DNA MICROARRAY AND MUTATIONAL ANALYSIS TO IDENTIFY VIRULENCE GENES IN *XYLELLA FASTIDIOSA*

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ABSTRACT
The development of successful management and control strategies of Pierces disease of grape requires the identification of virulence and pathogenicity genes and determining how they functions to control the disease development process. Based on the presumption that biofilm formation is a major pathogenicity factor of *Xylella* and that it may play a major role in the disease causing process, we have been studying the factors – genetic and environmental that affect biofilm formation by *Xylella fastidiosa.* We have identified that, Bovine serum albumen, a component of PW medium specifically inhibits biofilm formation in *X. fastidiosa* and that this inhibition is BSA concentration dependent. Because of its effect on the biofilm formation *in vitro*, we are studying the expression profiles of *X. fastidiosa* genes in the presence and absence of BSA in the media. We have also identified a global regulatory gene, *rsmA* (*rsm* = *r*egulator of *s*econdary *m*etabolism) that control biofilm. An *rsmA*-deficient strain of *X. fastidiosa* forms more biofilm *in vitro* than the wild type. In a preliminary nylon membrane DNA macroarray experiment using about a 100 select candidate pathogenicity genes, we have determined an increased expression of 15 genes in the mutant when compared to the wild type parent. We are now using full genome microarrays of *Xylella fastidiosa* to catalogue the genes whose expressions are controlled by either *rsmA* or BSA. The results from these ongoing analyses using both approaches should help us catalogue *X. fastidiosa* genes which may be involved in pathogenicity and biofilm formation. Subsequent genetic analysis of the genes to be identified should give us some understanding of not only how pathogenicity is regulated in this bacterium but also how to tackle the problems posed by Pierces disease.

INTRODUCTION
Although the exact mechanism of Piece's disease is not completely understood, infected grape plants show symptoms resembling those of water-stress. Moreover, the xylem-limited *Xylella fastidiosa* bacterium produces biofilm *in vitro* and *in planta* (4, 9, 10, 12). Putting these two observations together, it has been suggested that this biofilm clogs up the vascular tissues of the plant and occlude water and nutrient transport. Because of this assumed importance of biofilm formation in the disease mechanism of *Xylella fastidiosa*, we have been studying signals and factors affecting biofilm formation in a bit to identify the regulators of pathogenicity in *Xylella fastidiosa*. *rsmA* is a post-transcriptional regulatory gene that controls pathogenicity and secondary metabolism in a wide group of bacteria including Gram positive and negative organisms (1, 3, 11, 15). In *Erwinia* spp. and other related plant-associated bacteria, rsmA together with its regulatory noncoding RNA pair, *rsmB* control many phenotypes including pathogenicity, extracellular polysaccharide and enzyme production, and elicitation of hypersensitive response, pigment formation, motility and antibiotic biosynthesis). And in *E. coli* and related enterobacterial human pathogens, *csrA* and *csrB*, the homologues of *rsmA* and *rsmB* regulate, among others, glycogen biosynthesis and biofilm formation (6, 8, 17, 19). Because of the role of biofilm formation on the pathogenicity of many bacterial pathogens (5, 14), and the fact that *rsmA* or its homologs control both pathogenicity and biofilm formation in different bacteria, we wanted to determine the possible role of *rsmA* on biofilm formation in *Xylella*. We found that *Xylella fastidiosa* strains vary widely in their biofilm forming abilities and this is influenced by the culture medium in which the assay is carried out.

We report that BSA is the specific inhibitor of biofilm formation in PW medium and that the amount of biofilm the bacterium forms if inversely proportional to the concentration of BSA in the medium. Further, we show that biofilm formation is regulated by *rsmA* gene as *rsmA*$^-$ mutants form higher levels of biofilm than the wild type parent. We confirm this observation by showing that the heterogonous expression of *Xylella fastidiosa rsmA* in *E. coli* reduces biofilm formation in this bacterium. Put together, these suggest that *rsmA* may regulate pathogenicity in *Xylella fastidiosa* through its effects on factors such as biofilm formation in the plant.

