Final Report for CDFA Contract Number 02-0149

Project title: **Exploiting *Xylella fastidiosa* Proteins for Pierce's Disease Control**

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Period of Research Reported

This final report covers the period 1 July 2002 – 30 June 2009, which includes no-cost extensions.

Project History

CDFA 02-0149 was first awarded 1 July 2002 and was continued on 1 July 2003 and 1 July 2004. The title "Roles of *Xylella fastidiosa* Proteins in Virulence" was changed to the title given above to reflect the more targeted character of the 1 July 2005 award, which was for 3 years. A no-cost extension carried the project to 30 June 2009.

Abstract

The goal of this project has been to interfere with *Xylella fastidiosa* (Xf) infections and Xf-induced symptoms of Pierce's disease. The primary approach is to create gene constructs encoding anti-Xf proteins and introduce them as transgene products into xylem elements. Transformed rootstocks are to target the anti-Xf proteins to the xylem for transport into the xylem of the scion, where their action against infecting Xf is expected. An anti-Xf protein may be able to act merely by binding to the Xf cell surface, or a fusion of the binding protein to an anti-microbial peptide may be necessary. The prerequisite for this approach is the discovery or development of peptides and small proteins that have an affinity for the Xf cell surface. In the early phase of this project, we discovered that Xf cells, heat killed or not, induced a chlorotic reaction when pressure infiltrated into the leaves of *Chenopodium quinoa* plants. Treatment of the Xf cells with any of three proteases or other protein-damaging reagents inactivated the chlorosis-inducing activity, suggesting that Xf cells possess a protein elicitor that is recognized in the intercellular spaces of *C. quinoa*. A sodium dodecyl sulfate (SDS) extract of Xf cells was resolved by gel

electrophoresis. Protein extracted from one band of the gel was associated with the chlorosis-inducing activity, and the most abundant protein component of that protein extract was MopB, a protein previously known only as an annotated protein of the Xf genome sequence. We identified the amino-end residue of MopB from Xf cells as pyroglutamate, which allowed us to correct the original annotation of the MopB gene by properly locating its signal peptide. The abundance of MopB and its crystal packing density suggest that MopB accounts for about 10% of the Xf cell surface.

Procedures we developed for purifying and fractionating MopB did not remove the chlorosis-inducing activity which, however, was not coincident with the distribution of MopB in some fractionations. A minor trailing electrophoresis band from a Xf cell-membrane preparation was identified as containing the Xf translation elongation factor temperature unstable (EF-Tu). Results from subsequent experiments strongly suggest that Xf EF-Tu, not MopB, is the chlorosis-inducing factor of Xf cells infiltrated into *C. quinoa* leaves. Others demonstrated that the EF-Tu proteins of several eubacteria are MAMPs (microbial associated molecular patterns, recognizable by specific plant species as a signal of eubacterium invasion). In many bacteria, EF-Tu is an abundant, soluble protein. We found affinity purified *E. coli* EF-Tu to activate a promoter that has been demonstrated by others to be specifically activated by Xf infection of grapevine. Xf EF-Tu differs from a typical eubacterial EF-Tu because it is associated with insoluble, rather than soluble, fractions in extracts of the cell. The insolubility of Xf EF-Tu prevented us from obtaining this protein in highly purified form. The unusual character of Xf EF-Tu has been pursued in our subsequent work.

Mop B, EF-Tu, and any other putative surface component of the Xf cell are potential targets for anti-Xf proteins. Peptides were selected from a bacteriophage M13 random peptide library for their ability to bind to Xf cells. We found that, among these were peptides, some were able to distinguish Xf cells grown in liquid culture from Xf cells grown on and scraped from the surface of nutrient agar. Others distinguish wildtype cells from cells with mutant outer membrane proteins. We tested several tobacco lines for their ability to serve as an experimentally amenable surrogate for grapevine in the assay of peptides and proteins for possible anti-Xf activity. *Nicotiana tabacum* line SR-1 proved to be readily infected with Xf and to produce scorch symptoms that are characteristic of Xf infections in many species, including grapevine. Bacteriophage particles from several of the selected M13 lines, each bearing a different Xf-cell-binding peptide, were mixed with Xf cells prior to inoculation of SR-1 tobacco plants. The disease ratings were significantly reduced ($p<0.05$) for tobacco plants inoculated with Xf that had been exposed to the highest M13 concentration, 3×10^{12} pfu/mL, compared to plants inoculated with Xf cells alone. Although the protective effect of M13 peptides was weak, the results were statistically significant and showed that peptide binding to the Xf cell surface alone, without fusion of the peptide to an antimicrobial peptide, interfered with infection by Xf.

