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 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 (PD) of grapevine. As reported previously, we were able to select several *Xf*-cell-surface-binding peptides, and some of these showed marginal ability to interfere with *Xf* infection of grapevine. Although this result is encouraging, in this period we have aimed at discovering proteins, rather than peptides, that can bind to the *Xf* cell surface, in order to achieve greater affinity and more potent interference with *Xf* infectivity. Other observations revealed that an activity of *Xf* cells which results in the development of chlorosis in leaves of *Chenopodium quinoa* is due to the translation elongation factor EF-Tu, which in *Xf* is found in an unusual aggregated or conjugated form unlike the soluble form that EF-Tu takes in most other bacteria.

INTRODUCTION
It is likely that the development of grapevine cultivars resistant to *Xf* presents the best approach to long term, effective, economical and sustainable control of 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 is expected to provide protection against insect vector-delivered *Xf*. An effective anti-*Xf* protein may kill *Xf* cells or merely interfere with their ability to colonize or spread in the scion xylem. 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. We reported (Bruening et al. 2006) the selection of *Xf* cell-surface-binding peptides and showed that some selected peptides were able, marginally, to interfere with *Xf* infection when mixed with *Xf* cells prior to inoculation of grapevine. Stronger interaction, as likely can be provided by an *Xf* cell-surface-binding protein or protein-domain, should prove to be more potent in bioassays. We reported that amino acid sequences of motifs found in selected *Xf* cell-surface-binding peptides were consistent with interactions between *Xf* cell-surface proteins. Therefore, a strategy for identifying *Xf*-cell-surface-binding protein domains is to search a library of *Xf* cell-surface-protein domains. Given the power of panning procedures using bacteriophage M13 libraries and the small size of the *Xf* genome, there is no need to limit a library to protein domains from *Xf* cell-surface proteins; libraries from random fragments of total *Xf* DNA should be suitable. The mechanisms by which *Xf* induces symptoms in infected grapevine have not been established. In the second subsection of Results we extend earlier research on the reaction of plants to invasion by *Xf* to study potential virulence factors derived from *Xf*. Virulence factors are potential targets for interfering with *Xf* infection.

OBJECTIVES
1. Discover or develop peptides and proteins with high affinity for 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 grapevine 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 against *Xf* cells in culture and in plants.
4. In collaboration with the Dandekar laboratory, prepare transgenic tobacco and grapevine expressing and xylem-targeting the candidate anti-*Xf* proteins; test the transgenic plants for resistance to infection by *Xf*

RESULTS
Selection of protein domains with high affinity for the *Xf* cell surface
Figure 1 describes the system we have developed for displaying domains of *Xf* proteins in the P3 protein of bacteriophage M13. In bacteriophage M13 panning procedures, a population (library) of M13 virions is created such that small sets of individual virions have the same displayed peptide, protein domain or protein, but there are many (10^4 to 10^9) such sets, each with a different display encoded in the bacteriophage M13 genome of the set. The entire population is exposed to the target,

in this case *Xf* cells. After extensive washing, M13 virions are released by exposure of the cells to pH 2.2 buffer. The released M13 virions are increased by infection of *E. coli* cells, and the panning cycle is repeated. To the extent that the original M13 population can be limited to members whose infectivity is dependent on inserts, the process of selection becomes more efficient and the chances of success are greatly enhanced. In the scheme outlined in Figure 1, two steps were designed to prepare M13 library members with the desired characteristics. A version of the gene for the P3 protein was prepared with a frameshift mutation. Insertion of a *Xf* DNA fragment capable of restoring the reading frame register is necessary to create a fusion protein with a full length P3 sequence. No insert or any insert that does not restore the reading frame register should result in a non-functional P3. The P3 protein is mobilized into complete bacteriophage M13 virions by inoculation of *E. coli* cells bearing the P3-encoding plasmid with the helper bacteriophage M3 described in Figure 1. Treatment of the resulting M13 virions with 27K protease will remove the N and M domains of the P3 protein derived from the helper bacteriophage, leaving the virions dependent on the P3 fusion protein derived from the plasmid. Tests show reductions in titer to 10^{-4} of the control value for 27KPro treatment of bacteriophage M13 with two cleavage sites as described in Figure 1.

