EXTRACELLULAR GENOMIC DNA MEDIATES ENHANCEMENT OF *XYLELLA FASTIDIOSA* BIOFILM FORMATION *IN VITRO*

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SUMMARY

Xylella fastidiosa (*Xf*) produces extracellular DNA in PD3 liquid medium. This extracellular DNA could enhance biofilm formation, a factor in successful establishment of *Xf in planta.* The relative amounts of extracellular DNA were positively correlated with planktonic growth and biofilm formation *in vitro*, but were negatively correlated with cell viability. DNase I treatment of actively growing *Xf* cultures in PD3 medium decreased or inhibited biofilm formation. In contrast, addition of *Xf* genomic DNA promoted biofilm formation. These results suggest that biogenesis of extracellular DNA may play a role for *Xf* biofilm formation and could be a critical step in establishment of host-bacterium interaction.

Key words: Xylella fastidiosa, biofilm formation, extracellular DNA, bacterial ecology, Gram-negative bacteria.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a Gram negative, xylem-limited bacterium that causes Pierce's Disease (PD) 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 *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). Biofilms of *Xf* in the insect are composed of cells that are polarly attached to insect foregut tissue (Ramey *et al*., 2004). In the plant hosts, the levels of *Xf* population with biofilm vary from sparsely colonized xylem vessels in PD-resistant plants to densely populated vessels in highly susceptible plants (Fritschi *et al.*, 2007a, 2007b).

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*.

MATERIALS AND METHODS

Bacterial culture. Two *Xf* strains, Temecula-1 and Fetzer were maintained in liquid PD3 medium containing 15% glycerol, at -80°C until use. The *Xf* strains were re-grown by streaking and culturing on PD3 agar medium at 28°C for 15 days. Actively growing bacteria were collected and re-suspended in PD3 liquid medium. The suspension was diluted to approximately $7x10^8$ cells ml^{-1} with the same medium. One ml of *Xf* suspension was distributed into each well of a 12-well microtiter plate.

Treatment of bacterial culture. Either DNase I (Invitrogen, USA) or total *Xf* genomic DNA were used as experimental treatments after growth of bacteria for 3 days *in vitro*. *Xf* genomic DNA was obtained from actively growing *Xf* strains Temecula-1 and Fetzer in PD3 liquid cultures using a modified protocol of lysozyme and guanidium thiocyanate lysis followed by phenol/chloroform extraction and dissolved in TE buffer

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(Pitcher *et al*., 2008). Various amounts of DNase I (1-10 U, 1U/µl) or *Xf* genomic DNA (50-500 ng, 50 ng/µl) were mixed with 10 ml PD3 liquid medium before being added into each well (1 ml) of a *Xf* culture. The same amount of PD3 medium was added into the control group at the same time. The plates were then incubated at 28°C for 7 days.

Measurement of *Xf* growth and biofilm formation. Quantitative measurements of both *Xf* growth and biofilm formation were made as described previously (Cheng *et al*., 2009).

Extracellular DNA isolation and identification. After incubation of the plates for 1, 3, 5 and 7 days, respectively *Xf* suspension culture were centrifuged at 4,000 rpm for 10 min at room temperature. The supernatant was collected, filtered through a 0.22 µm filter to remove *Xf* cells, and used for extracellular DNA isolation. The *Xf* extracellular DNA was dissolved in TE buffer. The quality of the extracted DNAs was assessed by electrophoresis in 1.2% agarose gel in 1x TAE buffer using the *Xf* genomic DNA as a positive control reference. To confirm that the extracellular DNAs were derived from *Xf* and not from contamination, isolated extracellular DNA was randomly cloned. Ninety-six colonies were picked and sequenced. BLAST analysis was conducted to confirm the identity of *Xf*.

Cell viability assay. *Xf* cell viability in the treated suspension cultures was quantitatively analyzed using a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, USA) as described (Filoche *et al*., 2007). The ratio of total cell counts (green fluorescence, 530 nm)/dead cells (red fluorescence, 630 nm) was measured using Spectra Max Fluorescence Microplate Reader (Molecular Devices, USA).