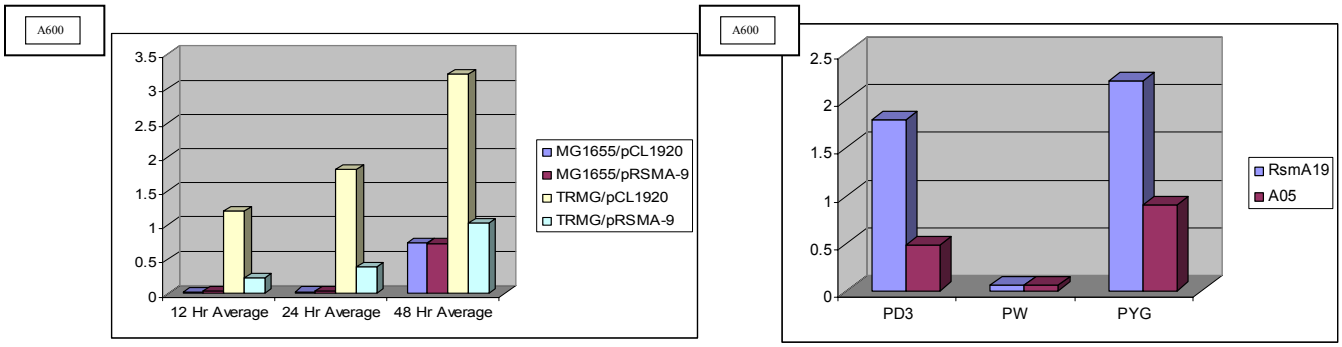
OBJECTIVES

1. Use DNA microarray analysis to identify virulence and pathogenicity genes in *Xylella fastidiosa* through coordinate regulation with a known virulence factor or expression *in planta* during infection.
2. Clone and mutate putative virulence genes and characterize virulence defects in a bid to understand the mechanism of virulence.

RESULTS

Cloning, Characterization of rsmA and the Construction of rsmA Mutant of Xylella fastidiosa

As mentioned above, three observations let us to investigate the role of *rsmA* in pathogenicity and biofilm formation in *Xylella fastidiosa*: 1, the homologues of the gene are widely distributed in the prokaryotic world; 2, the gene controls pathogenicity and virulence in many phytobacteria and 3; in *E. coli*, the gene controls biofilm formation. To determine the role of *rsmA* in *Xylella*, we the cloned the gene and characterized it. The authenticity of the cloned gene was confirmed with DNA sequencing. *Xylella fastidiosa rsmA* is a small gene that encodes a predicted product is 72-amino acid with a putative RNA-binding protein. Heterologous expression of *X. fastidiosa rsmA* in a biofilm overproducing *csrA* mutant of *E. coli* resulted in reduced biofilm formation indicating that the gene does have a role in biofilm formation (Figure 1). After confirming that the cloned gene is indeed *rsmA*, we determined the effect of the mutation on biofilm formation in *Xylella.* The mutant and wild type were assayed for their ability of form biofilm in vitro. Observation show that, the mutant formed more biofilm that the parent (Figure 1). Since the ultimate goal is to identify virulence genes, we tested whether *rsmA* mutants are pleiotropically affected in the expression of any genes. For this, we used the nylon membrane DNA macroarrays of about 100 select pathogenicity genes based on the published genomic sequences (7, 16, 18). Hybridization of ^{32}P-labelled total cDNA reveal 15 genes which were more than 10-fold induced in the mutant (Table 1).



MG1655: Wt type *E.coli*
TRMG: *csrA*$^-$ derivative
pCL1920: Spc^R pSC101 replicon plasmid
pRSMA-9: *X. fastidiosa rsmA* clone in pCL1920

A05: Wild type *X. fastidiosa*
RsmA19: *rsmA*$^-$ derivative of A05
PD3, PW, PYG: *Xylella fastidiosa* growth media

Figure 1. Left. Biofilm formation by *csrA*$^-$ *Ecoli* is suppressed by *X. fastidiosa rsmA* and (Right), *rsmA* mutant of *X. fastidiosa* form more biofilm than their wilt type *rsmA*$^+$ parents.

Identification of the PW Medium Component that Inhibits Biofilm Formation

Because of the increasing evidence of links between biofilm formation and pathogenicity in many biofilm forming bacteria (2, 13), we were interested in identifying any possible factors that control biofilm formation. We had long observed that *Xylella fastidiosa* make more biofilm when grown in PD3 medium than in PW medium. We explored this difference between the two media by adding different components of PW media to PD3 medium in order to identify the component responsible for the inhibition of biofilm formation. Our result show when Bovine serum albumen (BSA) was added to PD3 medium, biofilm formation was reduced; implying that BSA is the inhibitor. We then wanted to see of this inhibition depends on the concentration of BSA present in the medium. Different concentrations of BSA were again supplemented into PD3 basal medium and the bacterium was assayed again for biofilm formation. Our results (Figure 2) again show that the bacteria formed less biofilm with increasing concentration of BSA. These results clearly indicate that BSA is a specific inhibitor of biofilm formation. We are now utilizing this information in our full genome microarrays experiments to determine identify the genes which are coordinately regulated with biofilm as has been done for another strain of *Xylella fastidiosa* (4).