Compared to selected peptides, selected proteins would be expected to have greater affinity for the Xf cell surface. We selected bacteriophage lines from a bacteriophage library bearing single-chain antibodies by binding to Xf cells. Of >10 lines examined, all proved to have the same selected sequence. The target of the bacteriophage-borne single-chain antibody proved to be amorphous material in the Xf cell preparations, apparently formed by or from Xf cells but not corresponding to intact cells. The selected single-chain antibody appears to bind to a specific protein of the amorphous material, as evidenced by immunoblot analysis using the corresponding bacteriophage particles in place of the usual primary antibody. None of the peptides or single-chain antibodies appears to bind to MopB.

Objectives

This project grew out of the observation of Bruening and Civerolo that live or killed *Xylella fastidiosa* (Xf) strain Temecula 1 cells, when pressure infiltrated into *Chenopodium quinoa* leaves or leaflets, induce a chlorosis that is confined to the infiltrated area. The chlorotic reaction is the result of chloroplast

bleaching without significant loss of chloroplasts or chloroplast viability (observation of Prof. Judy Jernstedt, UC Davis Plant Sciences Department). The chlorosis-initiating activity was associated with a major band on polyacrylamide gels to which protein fractions extracted from Xf cells had been applied. This major band was demonstrated to have, as its most abundant component, the Xf outer membrane protein MopB. MopB previously had been known only as a protein predicted by the Xf genome sequence. *E. coli* has a major outer membrane protein designated OmpA, and the Xf MopB amino acid sequence places MopB in the OmpA class of outer membrane proteins. *E. coli* OmpA has been studied extensively and is known to display polypeptide loops on the cell surface. The surface-exposed portion of OmpA is known to interact with molecules in the environment of *E. coli*, including the adhesins of certain bacteriophages. That is, OmpA has been demonstrated to act as the *E. coli* cell surface absorption sites which specific bacteriophages use to initiate the process by which they infect the bacterium. Interactions of OmpA with other molecules, exterior to the cell, also have been demonstrated. MopB and OmpA show similar predicted (and, in the case of OmpA, experimentally determined) secondary structure for their N-terminal domain, which includes the externally displayed portions. Therefore, MopB is a reasonable candidate Xf cell surface protein that could be a target for agents that can inactivate Xf if introduced into the xylem of Xf host plants. The specific strategy was to transform the rootstock with a gene construction that would secrete the engineered anti-Xf protein into the rootstock xylem for transport to scion xylem where it would provide protection against insect vector-delivered Xf. The specific objectives were:

1. Discover or develop low molecular weight proteins (MopB binding protein, MBP) with high affinity for portions of the MopB protein that are displayed on the Xf cell exterior.
2. Test MopB-binding proteins for their ability to coat Xf cells, for possible bactericidal activity, and for interference with disease initiation following inoculation of grape 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 vitro*.
4. Evaluate gene constructions for desired anti-Xf phenotypes using a tobacco line as a test bed for rapid plant regeneration and testing
5. In collaboration with the Dandekar laboratory, prepare transgenic grapevine rootstock with xylem-targeted constructions favored by Objective 4 results and expressing the candidate anti-Xf proteins; test the transgenic rootstock for its ability to develop resistance to infection by Xf *in planta* (i.e., in the scion)

Activities

MopB, an abundant outer membrane protein of Xf

Three Xf strains were used in these studies, the wildtype Temecula 1 and transposon insertion mutants of Temecula 1, designated HxfA- and HxfB- (Guilhabert and Kirkpatrick, 2005). The latter have knock-out insertions in the two Xf hemaglutinin-like protein genes, *HxfA* and *HxfB*, respectively. The two mutant strains were used because they grow well in liquid culture and on the surface of nutrient agar and do not show extensive aggregation in culture, as wildtype Temecula does. The Xf mutant lines were the souce of most cell preparations grown in liquid culture (planktonic cells). Evidence for possible minor surface differences between these three strains is reported in the section, below, "Peptides with affinity for Xf cells."

