EXPLOITING *XYLELLA FASTIDIOSA* PROTEINS FOR PIERCE'S DISEASE CONTROL

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ABSTRACT

The aim of this project is to construct and express in test plants, and then in grapevine, a protein or protein chimera ("anti-*Xf* protein") capable of inactivating or otherwise interfering with the infectivity of *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease of grapevine. Several *Xf*-cell-surface-binding peptides were selected from a random peptide library. For some of these peptides, the *Xf* cell target of binding and the stoichiometry of binding have been tentative identified. Evidence was obtained for a biologically relevant interaction between the selected peptides and *Xf* cells.

INTRODUCTION

It is likely that the development of grapevine cultivars resistant to *Xylella fastidiosa (Xf)* presents the best approach to long term, effective, economical and sustainable control of Pierce's disease (PD). Our strategy is to create transgenic rootstock(s) that will secrete a protein or proteins into the xylem for transport to scion xylem, where it will provide protection against insect vector-delivered *Xf*. An effective protein may kill *Xf* cells or merely interfere with the ability of *Xf* cells to colonize or spread in the scion xylem. Regardless of the mode of action, such proteins are here referred to here as anti-*Xf* proteins. No protein of the desired activity exists, and it is the immediate aim of this project to create anti-*Xf* protein(s). Several approaches have been taken. The approach that has been most productive is the selection of *Xf* cell-surface-binding peptides, as we describe in this report. Such peptides may be incorporated into a protein scaffold so as to generate a *Xf*-cell-surface binding protein. We have identified as a promising scaffold a protein of a T2-like bacteriophage: the tail fiber adhesion gp38 (Riede et al. 1987).

OBJECTIVES

1. Discover or develop peptides and proteins with high affinity for portions of MopB or other macromolecule that is displayed on the *Xf* cell exterior.
2. Test surface-binding proteins for their ability to coat *Xf* cells, for possible bactericidal activity or for interference with disease initiation following inoculation of grape or model plant with *Xf*.
3. In collaboration with the Gupta laboratory, develop gene constructions for chimeric proteins designed to bind tightly to and inactivate *Xf* cells; express and test the chimeric proteins for their effects on *Xf* cells in culture.
4. In collaboration with the Dandekar laboratory, prepare transgenic tobacco and grape expressing and xylem-targeting the candidate anti-*Xf* proteins; test the transgenic plants for resistance to infection by *Xf*

RESULTS

Objective 1 (Discover peptides and proteins with high affinity for macromolecules on the *Xf* cell).

Selecting peptides that bind to Xf cells

Xf cell-binding peptides were obtained by a combinatorial biology approach: selection from a random peptide library. The source of the random peptide library was a commercial kit (New England Biolabs "Ph.D.-12 Phage Display Peptide Library," designated here RP-M13) incorporating 12 amino acid residue random peptides at the amino end of the bacteriophage M13 adhesin protein pIII (Figure 1) (Anonymous 2004). The RP-M13 (~2.7 x 10^9 peptide sequences, with ~55 particles displaying any single peptide in a 10µL aliquot) was applied using "panning," a procedures involving multiple rounds (typically four or more) of selection in which the filamentous M13 particles bearing random peptides were exposed to the target (*Xf* cells). The target was washed, typically 8 times, and any remaining bound M13 was eluted and recovered, typically at pH 2.0-2.2. The eluted M13 was titered and amplified by inoculation of male *E. coli*. M13 progeny were partially purified before initiating the next round of selection (Smith and Scott 1993, Barbas et al. 2001).

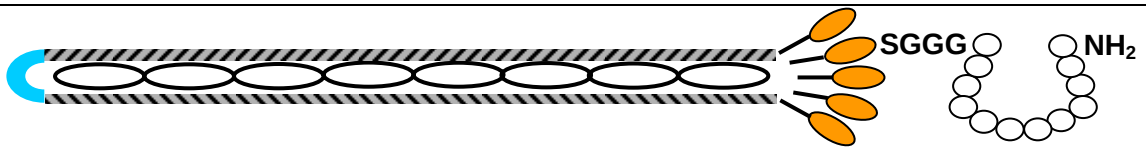
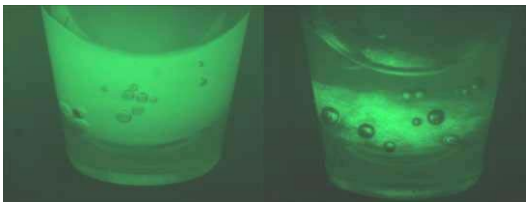


Figure 1. Diagram of a bacteriophage M13 random peptide library member. The random peptide 12-mer (open ovals) is incorporated at the amino end of the absorption protein (designated PIII or P3) of the M13 particle by a flexible tri-glycine-serine sequence and is encoded in the bacteriophage DNA. For simplicity only one of the 5 PIII proteins, orange oval, is shown with the random peptide amino end, although all five actually incorporate it.

