XYLELLA FASTIDIOSA EXTRACELLULAR GENOMIC DNA ENHANCES BIOFILM FORMATION *IN VITRO*

Principal Investigator:
Hong Lin
Crop Dis., Pests, & Genet. Res. Unit
USDA-ARS
Parlier, CA 93648
hong.lin@ars.usda.gov

Cooperators:
Davis Cheng
Crop Dis., Pests, & Genet. Res. Unit
USDA-ARS
Parlier, CA 93648

Edwin L. Civerolo
Crop Dis., Pests, & Genet. Res. Unit
USDA-ARS
Parlier, CA 93648

Reporting Period: The results reported here are from work conducted October 2008 to September 2009.

ABSTRACT
Xylella fastidiosa (*Xf*) produces extracellular DNA in PD3 liquid medium. This extracellular DNA may play a role in enhancing biofilm formation, a factor that is required by *Xf* to establish infection in host plants. Amounts of extracellular DNA generated by *Xf in vitro* were positively correlated with planktonic cell growth and biofilm formation, but were negatively correlated with cell viability. DNase I treatment of actively growing *Xf* cultures in PD3 medium resulted in decrease or inhibition of biofilm formation. In contrast, addition of *Xf* genomic DNA to *Xf* cultures promoted biofilm formation. These results support the hypothesis that biogenesis of extracellular DNA may play a role in *Xf* biofilm formation leading to successful host plant infection.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) generates extracellular DNA in PD3 culture medium. This extracellular DNA may enhance biofilm formation, the process of which the matrix of extracellular polymeric substance is formed, which facilitates establishment of *Xf* infection in plants. The planktonic *Xf* cell growth and its biofilm formation *in vitro* culture were positively correlated with extracellular DNA produced by *Xf*, but negatively correlated with *Xf* cell viability. Biofilm formation was decreased or inhibited when growing cells were treated with DNase I, an enzyme which degrades DNA. In contrast, addition of *Xf* genomic DNA to cultures promoted biofilm formation. These results suggest that biogenesis of extracellular DNA may play a role in *Xf* biofilm formation and, therefore, contribute to development of Pierce's disease.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a Gram negative, xylem-limited bacterium that causes Pierce's disease of grapevine, as well as other diseases of economically important crops and landscape plants (Hopkins, 1989). *Xf* is transmitted by xylem-feeding insects, including the polyphagous and invasive glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis* (Germar)) (Almeida and Purcell, 2003). The mechanism of *Xf* pathogenicity in host plants is not fully understood. It has been reported that a functional relationship exists among *Xf* planktonic growth, aggregation, biofilm formation and pathogenesis in *Vitis* species (Leite *et al*., 2004, Andersen *et al*., 2007). Previously, we reported that differences in xylem sap composition and cell wall properties among PD- resistant and -susceptible grapes may play a role in affecting PD development (Cheng *et al*., 2009). Bacterial biofilms are structured communities of cells enclosed in self-produced hydrated polymatrixes that adhere to inert or living surfaces (Costerton *et al*., 1999). The matrix, which holds bacterial biofilms together, is a complex mixture of macromolecules including exopolysaccharides, proteins and nucleic acids (Sutherland, 2001). A diffusible signal molecule is reportedly required for biofilm formation by *Xf* in the vector(s) and for vector transmission of *Xf* to plants (Newman *et al*., 2004). In addition, cell density-dependent exopolysaccharide synthesis (EPS) is required for virulent biofilm formation *in planta* (Koutsoudis *et al*., 2006). The objective of this study was to determine if extracellular genomic DNA is involved in the *Xf* biofilm formation *in vitro*.

OBJECTIVE
Determine if extracellular genomic DNA is involved in the *Xf* biofilm formation *in vitro*.