As described above, Xf MopB was identified as a candidate for the *C. quinoa* chlorosis-inducing factor based on its abundance in a chlorosis-inducing fraction extracted from Xf cells. We developed a procedure for partial purification of MopB based on retaining the precipitate seen after extracting Xf cells with pH 8.5 sodium dodecyl sulfate (SDS) solution for 30 min. Analysis of this precipitate, designated

SP, by electrophoresis through SDS-permeated polyacrylamide gel (SDS-PAGE) revealed a pattern dominated by one band, with a mobility corresponding to about 43K, corresponding approximately to the mobility expected for MopB. SP was then extracted with SDS solution at pH 8.8 in the presence of sodium perchlorate for 16-18 hr, retaining the supernatant. Most of the SDS and perchlorate were removed, and the protein was concentrated, by centrifugal filtration. This fraction, designated SS, was further enriched in the 43K band material. Fraction SS material apparently was dependent on small amounts of retained SDS for solubility, since further centrifugal filtration and dilutions with water resulted in the formation of a finely divided precipitate. The SS fraction, and similarly MopB-enriched fractions derived by chromatography of SP MopB preparations over 6% agarose beads in SDS solution, were used to raise rabbit polyclonal antibody. This antibody, fluorescently labeled with the Alexa 488 fluorescein reagent, stained planktonic Xf cells, apparently uniformly. However, Xf cells recovered from the surface of nutrient agar were not stained by this fluorescent antibody.

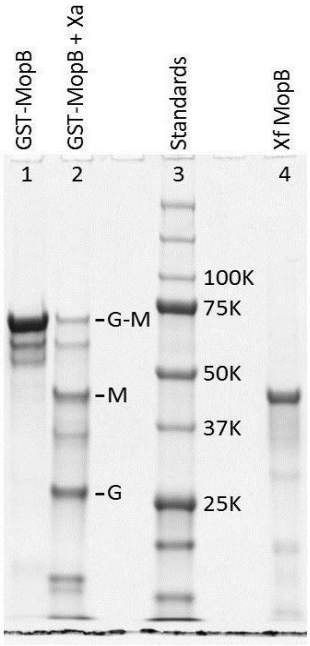
Given the abundance of MopB in Xf cells and Xf cell membrane preparations, and the ability of anti-MopB antibody to bind to planktonic Xf cells, MopB is a candidate for a Xf cell surface protein that may interact with the interior surface of xylem elements to immobilize Xf cells. As a surrogate for the xylem vessel interior, we tested balsa wood and filter paper for their ability be targeted by MopB. SS MopB preparations were mixed with bovine serum albumin (BSA), ovalbumin, or a mixture of four proteins (transferrin, BSA, glyceraldehyde phosphate dehydrogenase, and carboxyanhydrase). The protein mixtures were exposed to 25 mm square x 0.8 mm thick balsa wood or to two disks of 8 mm diameter Whatman 3MM filter paper in a buffered solution of 2 mg/mL NP-40 non-ionic detergent for 90 min at room temperature. The balsa wood and filter paper pieces were washed with the NP-40 solution and then were eluted with hot, alkaline SDS solution. Analysis of the solutions by SDS-PAGE revealed that only MopB was absorbed to the pieces. To assess the specificity of the absorption reaction, the experiment was repeated with BSA in the solution and with filter paper disks and with a similar mass of fabric disks of three distinct chemical compositions: polyamid, polyester, and a rayon/nylon mix. Very similar results were obtained in each case, with MopB but not BSA recovered in the solution eluted from the disks with hot, alkaline SDS. When Xf cells were substituted for the SS MopB preparation, the eluate was highly enriched in MopB and BSA was not absorbed. These results suggest that MopB has an affinity for porous surfaces, since this apparently is the only characteristic that balsa wood and the four woven and non-woven flat materials (cellulose, polyamid, polyester, and rayon/nylon mix) have in common. The significance of these observations for MopB function is not obvious.