Panning on the agar plate-derived and liquid culture (planktonic) cells proceeded differently. For the plate-derived cells, the titer increased about 10^2 at each cycle, and 14 of 20 clones obtained after 4 cycles were positive for binding to plate-derived *Xf* cells. For the planktonic cells, the titer increased only about 10^1 at each cycle, and only 1 of the first 20 M13 clones obtained after 5 cycles was found to be positive. Higher throughput methods for assaying M13 clones are in progress. Figure 2 presents an assay for *Xf*-cell-binding by selected M13 clones, and Table 1 presents results for several M13 clones.

Figure 2. *Xf* cells binding of 12-mer peptide-bearing M13. Each M13 clone preparation (~10^{11} particles, 2.7µg) was incubated with *Xf* cells, and the cells were washed three times to remove unbound M13 particles. Fluorescently labeled anti-M13 IgG was added. Left panel: no reaction detected; fluorescent antibody remains in solution (M13 clone 4N2). Right panel: binding detected by agglutination (M13 clone 4N1).



Of the nine M13 clones selected with plate-derived *Xf* cells, none were found to bind to planktonic cells. In contrast, the single M13 clone obtained with planktonic *Xf* cells as the target reacted with both plate-derived and planktonic cells. Thus we have isolated reagents that are plate-derived cell-specific (9 M13 clones of the 4N and 4T series), that are planktonic-cell-specific (antibody to MopB protein, an abundant outer membrane protein, data presented in our 2004 report), or that are able to recognize *Xf* cells of both origins (M13 clone 5-19). These results suggest that plate-derived and planktonic *Xf* cells have surface compositions that are largely, but not entirely, distinct.

Table 1. Binding of M13 peptide-bearing clones to *Xf* cells

Clone identifier (a)	Target	Amino acid sequence (b)	Wt *Xf* plate cells?	Wt *Xf* planktonic cells?	H*Xf*A-minus *Xf* plate cells? (c)	H*Xf*B-minus *Xf* plate cells? (d)
4N1, 4N5	A[d]	STLHRHTPDLRLGGGS	yes	no	no	yes
4N2	A	TLPPWITTMRYQGGGS	very weak	no	no	no
4N3	A	YDLWTMSPDFKLGGGS	yes	no	no	yes
4N4, 4T1, 4T7, 4T8	A	QIVTQNVPFILRGGGS	yes	no	ND	ND
4N6	A	IISHTPVIQLGRGGGS	yes	no	ND	ND
4T2, 4T6	A	NLVYTMSSDIPLGRGS	yes	no	no	yes
4T3, 4T9	A	WTLDLWAKPIDLGGGS	yes	no	no	yes
4T4a	A	TQMNLYTPALLLGRGS	yes	no	ND	ND
4T5	A	EAGNIVIRPFYAGGGS	yes	no	ND	ND
5-19	P[d]	ATSPTRLAALAQGGGS	weak	weak	no	no
FR	A	not a clone	no	ND	no	no

(a) Bold font designates the clone selected among duplicates for subsequent experiments; FR = first round selection, which presumably is only very poorly enriched in *Xf*-cell-binding proteins

(b) GGGS is the linker sequence between the 12-mer random peptide and the amino end of the M13 adhesin P3 (Fig. 1), although in two instances the sequence was found to be altered to GRGS

(c) H*Xf*A, H*Xf*B, products of *Xf* genes PD2118 and PD1792, respectively; *Xf* cell strains with inserts in these strains were provided by Tanja Voegel and Bruce Kirkpatrick (Guilhabert and Kirkpatrick 2005)

(d) A = cells cultured on and recovered from agar plates ("plate cells"; PD3 medium); P = planktonic cells from liquid culture (PD3)