RESULTS AND DISCUSSION
Bacteria produce substantial quantities of extracellular DNA through a mechanism that is thought to be independent of cellular lysis and that appears to involve the release of small vesicles from the outer membrane (Muto and Goto 1986, Kadurugamuwa and Beveridge 1995). We assayed cell-free PD3 liquid culture medium following growth of *Xf in vitro* for extracellular DNA. As shown in **Figure 1A**, extracellular genomic DNA was present in cell-free PD3 liquid medium of *Xf* cultures with fragment sizes ranging from less than 100 bp to 10 kb approximately. The most abundant *Xf* extracellular DNA size was ~100 bp detected by agarose gels and was readily distinguished from the intact *Xf* genomic DNA.

We investigated the effect(s) of extracellular genomic DNA produced by *Xf* in PD3 liquid culture medium on *Xf* planktonic growth, biofilm formation and cell viability *in vitro*. As shown in **Figure 1B**, the production of extracellular DNA in the *Xf* culture medium at different time periods of growth was positively correlated with *Xf* planktonic growth and biofilm formation, but was negatively correlated with the *Xf* cell viability (R^2 = - 0.9947). *Xf* cell viability decreased with the increase of Xf planktonic growth and biofilm formation (R^2 = - 0.9967 and R^2 = - 0.9997, respectively). In contrast, *Xf* planktonic growth and biofilm formation increased during growth. To confirm that there was no contamination, 96 cloned DNAs were randomly picked for sequencing. BLAST reports showed that all DNA sequences matched *Xf* genome sequences in the NCBI *Xf* database, indicating that there was no contamination in the *Xf* culture by other bacteria. In addition, no specific DNA sequence may be required for the enhancement of *Xf* biofilm formation *in vitro*. This result is consistent with a previous study in which no specific DNA sequences were found in the population of extracellular DNAs (Allesen-Holm *et al*., 2006). The occurrence of extracellular DNA in the *Xf* growing medium may result from *Xf* cell death during growth *in vitro* and biofilm formation. However, it is not clear if there are other mechanisms also involving secretion of extracellular DNA by *Xf*. Whitchurch *et al* (2002) reported that extracellular DNA derived from membrane vesicles promotes biofilm formation by *Pseudomonas aeruginosa*. Characterization of DNA release in *P. aeruginosa* cultures and biofilms provided evidence that extracellular DNA was generated via lysis of a subpopulation of the bacteria (Allesen-Holm *et al*., 2006). The results of a more recent study also suggested that extracellular DNA was generated in *Staphylococcus epidermidis* cultures through autolysin AtlE-mediated lysis of a subpopulation of the bacteria (Qin *et al*., 2007).

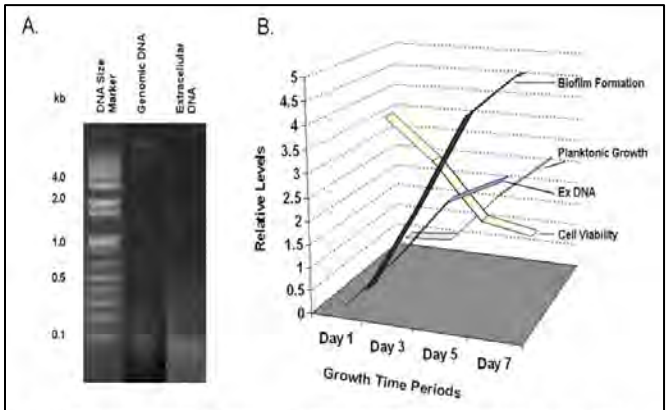

Fig. 1. A. Comparison between *Xf* (strain Temecula-1) genomic DNA and extracellular DNA extracted from PD3 liquid culture medium; B. Relationships of *Xf* growth, biofilm formation, cell viability and extracellular DNA biogenesis in PD3 liquid medium *in vitro*.