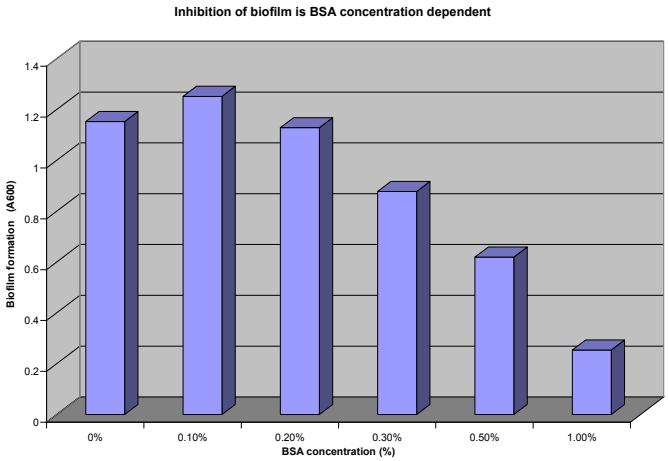


Figure 2. Inhibition of biofilm formation is BSA concentration dependent

Table 1. List of genes overexpresses at least 10-fold in RsmA19.

Gene name	Function	Volume Ratio (A19/A05)
brk	BrkB protein	**14.4**
pilE	Type IV pilin	**10.8**
chi	Chitinase	**12.0**
pcp or lpp	Peptidoglycan-associated outer membrane lipoprotein precursor	**25.7**
pilU	Twitching motility protein	**10.6**
vacB	VacB protein	**14.0**
algH	Transcriptional regulator	**18.3**
algU or algT	RNA polymerase sigma-H factor	**21.8**
ccmA	Heme ABC transporter ATP-binding protein	**14.5**
colS	Two-component system, sensor protein	**10.7**
tapB	Temperature acclimation protein B	**69.8**
fucA1	Alpha-L-fucosidase	**10.9**
pilT	Twitching motility protein	**13.3**
gcvR	Transcriptional regulator	**12.9**
clpP or lopP	ATP-dependent Clp protease proteolytic subunit	**13.2**

CONCLUSIONS

In conclusion, we have identified a genetic factor and an environmental factor, both of which control the important phenomenon of biofilm formation; a process that is tightly linked to pathogenicity of *Xylella fastidiosa*. *rsmA*$^-$ mutants of *Xylella fastidiosa* form more biofilm that the parents and the presence of BSA in the medium suppresses biofilm formation by the bacterium. We have identified 15 preliminary genes which are coordinately regulated with *rsmA* mutation and possibly, biofilm formation. We are using high density DNA microarrays to catalogue *Xylella fastidiosa* genes which are up- or down-regulated with *rsmA* mutation and reduced biofilm formation due to BSA in the medium. This work will contribute significantly to fundamental information on the genetics and pathogenicity of *Xylella fastidiosa.* This information is essential for any attempt to design a management strategy for PD based on the disease mechanism. The identification of previously unknown virulence genes can also lead to recognition of new unforeseen targets for management strategies. In addition, the construction of a DNA microarray for this pathogen, and identification of genes differentially expressed during infection, will complement work by others on differential expression of grapevine genes during infection. This will open the door to "interactive genomic" studies that will enhance our understanding of the bacterial-plant interaction that leads to Pierce's disease, and in the future, studies of interactions with its insect vectors.

Work in Progress

We have developed whole genome arrays of *Xylella fastidiosa* and are presently analyzing gene expression levels between the wild type and *rsmA* mutant, growth with and without BSA and *in vivo* versus *in vitro* conditions. We hope to catalogue the genes whose expressions are associated with biofilm formation, *rsmA* mutation and infection. Those genes which will overlap with more than one approach will be especially interesting for further analysis. Genetic analysis of these genes therefore should open a window for us into what goes on during the infection process. The *rsmA* mutant together with its parent is also being assayed for pathogenicity on grapes. In addition, we have constructed several mutants in a select candidate pathogenicity genes and are in the process of analysis these for the effects of the mutations and hence the roles of these genes in the bacterium.

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