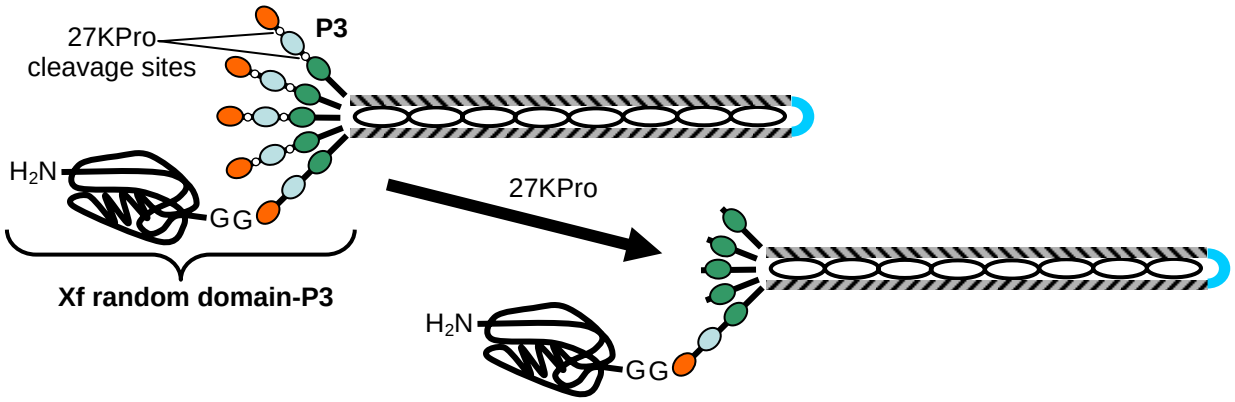

Figure 1. A solution to the problem of background infectivity during panning experiments with protein domains substituted into bacteriophage M13 protein P3. Random fragments of *Xf* DNA were inserted into a site in an out-of-reading-frame register (not infectious) version of bacteriophage M13 protein P3. About 1 in 18 such inserts can be expected to restore the reading frame with sequence of the sense polarity, and a fraction of these will contain *Xf* protein domain-encoding sequences possibly compatible with M13 infectivity. There are five P3 proteins per wildtype bacteriophage particle. To avoid steric and avidity effects from multiple copies of the *Xf* protein domain, ideally there would be only a single copy of that domain per M13 particle, and infectivity of the particle would be dependent on the presence of that P3 protein molecule. Conditions can be arranged so that most of the P3 molecules of a bacteriophage M13-infected cell are provided by a helper bacteriophage M13. The helper bacteriophage M13 was engineered to insert, into the P3 gene, sequences encoding two cleavage sites for the *Tobacco etch virus* 27K protease (27KPro), one site between the N domain (orange) and the M domain (blue), and one between the M domain and the C domain (green). Infectivity of M13 requires at least one P3 protein with intact N and M domains. A library of *E. coli* cells bearing a plasmid with *Xf* DNA fragment inserts is inoculated with the engineered helper bacteriophage M13. The progeny bacteriophage virions are treated with 27KPro to inactivate virtually all of the P3 molecules that do not bear an insert derived from *Xf* DNA.