In this study, while there was no direct evidence to determine whether the extractcellular DNA in liquid PD3 *Xf* culture was derived mainly from membrane vesicles rather than by cell lysis, *Xf* genomic DNA added into culture medium to mimic extracellular *Xf* DNA resulted in enhancement of biofilm formation. Moscoso *et al*. reported that simultaneous inactivation of *Streptococcus pneumoniae*'s LytA amidase and LytC lysozyme abolished DNA release in liquid culture (Moscoso and Claverys, 2004, Moscoso *et al*., 2006). A choline-binding protein D (CbpD) is essential for competence-induced cell lysis in *S. pneumoniae*, but DNA release is also strongly attenuated in its (cbpD) mutant (Kausmally *et al*., 2005). It is, therefore, possible that biogenesis of extracellular DNA could be a genetically regulated process in bacteria including *Xf*. It is not clear if extracellular DNAs released from host cells could also function in regulating bacterial biofilm formation *in vivo*. In this regard, it would be interesting to evaluate whether the host extracellular DNA released in PD-resistant and -susceptible grapevines could differentially affect cell attachment, aggregation and biofilm formation *in planta*. This may provide insight into understanding the role of extracellular DNA in regulation of host-pathogen interactions toward genetic resistance or susceptibility *in planta*.

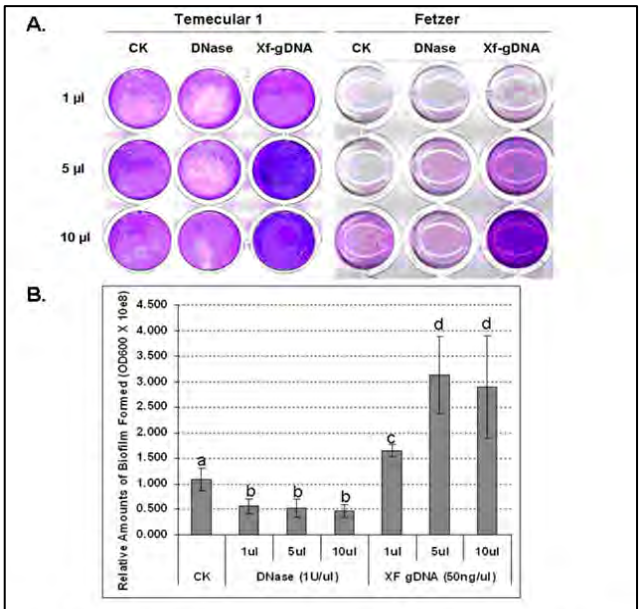

Fig. 2. Effects of DNase I and *Xf* (strain Temecula-1) genomic DNA on *Xf* biofim formation. A. Bioassay for *Xf* biofilm formation in the presence of various amounts of DNase I and *Xf* genomic DNA; B. Quantitative analysis of the effects of DNAse I and *Xf* genomic DNA treatment on *Xf* biofilm formation. The statistic significances (a, b, c and d) indicate that the differences are very significant among different groups, where no significant statistic difference exists within the same groups (b and d).

Xf bacterial planktonic growth, biofilm formation and cellular aggregation are dependent on the chemistry of xylem sap and can be manipulated by altering xylem chemistry (Andersen *et al*, 2007, Leite *et al*., 2004). *Xf* biofilm formation likely plays a key role in xylem vessel occlusion and is a key virulence factor probably required for *Xf* pathogenicity (de Souza *et al*., 2005, Marques *et al*., 2002, Newman *et al*., 2004). We examined a potential role of extracellular DNA as an additional factor in *Xf* biofilm formation *in vitro* by adding different amounts of DNase I and *Xf* genomic DNA into the pre-cultured *Xf* growing medium. DNase I treatments

diminished the effect of *Xf* biofilm formation in both *Xf* strains, Temecula-1 and Fetzer (kindly provided by Dr. Bruce Kirkpatrick) (**Figure 2A**). In contrast, addition of Xf genomic DNA (Temecula-1) greatly enhanced biofilm formation by both *Xf* strains. Quantitative analyses of the effects of DNase I and *Xf* genomic DNA on biofilm formation revealed that all DNase I treatments decreased the cell density of *Xf* biofilm by nearly 50%, although there were no significant differences between the DNase I concentrations used. As expected, addition of *Xf* genomic DNA significantly increased the cell density of *Xf* biofilms 1.5- to 3-fold depending on the concentrations of *Xf* genomic DNA added (**Figure 2B**).

