and competition within the xylem. Similar experiments will be conducted to examine differential expression of the *algU::nptII* mutant and wild type *in planta*. In addition, several select candidate pathogenicity genes that were regulated by AlgU will be mutated, and the effects of the mutations on phenotype and virulence will be assessed. The intent of this research is to identify essential virulence factors that may serve as targets for novel control approaches.

REFERENCES

Blumer, C., and D. Haas. 2000. Multicopy suppression of a gacA mutation by the infC operon in *Pseudomonas fluorescens* CHA0: competition with the global translational regulator RsmA, FEMS Microbiol. Lett. 187:53-58.

Bruening G., E. L. Civerolo, Y. Lee, J. M. Buzayan, P. A. Feldstein, and E. Re. 2005. A major outer membrane protein of *Xylella fastidiosa* induces chlorosis in *Chenopodium quinoa*. Phytopathology 95:S14.

Cooksey, D. A. 2004. DNA Microarray and Mutational Analysis to Identify Virulence Genes in *Xylella fastidiosa*. Pages 174-177 IN: Proceedings of the Pierce's Disease Research Symposium, December 7-10, 2004, San Diego, CA, California Department of Food and Agriculture. Sacramento

Coyne, M. J., K. S. Russell, C. L. Coyle, and J. B. Goldberg. 1994. The *Pseudomonas aeruginosa algC* gene encodes phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core. J. Bacteriol. 176:3500–3507.

Hoch, H. C., and T. J. Burr. 2005. Evaluating the Roles of Pili in Twitching and Long Distance Movement of *Xylella fastidiosa* in Grape Xylem and in the Colonization of Sharpshooter Foregut. Pages 162-177 IN: Proceedings of the Pierce's Disease Research Symposium, December 5-7, 2005, San Diego, CA, California Department of Food and Agriculture, Sacramento

Martin D. W., M. J. Schurr, H. Yu, and V. Deretic. 1994. Analysis of promoters controlled by the putative sigma factor AlgU regulating conversion to mucoidy in *Pseudomonas aeruginosa*: relationship to σE and stress response. J. Bacteriol. 176:6688–6696.

May, T. B., and A. M. Chakrabarty. 1994. Isolation and assay of *Pseudomonas aeruginosa alginate*. Methods Enzymol. 235:295–304.

Meng Y. Z., Y. X. Li, C. D. Galvani G. X. Hao, J. N. Turner, T. J. Burr, and H. C. Hoch. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility J. Bacteriol. 187:5560-5567.

Mukherjee, A., Y. Y. Cui, Y. Liu, C. K. Dumenyo, and A. K. Chatterjee. 1996. Global regulation in *Erwinia* species by *Erwinia carotovora* rsmA, a homologue of *Escherichia coli* csrA: Repression of secondary metabolites, pathogenicity and hypersensitive reaction. Microbiology 142:427-434.

Pashalidis S., L. M. Moreira P. A. Zaini, J. C. Campanharo, L. M. C. Alves, L. P. Ciapina, R. Z. N. Vencio, E. G. M. Lemos, A. M. da Silva, A. C. R. da Silva. 2005. Whole-genome expression profiling of *Xylella fastidiosa* in response to growth on glucose. Omics-A Journal of Integrative Biology 9:77-90.

Schnider-Keel, U, K. B. Lejbolle, E. Baehler, D. Haas, and C. Keel. 2001. The sigma factor AlgU (AlgT) controls exopolysaccharide production and tolerance towards desiccation and osmotic stress in the biocontrol agent *Pseudomonas fluorescens* CHA0. Appl Environ Microbiol. 67:5683-93.

Simpson A. J., F. C. Reinach, P. Arruda, F. A. Abreu, M. Acencio, et. al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151–157.

Van Sluys M. A., M. C. de Oliveira, C. B. Monteiro-Vitorello, et al., 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. J. Bacteriol. 185:1018–1026.

Yu, H., M. J. Schurr, and V. Deretic. 1995. Functional equivalence of *Escherichia coli* and *Pseudomonas aeruginosa* AlgU: *E. coli* rpoE restores mucoidy and reduces sensitivity to reactive oxygen intermediates in algU mutants of P. aeruginosa. J. Bacteriol. 177:3259–3268.