Potential virulence factor(s) of *Xf*

Based on electrophoretic mobility, MopB, which likely is the major outer membrane protein of *Xf*, was tentatively identified as a chlorosis-inducing factor in pressure-infiltrated *Chenopodium quinoa* leaves. A procedure for partial purification of MopB was developed. When the MopB preparations were analyzed on a 10% or 11% polyacrylamide gel, rather than the more usual 12.5% gel, a faint band was observed trailing the main MopB band and was found to contain the *Xf* protein-synthesis-elongation-factor designated "temperature unstable" (EF-Tu). Material from this trailing band induced chlorosis in *C. quinoa*, and *Xf* MopB produced in transformed *E. coli* failed to induce chlorosis in *C. quinoa*. These observations suggest that the chlorosis-inducing factor in our MopB preparations may be *Xf* EF-Tu and not MopB. EF-Tu is one of a small number of highly conserved eubacterial proteins ("pathogen-associated molecular pattern," PAMP) that have been discovered to induce defense responses in a variety of plants (Jones and Dangl 2006). Subnanomolar *E. coli* EF-Tu was found to induce alkalization in the medium of cultured *Arabidopsis thaliana* cells and to induce at 1 μM in pressure-infiltrated Arabidopsis leaves resistance to *Pseudomonas syringae* and the accumulation of defense gene mRNAs (Kunze et al. 2004). *E. coli* EF-Tu has been demonstrated to be amino-end-N-acetylated, which is unusual for a prokaryotic protein. (Kunze et al. 2004) also demonstrated that peptides corresponding to the first 18-26 amino acid residues of *E. coli* EF-Tu had the biological activities of the intact protein. Subnanomolar concentrations of EF-Tu amino end peptides from four plant pathogenic bacteria all induced alkalinization of Arabidopsis cell medium, whereas the corresponding peptides from *P. syringae* and *Xf* required a ~100X greater concentration. The recognition of EF-Tu was specific for brassicas among the plants tested (Kunze et al.

2004). The gene for the Arabidopsis EF-Tu receptor, ERF, when expressed in *N. benthamiana* leaves, conferred the ability to bind the EF-Tu amino-end epitope (Zipfel et al. 2006). Arabidopsis *erf* mutants showed enhanced disease susceptibility.

E. coli EF-Tu and *Xf* EF-Tu are 77% identical and 88% similar in amino acid sequence. The regions of identity between the *E. coli* and *Xf* EF-Tu sequences also showed >90% identity with >100 eubacterial sequences (Kunze et al. 2004). We found purified *E. coli* EF-Tu to be a strong inducer of chlorosis in pressure-infiltrated *C. quinoa* leaves (Figure 2, A-C). However, when we attempted to purify EF-Tu from *Xf*, we found that the bulk of the *Xf* EF-Tu and the bulk of the *E. coli* EF-Tu have different states of association. Procedures for purifying *E. coli* EF-Tu are not applicable to *Xf* EF-Tu. When *E. coli* cell suspension or crude extract of *E. coli* cells was heated in Laemmli SDS/mercaptoethanol disruption solution, as for SDS gel electrophoresis, antibody against *E. coli* EF-Tu detected a band with a mobility corresponding to the expected molecular weight of 43K (Figure 3, lanes 3', 4 and 5). Contrary to our expectation, we did not observe a band at 43K in immunoblots to which extract of *Xf* cells had been applied (Figure 3, lanes 1' and 2'). Rather, we found immunoreactive material in the gel well that adhered so poorly that streaks sometimes were observed emanating from the well. Weakly alkaline 10 M urea-SDS-DTT was more effective than Laemmli disruption solution in extracting the anti-EF-Tu immuno-positive material; prior treatment of the *Xf* cells with lysozyme did not increase the amount or mobility of immuno-positive material (data not shown).