Statistical analysis. The experiment was repeated 4 times. Student's T-test was used to analyze the statistical significance at $p<0.01$ and $p<0.05$.

RESULTS AND DISCUSSION

Cellular production of extracellular DNA. Bacteria produce substantial quantities of extracellular DNA via 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 the cell-free PD3 liquid culture medium of *Xf* cul-

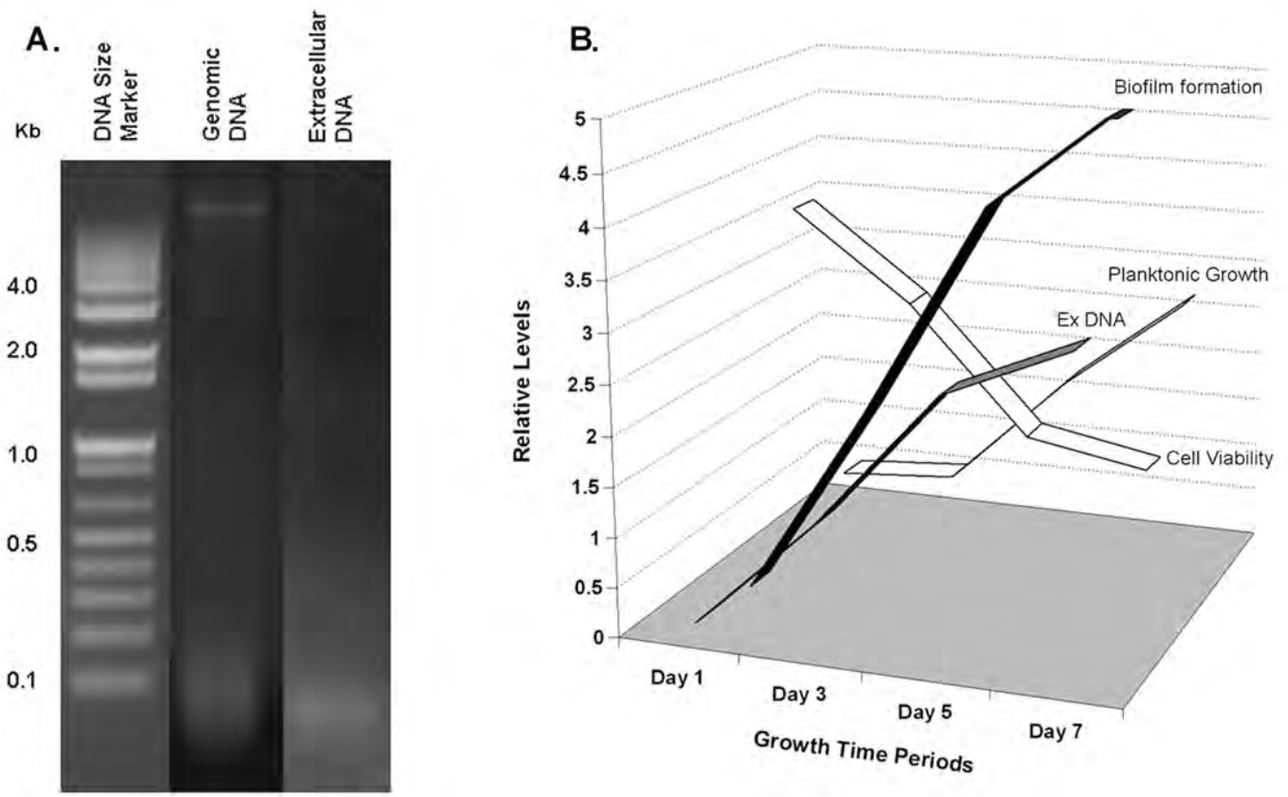

Fig. 1. A. Comparison between *Xf* (strain Temecula-1) genomic DNA and extracellular DNA extracted from PD3 liquid culture medium; B. Relationships of *Xf* growth, biofilm formation, cell viability and extracellular DNA biogenesis in PD3 liquid medium *in vitro*.

ture for extracellular DNA. As shown in Figure 1A, extracellular genomic DNA was present in PD3 liquid medium *Xf* cultures with fragment sizes ranging from less than 100 bp to 10 kb approximately. The most abundant *Xf* extracellular DNA size was *ca.* 100 bp as shown in the agarose gels and was readily distinguished from the intact *Xf* genomic DNA.