Attributing the chlorosis-inducing capability of MopB preparations to MopB itself is very difficult because a minor but potent component of the MopB preparation could be the true chlorosis-inducing factor. Therefore, we attempted to obtain Xf MopB from a non-Xf source, transformed *E. coli*, and in a form that should allow MopB to be highly purified in microgram amounts. Plasmid constructions were prepared bearing the Xf MopB sequence or portions thereof, with and without fusion to other peptide or protein sequences, using the pET160 plasmid system. This system employs a bacteriophage T7 promoter for high-level expression (Dubendorff and Studier, 1991). A chimeric, hexahistidine-tagged OmpA-MopB protein was synthesized in the pET160 system and was purified by chromatography on a nickel chelate column using 8 M urea to keep the protein in solution. Urea was removed and the product was concentrated using centrifugal filtration. Pressure infiltration of this chimeric OmpA-MopB protein preparation into *C. quinoa* leaves failed to induce chlorosis. Control MopB preparations from Xf cells induced the usual chlorotic reaction, suggesting that chlorosis-inducing activity does not reside in the carboxyl half of MopB. In other experiments, *E. coli*-expressed hexahistidine-MopB (the full sequence) was purified by nickel column chromatography in 8 M urea. In a different approach, a MopB fusion to glutathione-S-transferase (GST) was electrophoretically purified and cleaved with a protease to release MopB (Fig. 1). None of these forms and preparations of MopB induced chlorosis after pressure infiltration into *C. chenopodium* leaves.

Xf EF-Tu (elongation factor, temperature unstable) as a chlorosis-inducing factor in *Chenopodium quinoa*

Evidence and information presented in this section and the three succeeding sections supports EF-Tu, rather than the previous candidate MopB, as the Xf protein that is recognized by *C. quinoa* leaves. Recognition is evidenced by development of chlorosis in infiltrated areas of the leaf (Fig. 2). Unusual properties of Xf EF-Tu, documented below, suggest outer membrane association for at least a portion of the Xf cell's complement of EF-Tu molecules, rather than the typical cytoplasmic and cytoskeletal (Mayer, 2006) locations for eubacterial EF-Tu. In parallel with efforts aimed at recovering MopB in various forms from *E. coli*, MopB preparations from Xf cells, prepared as described above, were fractionated in attempts to discover chlorosis-inducing minor components. When the MopB preparations were analyzed on a 10% or 11% polyacrylamide gel, rather than the more usual 12.5% gel, and stained with zinc and imidazole, a faint band was observed trailing the main MopB band. Material from this trailing band induced chlorosis in *C. quinoa* (Fig. 2). The major component of this minor band was found, by trypsin digestion and mass spectrometry, to be Xf EF-Tu. These observations strongly suggest that EF-Tu, rather than MopB, is the Xf factor inducing chlorosis in *C. quinoa*.

Figure 1. Proteolytic release of MopB from a glutathione-S-transferase-MopB fusion protein. The construction incorporated a clotting factor Xa protease cleavage site between the glutathione-S-transferase (GST) and MopB sequences. *E. coli* transformed to express the GST-MopB fusion protein was induced with IPTG. Coupling glutathione-S-transferase (GST) to a protein of interest is intended to facilitate purification of the resulting fusion protein by adsorption to immobilized glutathione. However, the insolubility of MopB and GST-MopB required a different approach. Collected *E. coli* cells were treated with lysozyme and nuclease. The washed residual material was disrupted by heating in SDS and reducing agent and was applied to a 10% polyacrylamide gel for electrophoresis. The central portion of the major band migrating at a position corresponding to a molecular weight of ~70K was located using a zinc-imidazole negative stain (Hardy and Castellanos-Serra, 2004), and protein was recovered from the gel. The electrophoretic purification was repeated once. The recovered GST-MopB was concentrated and depleted in SDS before further analysis. An aliquot was applied to a 10%, SDS-permeated polyacrylamide gel (lane 1). Another aliquot was incubated with factor Xa protease and applied to lane 2. Lane 3 received protein standards, and lane 4 received Xf MopB purified by SDS-extraction of Xf cells as indicated in the text. Expected locations of GST-MopB (G-M), MopB derived from G-M (M), and GST (G) are indicated beside lane 2. Molecular weights of some of the standard proteins are indicated to the right of lane 3. The gel was stained with Coomassie brilliant blue. M is expected to have 3 additional amino acid residues, derived from the inserted Xa cleavage site, at its amino end



Relevant characteristics of EF-Tu

EF-Tu is one of a small number of highly conserved eubacterial proteins ("pathogen-associated molecular patterns," PAMPs, or microbe-associated molecular patterns, MAMPs) that have been discovered to induce defense responses in a variety of plants (Jones and Dangl, 2006). A MAMP is a macromolecule of the microbe that is sensed by the plant as a signal of the presence of any of many microorganism species of a phylum or other group, e.g., eubacteria. 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), and exposure of arabidopsis to bacterial flagellin or EF-Tu resulted in an increase in binding sites for EF-Tu. This observation allowed the gene for the arabidopsis EF-Tu receptor, *ERF*, to be cloned and expressed in *N. benthamiana* leaves, where it conferred the ability to bind the EF-Tu amino-end epitope and develop resistance to several bacterial pathogens (Lacombe et al., 2010; 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).