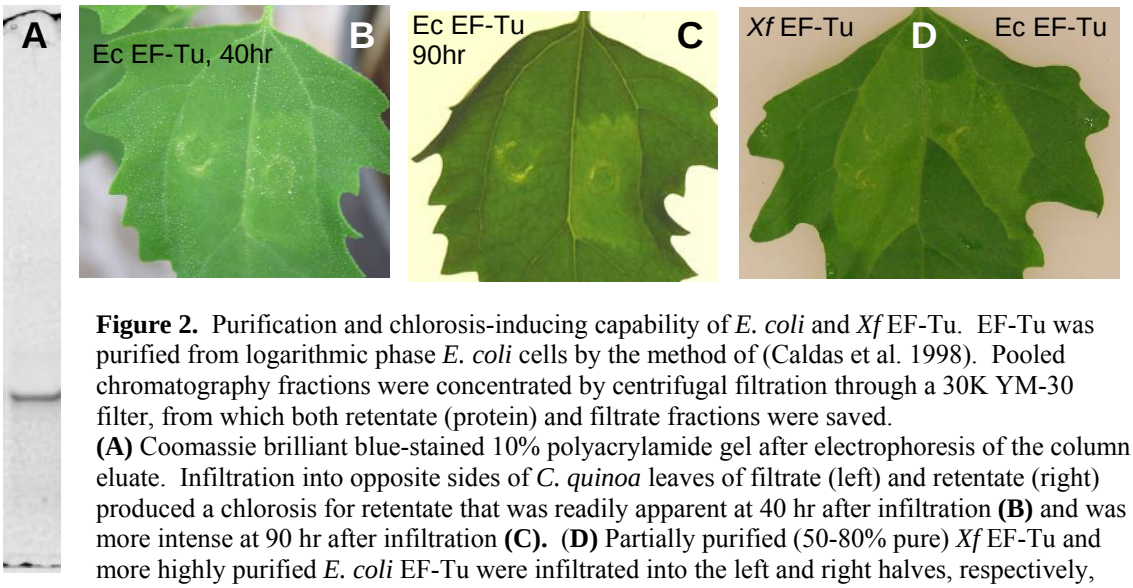

Figure 2. Purification and chlorosis-inducing capability of *E. coli* and *Xf* EF-Tu. EF-Tu was purified from logarithmic phase *E. coli* cells by the method of (Caldas et al. 1998). Pooled chromatography fractions were concentrated by centrifugal filtration through a 30K YM-30 filter, from which both retentate (protein) and filtrate fractions were saved.
(A) Coomassie brilliant blue-stained 10% polyacrylamide gel after electrophoresis of the column eluate. Infiltration into opposite sides of *C. quinoa* leaves of filtrate (left) and retentate (right) produced a chlorosis for retentate that was readily apparent at 40 hr after infiltration **(B)** and was more intense at 90 hr after infiltration **(C).** (**D)** Partially purified (50-80% pure) *Xf* EF-Tu and more highly purified *E. coli* EF-Tu were infiltrated into the left and right halves, respectively, inducing a similar chlorosis. The infiltrated protein corresponded to about 20 pmole of EF-Tu.

Centrifugation at 90,000 rpm for 90 min distributed the immuno-positive material between the supernatant and a clear, gelatinous precipitate (compare lanes 1' and 2', Figure 3). The fact that part of the immuno-positive material sedimented suggests that buoyant density is not impairing sedimentation. Therefore, *Xf* EF-Tu may be part of a very asymmetric structure of aggregated EF-Tu molecules or EF-Tu molecules conjugated to other molecules. These results suggest that a fundamental difference exists between the *Xf* and *E. coli* EF-Tu molecules. We postulate that *Xf* EF-Tu may occur predominantly as an aggregate that is exposed on the cell surface, where it can be detected by a plant equipped with the appropriate receptor(s). *Mycoplasma pneumoniae* and *Lactobacillus johnsonii* appear to use EF-Tu as an adhesion that is responsible for the binding of these bacteria to human cells, and, in the case of *M. pneumoniae*, antibody to EF-Tu was demonstrated to interfere with attachment to human cells (Dallo et al. 2002, Granato et al. 2004). *Xf* EF-Tu may have a similar function. As a model for EF-Tu on the surface of an *Xf* cell, we prepared a construction designed to place *Xf* EF-Tu on the surface of *E. coli* cells. The plasmid construction encodes an EF-Tu-P3 (Figure 1). In the absence of other bacteriophage M13 components, P3 expressed alone accumulates in outer membrane. In *E. coli* cells transformed to express P3 (calculated molecular weight 44.6K), a band of the appropriate mobility was observed after gel electrophoresis, immunoblotting and incubation with monoclonal antibody to P3, whereas no band was detected in the gel for the EF-Tu-P3 (calculated molecular weight 87.5K) fusion construction (data not shown). *Xf* EF-Tu-P3 fusion behaved like *Xf* EF-Tu from *Xf* cells (Figure 3, compare lane 5 with lane 4), whereas *E. coli* EF-Tu showed its expected mobility in the gel (Figure 3, lanes 3', 4 and 5). Partially purified *Xf* EF-Tu and nearly pure *E. coli* EF-Tu induced a similar chlorosis in *C. quinoa* (Figure 2, D).