Yu, J., A. Pen˜aloza-Va´zquez, A. M. Chakrabarty, and C. L. Bender. 1999. Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae* pv. *syringae*. Mol. Microbiol. 33:712–720.

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GRAPE ROOTSTOCK VARIETY INFLUENCE ON PIERCE'S DISEASE SYMPTOMS IN CHARDONNAY

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ABSTRACT
Chardonnay is a *Vitis vinifera* scion variety that is susceptible to Pierce's disease (PD). We are evaluating the effect of rootstock variety on PD symptom expression in Chardonnay grown in an experimental vineyard at Weslaco, Texas with high natural PD pressure and abundant vectors, including glassy-winged sharpshooter. The rootstocks under evaluation are Dog Ridge, Florilush, Tampa, Lenoir, and Freedom. Natural *Xylella fastidiosa* infection will be permitted to test the effect of rootstock variety on PD in the Chardonnay scions.

INTRODUCTION
Rootstocks are widely in use in viticulture to manage damage from soil-borne pests and provide adaptation to soils. In citrus (He et al. 2000) and peach (Gould et al. 1991), rootstock variety has been reported to impact expression of *Xylella fastidiosa* (*Xf*) diseases in scions. Pierce (1905) reported that rootstock variety affected expression of "California vine disease" (PD) in grape. Grape rootstock trials in Mississippi showed a large effect of rootstock trial on vine longevity in a region recognized for high Pierce's disease pressure (Loomis 1952, 1965; Magoon and Magness 1937). If grape rootstocks could contribute Pierce's disease resistance or tolerance to their scions, this would be a major benefit to viticulture in PD prone areas. Elite wine, juice, and table grape varieties could be grown in areas where viticulture is currently restricted to PD resistant and tolerant varieties whose consumer appeal is low.

The Rio Grande Valley is an excellent location for the field evaluation of PD resistant plant germplasm and PD management techniques. Many insect vectors of *Xf* are native to the region, including the glassy-winged sharpshooter. Susceptible grapevine varieties are infected naturally with *Xf* in the vineyard and demonstrate characteristic PD symptoms and decline. The Rio Grande Valley is similar to many viticultural regions in California; the region is flat, irrigated, and supports multiple types of crops (citrus, grains, vegetables) in close proximity. The Rio Grande Valley is an ideal test environment due to heavy PD pressure, with abundant vectors and inoculum, in contrast to many other locations, especially California, which demonstrate substantial cycling of PD incidence. The USDA Agricultural Research Service Kika de la Garza Subtropical Agricultural Research Center in Weslaco, Texas is located in the heart of the Rio Grande Valley and provides an ideal experimental location for the evaluation of PD management practices, including rootstock evaluation.

Five rootstocks were chosen for evaluation in this project. Freedom is a complex interspecific hybrid developed as a root-knot nematode resistant rootstock by the USDA ARS, Fresno, California (Clark 1997); its parentage includes *Vitis vinifera, V. labrusca, V. x champinii, V. solonis,* and *V. riparia.* Freedom is widely used in California viticulture. Dog Ridge is a *V. x champinii* selection recognized for its nematode resistance and resistance to PD, but it is rarely used as a rootstock. Lenoir, most probably a *V. aestivalis*/*V. vinifera* hybrid, was used historically as a rootstock and presently is cultivated as a wine grape in PD prone regions (including some parts of Texas) (Galet 1988). Tampa (Mortensen and Stover 1982) includes a *V. aestivalis* selection and the juice grape Niagara (a *V. labrusca* hybrid) in its parentage. Florilush (Mortensen et al. 1994) is a selection from the cross Dog Ridge x Tampa. Both Florilush and Tampa were selected by the University of Florida as PD resistant rootstocks for bunch grapes. PD resistance is necessary for rootstock mothervines to thrive in Florida, so the PD resistance of Florilush and Tampa should not be construed necessarily as contributing to the PD response of the scions.

OBJECTIVE
1. Evaluate the impact of rootstock variety on expression of PD symptoms in naturally infected PD susceptible *Vitis vinifera* scion varieties Chardonnay.

RESULTS AND CONCLUSIONS
Grafted vines of Chardonnay on five rootstocks (Freedom, Tampa, Dog Ridge, Florilush, and Lenoir) were planted at the Kika de la Garza Subtropical Agricultural Research Center in Weslaco, Texas in July, 2006. Evaluation of PD response of the vines will begin in 2007.