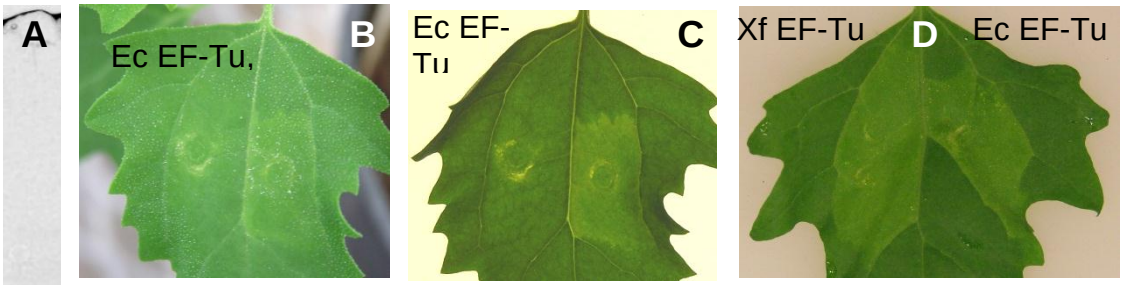


Figure 2. Purification and chlorosis-inducing capability of *E. coli* and Xf EF-Tu. EF-Tu was purified from logarithmic phase *E. coli* cells by the method of (Caldas et al., 1998). Pooled chromatography fractions were concentrated by centrifugal filtration through a 30K YM-30 filter, from which both 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 and confined to the infiltrated area (**B**). The chlorosis became more intense at 90 hr after infiltration (**C**). **D**. Partially purified (~50% 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.

Although EF-Tu proteins of eubacteria typically are soluble and abundant components of the bacterial cell cytoplasm, where they have an essential role in protein synthesis, some eubacterial EF-Tu proteins have at least one other cell location and function. *Mycoplasma pneumoniae* and *Lactobacillus johnsonii* appear to use EF-Tu as an adhesin 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.

Association of Xf EF-Tu with insoluble subcellular fractions

The MopB purification procedure, outlined above, was initiated with SDS-extraction of Xf cells, retaining the precipitate, which was shown to contain EF-Tu. Therefore, it is likely that at least some EF-Tu of Xf is in an insoluble form. Results presented in Fig. 3 show that EF-Tu is associated with an insoluble fraction prepared by a distinct method. HxfA- cells were exposed to a proprietary mild detergent solution, "BugBuster®," or they were exposed to lysozyme, or to both. Prior exposure of HxfA- cells to BugBuster® solution reduced the intensity of, or eliminated, a few protein bands (Fig. 3, lanes 1 and 2), whereas treatment with lysozyme had no apparent effect on the pattern of protein bands (lane 4 compared to lane 1). Incubating the cells with BugBuster® and lysozyme together resulted in diminution or elimination of several protein bands (lane 3 compared to lane 1), including a prominent band with a mobility corresponding to an apparent molecular weight of about 43K, i.e., to the expected mobility of Xf

EF-Tu (and of Xf MopB). Immunoblot analysis using chemiluminescense detection revealed that most of the EF-Tu remained in the insoluble fraction after treatment with BugBuster® and lysozyme (Fig. 3, compare lanes 7 and 5).