CONCLUSIONS

Based on results with *Xf*-cell-binding peptides, selection of *Xf*-cell-binding proteins likely will provide reagents capable directly, or as conjugates with other protein motifs, of interfering with *Xf* replication. These proteins are to be expressed in grapevine rootstock for transport into the xylem for targeting of *Xf* cells. The bulk of the protein EF-Tu of *Xf* accumulates in

an aggregated or conjugated form that presumably is incompatible with EF-Tu function in protein synthesis. A few eubacteria enlist EF-Tu for cell-surface adhesion, and some plant species recognize the presence of bacteria by detecting EF-Tu. *C. quinoa* apparently recognizes EF-Tu from *Xf* and *E. coli*. Therefore, it is reasonable to suggest that the unusual form of EF-Tu in *Xf* may mediate pathogenesis or symptom induction in grapevine.

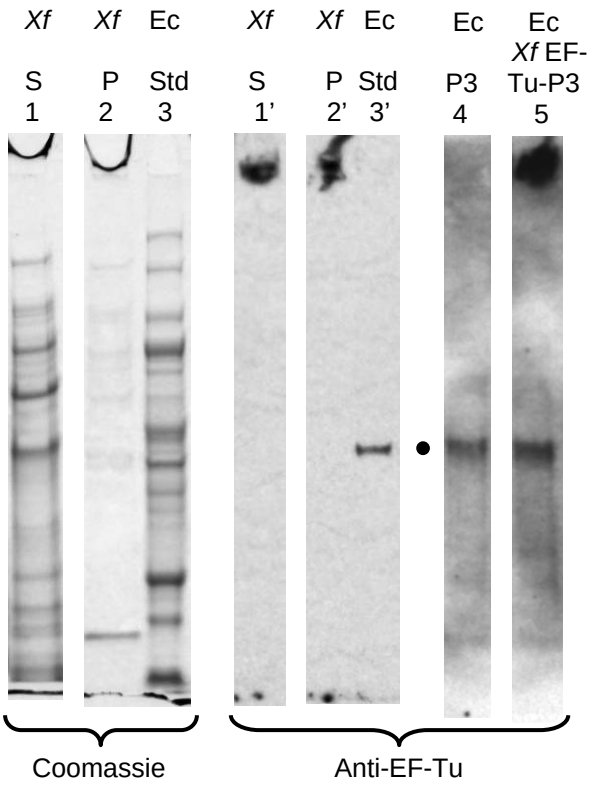

Figure 3. *Xf* EF-Tu and of *E. coli* EF-Tu show distinct properties. Lanes 1-3 are from a Coomassie brilliant blue-stained gel; immunoblots (lanes 1'-3', 4 and 5) were visualized using goat anti-EF-Tu (*E. coli*) IgG (gift from Dr. David Miller, Institute for Basic Research in Developmental Disabilities, Staten. Island, NY), rabbit anti-goat IgG coupled to horse radish peroxidase, and "supersignal" (Pierce) substrate. Mutant HxfB-*Xf* cells grown in liquid culture were extracted with 10 M urea, 0.1 M dithiothreitol (DTT), 6 mg/mL SDS, 96 mM Tris-HCl buffer, pH~8.7, at 90-95˚C for 4 min. Lanes 1 and 1' received an aliquot of the supernatant, and lanes 2 and 2' the precipitate, from a 90,000 rpm, 90 min centrifugation of the extract. Lanes 3 and 3' received colored protein standards mixed with a 1 hr, 100,000 x g supernatant of lysozyme-treated *E. coli* cells (Cull and McHenry 1990, Caldas et al. 1998). Samples for lanes 4 and 5 were *E. coli* cells collected by centrifugation. The *E. coli* strains for lanes 4 and 5, respectively, were transformed to express bacteriophage M13 protein P3 and a *Xf* EF-Tu-P3 fusion. All samples were heated in Laemmli SDS/mercapto-ethanol disruption buffer before electrophoresis through 10% (lanes 1-3; 1'-3', gels run simultaneously) or 12.5% (lanes 4-5, same gel) polyacrylamide gel. The dot between lanes 3' and 4 locates the expected position for EF-Tu. *Xf* EF-Tu from wildtype *Xf* cells grown on solid medium gave results similar to those for HxfB- cells from liquid culture (data not shown).

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