ND: not determined

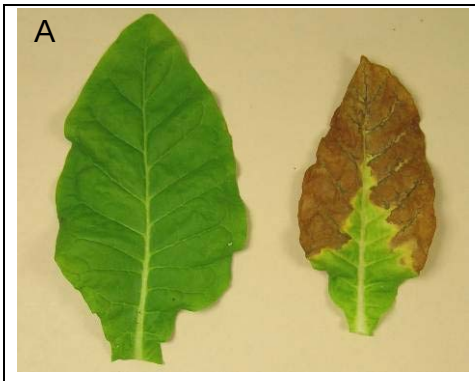
Investigations of the possible Xf cell surface target and binding extent for M13 clone-Xf cell interactions.
Plate-derived *Xf* cells may be "exopolysaccharide coated." Therefore, we tested for the ability of "fastidian gum" (gift from L. Ielpi via C. Roper and B.C. Kirkpatrick), the postulated exopolysaccharide material of *Xf* (da Silva et al. 2001), to interfere with the agglutination assay for M13 binding (Figure 2). M13 bacteriophage (10^{11} particles, about 2.7μg) was exposed to 25μg of fastidian gum in 100μL of buffer for 45 minutes before addition of *Xf* cells. No interference in the agglutination reaction was observed, suggesting that fastidian gum is not involved in the *Xf* cell interactions with the selected M13 clones. Results from other experiments suggest that MopB, likely the major outer membrane protein of *Xf*, also is not the target on the *Xf* cell surface to which any of our M13 clones bind. Other potential targets for the M13 clones are two hemagglutinin-like proteins of *Xf*, H*Xf*A and H*Xf*B (Guilhabert and Kirkpatrick 2005). We did not detect binding of any of our M13 clones to mutant *Xf* cells with an inactivated *HXfA* gene, whereas *Xf* cells with an inactivated *HXfB* gene bound to all of the tested M13 clones that exhibited strong binding to wildtype *Xf* cells (Table 1).

In order to obtain a lower bound estimate of the number of M13 particles bound to an *Xf* cell, a suspension was prepared of 2 x 10^8 *Xf* cells from agar plates and 2 x 10^{12} pfu/mL of either M13 clone 4N1 or M13 clone 4N2. The suspension was incubated for 1 hr at room temperature, and the cells were recovered and washed three times with the buffer that was used in the panning experiments. Plaque assays were performed on the last wash and after a pH 2.2 elution. For 4N1, about 1000 pfu of M13 was recovered per *Xf* cell after pH 2.2 elution, whereas the value for 4N2 (known to bind to *Xf* cells poorly in the Figure 2 assay) was under 100.

Objective 2 (test proteins for interference with disease initiation)
The usual approach for the application of selected, target-binding random peptides is to incorporate them into a scaffold protein for testing against the target. We expect to have a large number of selected peptides and therefore need a less elaborate approach to evaluating their potential efficacy under biologically relevant conditions. Others have selected RP-M13 clones that bind to bacterial cells by panning and have observed the binding of the M13 bacteriophage particles to the target cells by electron microscopy (Petrenko and Sorokulova 2004). *Xf* cells were mixed with each of the six M13 clones (4N1, 4N2, 4N3, 4T2, 4T3, 5-19) as described above, incubated, and then inoculated to SR-1 tobacco plants. No M13 clone was able to prevent infection of the tobacco plants. However, interference with *Xf* infection, i.e., a potentially relevant biological activity, was observed for the set of M13 clones taken as a group (Figure 3). At the greater of two concentrations of M13 tested (3 x 10^{12} pfu/mL), the disease rating was significantly reduced ($p=0.0003$ assuming null hypothesis) compared to the average disease rating for plants receiving *Xf* alone. The M13 molar excess over *Xf* cells, about 10^4-fold, corresponds in magnitude to the number of copies of some abundant bacterial cell-surface proteins that could be sites for binding and is only 10-fold greater than the estimate for 4N1 M13 clone binding to *Xf* cells as observed above.

The observed interference with *Xf* infectivity supports the feasibility of the overall approach being taken in this project. However, it is important to note that Figure 3 reports results from a single experiment and, though the results are statistically significant, the experiment must be repeated to be convincing. The observed effect is small and presumably will require more effective peptides and/or incorporation of binding peptides into bactericidal constructions to create a more powerful anti-*Xf* technology.