We investigated the effect(s) of extracellular 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). The relative *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. Subsequently, we cloned *Xf* extracellular DNA fragments. Ninety six cloned DNAs were randomly picked up for sequencing confirmation. 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 and 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 was found in the population of extracellular DNAs (Allesen-Holm *et al*., 2006).

Apparently, at least some of the extracellular DNA in the culture medium may be derived from dead cells as the *Xf* cultures aged. This observation suggests that occurrence of extracellular DNA in the *Xf* growing medium may result from *Xf* cell death during growth *in vitro* and from 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). A more recent study also suggested that extracellular DNA was generated in *Staphylococcus epidermidis* through autolysin AtlE-mediated lysis of a subpopulation of the bacteria (Qin *et al*., 2007).

In this study, while there was no direct evidence to support the hypothesis that the extracellular DNA in liquid PD3 *Xf* culture was derived mainly from membrane vesicles rather than cell lysis, *Xf* genomic DNA added into culture medium to mimic extracellular *Xf* DNA resulted in enhancement of biofilm formation. It was previously reported that simultaneous inactivation of *Streptococcus pneumoniae* LytA amidase and LytC lysozyme abolishes 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*.

Extracellular DNA required for enhancement of *Xf* biofilm formation. *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 by both *Xf* strains (Figure 2A). In contrast, addition of *Xf* genomic DNA greatly enhanced biofilm formation by both *Xf* strains. Quantitative analysis of the effects of DNase I and *Xf* genomic DNA on biofilm formation by *Xf* strain Temecula-1 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* biofilm 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 formation in 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 multiprotein 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