Extracellular DNA plays a role in the maintenance of biofilms formed by gram-positive and gram-negative bacteria (Tetz *et al*., 2009). Digestion of *P. aeruginosa* and *S. pneumoniae* extracellular DNA changed the properties of the biofilms formed by these bacteria (Whitchurch *et al*., 2002, Izano *et al*., 2008, Moscoso *et al*., 2006). However, the mechanism(s) of how extracellular DNA functions in *Xf* biofilm formation is not clear. A functional DNA binding and uptake system was suggested to be involved in the biofilm formation by *S. mutans*, where the presence of synthetic competence-stimulating peptide significantly promoted the release of DNA and enhancement of biofilm formation (Petersen *et al*., 2005). The DNA binding-uptake system is a multi-protein complex that is required for the assembly of type IV pili and for the secretion of certain proteins in gram-negative bacteria (Chung and Dubnau 1998). In addition, pseudopili cross the cell wall and allow the extracellular DNA to access a membrane-bound receptor in *B. subtilis* (Chen and Dubnau, 2004). Type IV pili, flagellum-mediated motility and quorum sensing-controlled DNA releases are involved in the formation of mature multicellular structures in *P. aeruginosa* biofilms (Barken *et al*., 2008). Based on a biophysical study of the bacterial organization in a model extracellular DNA matrix, bacteria can spontaneously become ordered in a matrix of aligned concentrated DNA, in which rod-shaped cells of *P. aeruginosa* follow the orientation of extended DNA chains (Smalyukh, 2008). It is likely, therefore, that extracellular DNA may function through interaction with specific proteins on the bacterial membrane that would favor or facilitate biofilm formation. This process may be coordinately regulated by bacterial pili and/or flagellum-mediated motility and/or quorum sensing systems.

CONCLUSIONS

The present study suggests that biogenesis of extracellular DNA may be a result of autolysis of *Xf* cells or other cellular mechanism(s) or both during the planktonic growth that was associated with enhanced biofilm formation *in vitro*. Further research is needed to assess the role of extracellular *Xf* DNA in biofilm formation and pathogenesis *in planta.*

REFERENCES CITED

Allesen-Holm M., Barken K.B., Yang L., Klausen M., Webb J.S., Kjelleberg S., Molin S., Givskov M., Tolker-Nielsen T., 2006. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Molecular Microbiology*, 59:1114-28.

Almeida R.P., Purcell A.H., 2003. Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). *Journal Economic Entomology*, 96:264-71.

Andersen P. C., Brodbeck B. V., Oden S., Shriner A., Leite B., 2007. Influence of xylem fluid chemistry on planktonic growth, biofilm formation and aggregation of *Xylella fastidiosa*. *FEMS Microbiology Letter*, 274:210-217.

Barken K.B., Pamp S.J., Yang L., Gjermansen M., Bertrand J.J., Klausen M., Givskov M., Whitchurch C.B., Engel J.N., Tolker-Nielsen T., 2008. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environmental Microbiology*, 10:2331-43.

Chen I., Dubnau D., 2004. DNA uptake during bacterial transformation. *Nature Reviews Microbiology*, 2:241-249.

Cheng D.W., Lin H., Walker A.M., Stenger D.C., Civerolo E.L., 2009. Effects of Grape Xylem Sap and Cell-Wall Constituents on *In Vitro* Growth, Biofilm Formation and Cellular Aggregation of *Xylella fastidiosa. European Journal of Plant Pathology*, 115:203-213.

Chung, Y. S., D. Dubnau., 1998. All seven comG open reading frames are required for DNA binding during transformation of competent Bacillus subtilis. *Journal of Bacteriology*, 180:41-45.