CONCLUSIONS
The approach to solving the PD problem that is taken in this project is to create transgenic grape rootstock that will confer, on the scion, protection against infection by *Xf*. Otherwise, it is expected, the new rootstock will cause no alteration in the agronomic or quality traits of the scion compared to the situation of scion propagation on conventional rootstock. Rootstock-conferred protection is to be accomplished by expression of a xylem-targeted anti-*Xf* protein, the creation of which is the current focus of the project. Results reported here suggest that we have selected *Xf*-cell-surface-binding peptides, that these peptides have a perceptible capability for interfering with *Xf* infection of SR-1 tobacco plants (Figure 3), and therefore that a biologically relevant and at least transient interaction can occur between the selected peptides and *Xf* cells *in planta*. Our results also (i) suggest that a single *Xf* cell can bind at least 1000 peptide molecules, (ii) tentatively identify the target molecule for several of our selected peptides as the hemagglutinin-like *Xf* surface protein H*Xf*A, (iii) reveal peptide consensus sequences possibly involved in the interaction (blue font and blue underline in Table 1), and (iv) suggest that planktonic cells cultured under at least one condition do not have a significant population of exposed H*Xf*a molecules on their surface (compare columns 5 and 6 of Table 1). It is likely that the form of *Xf* cell that is released by the sharpshooter as it inoculates the plant resembles planktonic *Xf* cells more than it resembles plate-derived *Xf* cells. Therefore, we plan to discover additional peptides with affinity for planktonic cells, to identify those showing activity *in planta*, and to incorporate these peptides into suitable scaffold/fusion proteins for generation of more effective anti-*Xf* activity.



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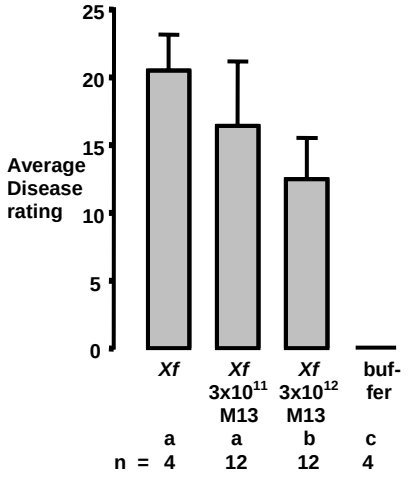

Figure 3. Biological activity of M13 clones selected for their affinity to the surface of *Xf* cells. Six M13 clones were tested for their ability to interfere with *Xf* infection of SR-1 tobacco, using six M13 clones and two plants per clone. The SR-1 plants were cut back to three leaves per plant. Temecula 1 *Xf* cell suspension from agar plates at ca. 2.4 x 10^8 cells/mL was mixed in 0.5X TE buffer (5 mM Tris-HCl pH7.5, 0.5 mM sodium EDTA) with M13 at 3 x 10^{11} or 3 x 10^{12} pfu/mL. After one hour, 20 µL of cell suspension was inoculated to the base of the petiole of each of the three leaves. The course of disease development was observed. Chlorosis on some leaves was observed at 3-4 weeks after inoculation, and symptoms typical of *Xf* diseases developed: scorching expanding from the leaf tip or other localized areas and separated from green tissue by a bright yellow halo. Symptoms were demonstrated to be correlated with the presence of *Xf* by quantitative PCR. At three months after inoculation, each leaf was assigned a disease rating as follows: scores of 1, 2 (leaf on right, panel A) or 3 for scorching symptoms accounting for <50%, 50-75%, and >75% of the leaf lamina area, respectively; no symptoms characteristic of *Xf* infection (leaf on the left, panel A), score of zero. The scores of all of the leaves of each plant were summed to give the disease rating of the plant. The average and standard deviation for four groups of plants are presented in panel B, with the number of plants per group at the bottom of the panel. Plants inoculated with buffer only or untreated showed no symptoms characteristic of *Xf* infection. As would be expected, the disease ratings for two plants receiving the same inoculum (root-mean-square difference 3.2) were more similar than they were for a pair of plants selected at random (standard deviation 4.7). The disease ratings were significantly different ($p<0.05$, Student's t-test) for plants receiving *Xf* exposed to 3 x 10^{12} Pfu/mL M13 as compared to the other three groups (lower case letters below bars).

REFERENCES

Anonymous. 2004. Ph.D.™ Phage Display Peptide Library Kits, Tech Bulletin E8111, pp. 4, http://www.neb.com/nebecomm/TechBulletinFiles/techbulletinE8111.pdf. New England BioLabs, Ipswich, MA 01938-2723.

Barbas, C. F., J. K. Scott, G. Silverman, and D. R. Burton. 2001. Phage Display: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, NY.

da Silva, F. R., A. L. Vettore, E. L. Kemper, A. Leite, and P. Arruda. 2001. Fastidian gum: the *Xylella fastidiosa* exopolysaccharide possibly involved in bacterial pathogenicity. Fems Microbiology Letters 203: 165-171.