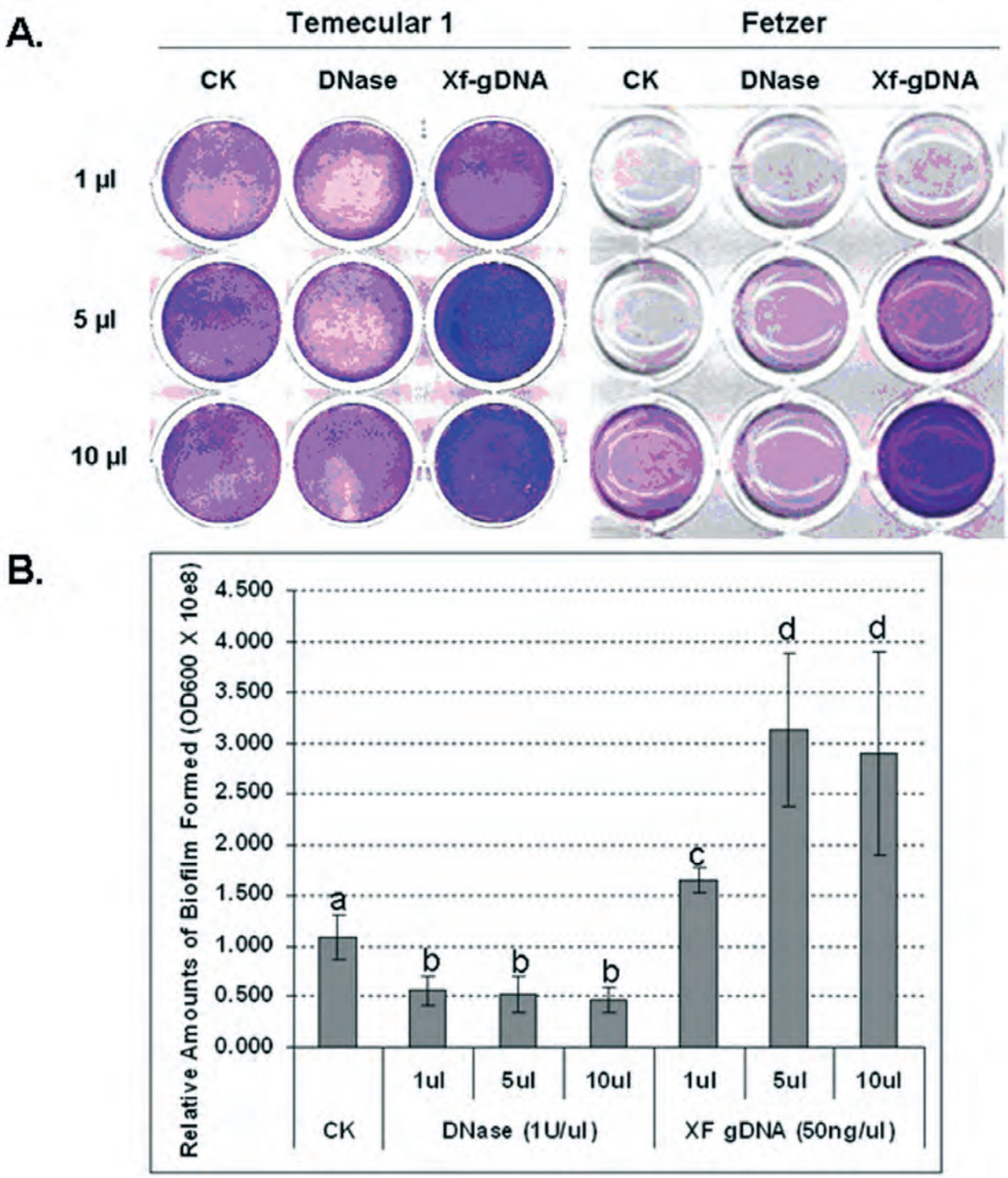

Fig. 2. Effects of DNase I and *Xf* genomic DNA on *Xf* biofim formation. A. Bioassay for *Xf* biofilm formation in the presence of various amounts of DNase I and *Xf* genomic DNA (*Xf*-gDNAs from two *Xf* strains Temecula-1 and Fetzer); CK, control. B. Quantitative analysis of the effects of DNase I and *Xf* genomic DNA treatment on *Xf* biofilm formation for *Xf* strain Temecula-1. The statistical significances (a, b, c and d) indicate that the differences are very significant among different groups.

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 the bacterial pili, flagellum-mediated motility and/or quorum sensing systems.

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

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REFERENCES

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**:1114-1128.

Almeida R.P., Purcell A.H., 2003. Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). *Journal of Economic Entomology* **96**: 264-271.

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**: 2331-2343.

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., Dubnau D., 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

Fritschi F.B., Lin H., Walker M.A., 2007a. *Xylella fastidiosa* Population dynamics in grapevine genotypes differing in susceptibility to Pierce's disease. *American Journal of Viticulture and Enology* **58**: 326-332.

Fritschi F.B., Lin H., Walker M.A., 2007b. Scanning electron microscopy reveals different plant-pathogen interaction pattern in four *Vitis* genotypes infected with *Xylella fastidiosa*. *Plant Disease* **92**: 276-286.

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 and 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**: 7785-7795.

Muto Y., Goto S., 1986. Transformation by extracellular DNA

produced by *Pseudomonas aeruginosa*. *Microbiology and Immunology* **30**: 621-628.

Newman K.L., Almeida R.P.P., Purcell A.H., Lindow S.E., 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**: 4392-4400.

Pitcher D.G., Saunders N.A., Owens 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**: 2083-2092.

Ramey B.E., Koutsoudis M., von Bodman S.B., Fuqua C., 2004. Biofilm formation in plant–microbe associations. *Current Opinion in Microbiology* **7**: 602-609.

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 E* **78**: 030701 (R).

Sutherland I.W., 2001. The biofilm matrix-an immobilized but dynamic microbial environment. *Trends in Microbiology* **9**: 222-227.

Tetz G.V., Artemenko N.K., Tetz V.V., 2009. Effect of DNase and antibiotics on biofilm characteristics. *Antimicrobial Agents Chemotherapy* **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.

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