Costerton J.W., Stewart P.S., Greenberg E.P., 1999. Bacterial biofilms: a common cause of persistent infections. *Science,* 248:1318-1322.

de Souza A. A., Takita M. A., Pereira E. O., Coletta-Filho H. D., Machado M. A. 2005. Expression of pathogenicity-related genes of *Xylella fastidiosa in vitro* and in planta. *Current Microbiology,* 50: 223-228.

Filoche S.K., Coleman M.J., Angker L., Sissons C.H., 2007. A fluorescence assay to determine the viable biomass of microcosm dental plaque biofilms. *Journal of Microbiological Methods,* 69:489–496.

Hopkins D. L., 1989. *Xylella fastidiosa*: xylem-limited bacterial pathogen of plants. *Annual Review of Phytopathology,* 27: 271-290.

Kausmally L., Johnsborg O., Lunde M., Knutsen E., Håvarstein L. S., 2005. Choline-binding protein D (CbpD) in *Streptococcus pneumoniae* is essential for competence-induced cell lysis. *Journal of Bacteriology*, 187:4338-4345.

Kadurugamuwa J.L., Beveridge T.J., 1995. Virulence factors are released from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *Journal of Bacteriology*, 177:3998-4008.

Koutsoudis, M. D., Tsaltas D., Minogue T. D., von Bodman S. B., 2006. Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. *Proceedings of the National Academy of Sciences, USA*, 103:5983-5988.

Leite B., Andersen P. C., Ishida M. L. 2004. Colony aggregation and biofilm formation in xylem chemistry-based media for *Xylella fastidiosa*. *FEMS Microbiology Letter,* 230:283-290.

Izano, E. A., Amarante M. A., Kher W. B., Kaplan J. B., 2008. Differential roles of poly-*N*-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Applied Environmental Microbiology,* 74:470-476.

Marques L. L. R., Ceri H., Manfio G. P., Reid D. M., 2002. Characterization of biofilm formation by *Xylella fastidiosa in vitro*. *Plant Disease,* 86: 633-638.

Moscoso, M., Claverys J. P., 2004. Release of DNA into the medium by competent *Streptococcus pneumoniae:* kinetics, mechanism and stability of the liberated DNA. *Molecular Microbiology*, 54:783-794.

Moscoso M., García E., López R., 2006. Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *Bacteriology*, 188:7785-95.

Muto Y., Goto S., 1986. Transformation by extracellular DNA produced by *Pseudomonas aeruginosa*. *Microbiology Immunology*, 30:621-8.

Newman K.L., Almeida R.P.P., Purcell A.H., Lindow SE., 2004. Cell-cell signaling controls *Xylella fastidiosa* interactions with both insects and plants. *Proceedings of the National Academy of Sciences, USA*, 101:1737-1742.

Petersen F.C., Tao L., Scheie A.A., 2005. DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation. *Journal of Bacteriology*, 87:4392-400.

Pitcher D.G., Saunders N.A., Owen R.J., 2008. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Letters in Applied Microbiology*, 8:151 – 156.

Qin Z., Ou Y., Yang L., Zhu Y., Tolker-Nielsen T., Molin S., Qu D., 2007. Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. *Microbiology*, 153:2083-92.

Smalyukh I.I., Butler J., Shrout J.D., Parsek M.R., Wong G.C., 2008. Elasticity-mediated nematiclike bacterial organization in model extracellular DNA matrix. Physical Review, 78: 030701.

Sutherland I.W., 2001. The biofilm matrix--an immobilized but dynamic microbial environment. *Trends in Microbiology*, 9:222-7.

Tetz G.V., Artemenko N.K., Tetz V.V., 2009. Effect of DNase and antibiotics on biofilm characteristics. *Antimicrob. Agents Chemother,* 53:1204-1209.

Whitchurch C.B., Tolker-Nielsen T., Ragas P.C., Mattick J.S., 2002. Extracellular DNA required for bacterial biofilm formation. *Science*, 295:1487.

FUNDING AGENCIES

Funding for this project was provided by the USDA Agricultural Research Service.