Guilhabert, M. R., and B. C. Kirkpatrick. 2005. Identification of *Xylella fastidiosa* antivirulence genes: Hemagglutinin adhesins contribute to *X-fastidiosa* biofilm maturation and colonization and attenuate virulence. Molecular Plant-Microbe Interactions 18: 856-868.

Petrenko, V. A., and I. B. Sorokulova. 2004. Detection of biological threats. A challenge for directed molecular evolution. Journal of Microbiological Methods 58: 147-168.

Riede, I., K. Drexler, H. Schwarz, and U. Henning. 1987. T-even-type bacteriophages use an adhesin for recognition of cellular receptors. Journal of Molecular Biology 194: 23-30.

Smith, G. P., and J. K. Scott. 1993. Libraries of peptides and proteins displayed on filamentous phage. Methods in Enzymology 217: 228-257.

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HOST PLANT PREFERENCE AND NATURAL INFECTIVITY OF INSECT VECTORS ON COMMON WEEDS KNOWN TO HOST *XYLELLA FASTIDIOSA*

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ABSTRACT
Common weed species can harbor *Xylella fastidiosa* (*Xf*) and its insect vectors. Should weed control be part of a Pierce's disease control program? To address this question, we will survey weed species in agricultural areas known to host *Xf,* and determine the level of insect vector activity and the proportion of potential vectors that carry *Xf*. In the laboratory, we will compare three techniques to detect *Xf* in insects. Current methods produce mixed results; assessment of each method's accuracy will improve comparison of research projects and field survey results. This project will provide information for control decisions by investigating the importance of vegetation management in reduction of insect populations and inoculum potential.

INTRODUCTION
The emergence of Pierce's disease (PD) of grape in the General Beale Road area in Bakersfield in 2001 and 2002 exemplifies the threat posed by the glassy-winged sharpshooter (GWSS). While GWSS populations and PD are currently managed by an area-wide insecticide spray program and diseased vine removal, endemic GWSS populations are still present. This may be especially true in weedy fields, abandoned vineyards, and along roadsides and windbreaks where PD and GWSS are not managed (J. Hashim - personal communication). Numerous common weeds and windbreak species are hosts of *Xylella fastidiosa* (*Xf*) in greenhouse studies (Purcell and Saunders 1999, Costa et al. 2004, Wistrom and Purcell 2005). More importantly, nine weed species common in the Central Valley were found to be naturally infected with *Xf* (Shapland et al. 2006). Since so many different plants can harbor *Xf* to some extent, more information on sharpshooter host plant use in the field is required before those studies can be translated into concrete recommendations to growers. While GWSS have been observed feeding on a wide range of ornamental and weedy species (CDFA host list at www.cdfa.ca.gov/phpps/pdcp/index), the quantitative data on the numbers and host plant preference of GWSS in agricultural settings focused mainly on presence on citrus, grapes and urban areas, with insect choice determined on plants provided in pots (Naranjo and Toscano 2003; Perring and Gispert 2004; Daane and Johnson 2004; Phillips et al. 2004).

The first objective of this study is the identification of preferred feeding and oviposition hosts by GWSS, among plants already identified as hosts of *Xf* in agricultural areas. Year-round information on sharpshooter presence on host plants would provide information about the need for vegetation removal or modification in and around vineyards. For example, the identification of major breeding hosts of blue-green sharpshooters (BGSS) in northern California enabled the development of a riparian management plan. When the major breeding and feeding hosts of BGSS were removed and replanted with other plants less attractive to BGSS, large reductions in sharpshooter populations and Pierce's disease in adjacent vineyards resulted (Purcell et al. 1999). Similarly, in Central Valley almond orchards affected by almond leaf scorch, the identification of common sharpshooter, treehopper, and spittlebug insect vectors has just been completed (Daane et al. – unpublished data), as well as concurrent assessment of *Xf* presence in sampled weeds and sharpshooters (Shapland et al. 2006).

Field-based data is critical for practical application of treatment thresholds in development (Perring 2004), for GWSS control in areas with endemic sharpshooter populations. One important variable in the infectivity model is the proportion of vectors carrying the pathogen. This second objective of this study will determine the proportion of field-collected sharpshooters, in the San Joaquin Valley, that carry *Xf* in agricultural areas. With a functional treatment threshold, growers can predict the relationship between GWSS population and Pierce's disease potential, and better plan insecticide applications for GWSS control. The natural infectivity of BGSS captured in riparian area was highly variable, ranging from 5 to >40% (A. Purcell -