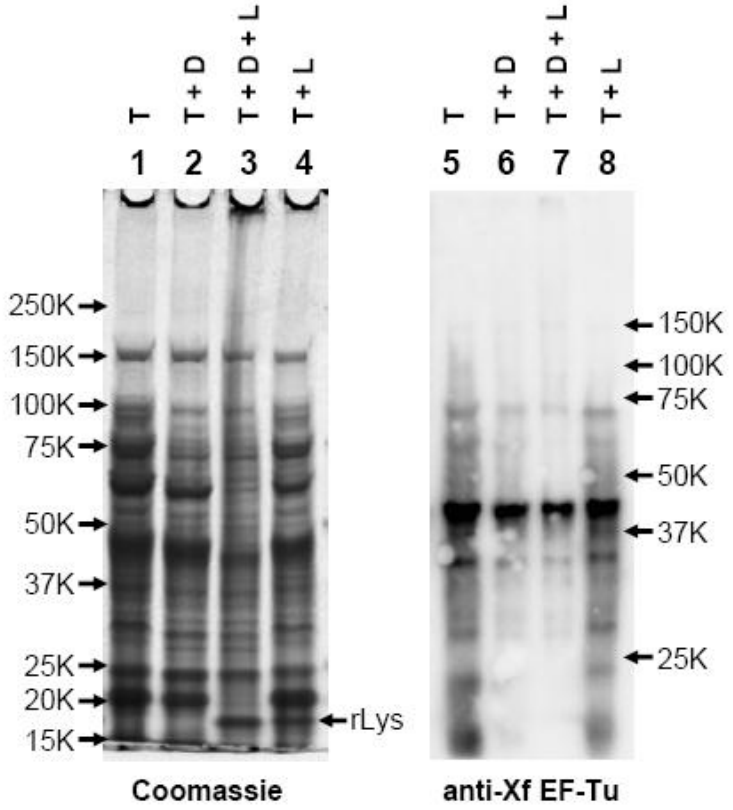


Figure 3. Effects of lysozyme treatment on the recovery of EF-Tu from an insoluble fraction of Xf cells. Lanes 1-4, and 5-8 were derived from different 12% polyacrylamide gels after electrophoresis of extracts of the relatively rapidly growing Xf strain HxfA-. Cells were harvested from liquid culture in late logarithmic phase. Cells were washed with water and cell pellets were quick-frozen in liquid nitrogen and stored at -70˚C. Rapidly thawed cell suspensions were approximately 0.3 µg/µL in total protein in 20 mM Tris-HCl, pH 8, alone (T, lanes 1 and 5), in Tris-buffered "BugBuster®" (EMD Biosciences, proprietary detergent solution, T+D, lanes 2 and 6), in Tris-buffered BugBuster® containing 45 U/µL recombinant lysozyme (EMD Biosciences, T+D+L, lanes 3 and 7), and in Tris-buffered 45 U/µL recombinant lysozyme (T+L, lanes 4 and 8). Samples were incubated at room temperature for 15 min with mixing, and insoluble material was collected by centrifugation at 14K rpm for 10 min. Precipitates were suspended in one-eighth the original volume of water, were mixed with SDS-mercaptoethanol-dithiothreitol disruption solution and were heated before loading the equivalent of 27 µg (lanes 1-4) or 8.5 µg (lanes 5-8) of starting protein on the gels. Detection was by staining with Coomassie Brilliant Blue (lanes 1-4) or by immunoblotting using anti-EF-Tu peptide polyclonal antibody (lanes 5-6). Horse radish peroxidase-conjugated goat-anti-rabbit secondary antibody was located using the DuraSignal (Thermo-Pierce) chemiluminescense system. The locations of bands for protein standards and a presumed band for recombinant lysozyme (rLys) are indicated by arrows.

The insoluble product of the BugBuster® and lysozyme incubation (Fig. 4A, fraction P) was applied to a 50%-to-80% (w/v) sucrose gradient, which was centrifuged under conditions which should result in the various insoluble components of the digest moving through the gradient and reaching their own density (isopycnic centrifugation). The main band of material (Fig. 4B, fraction MB) was retained and enriched in EF-Tu according to immunoblot results (Fig. 4C). The centrifugation process, in effect, involves multiple washing steps as the main band material moves from the top of the tube to its isopycnic density position, suggesting that the association of EF-Tu with the insoluble material is strong. These results also suggest that the bulk of the Xf EF-Tu is tightly associated with, a presumably insoluble, component of the

cell wall that is cleaved by lysozyme to release the white MB material. This insoluble EF-Tu may or may not be participating in protein synthesis but, as has been found for the EF-Tu of other systems (Dallo et al., 2002; Granato et al., 2004), Xf EF-Tu may have functions in addition to participating in protein synthesis.

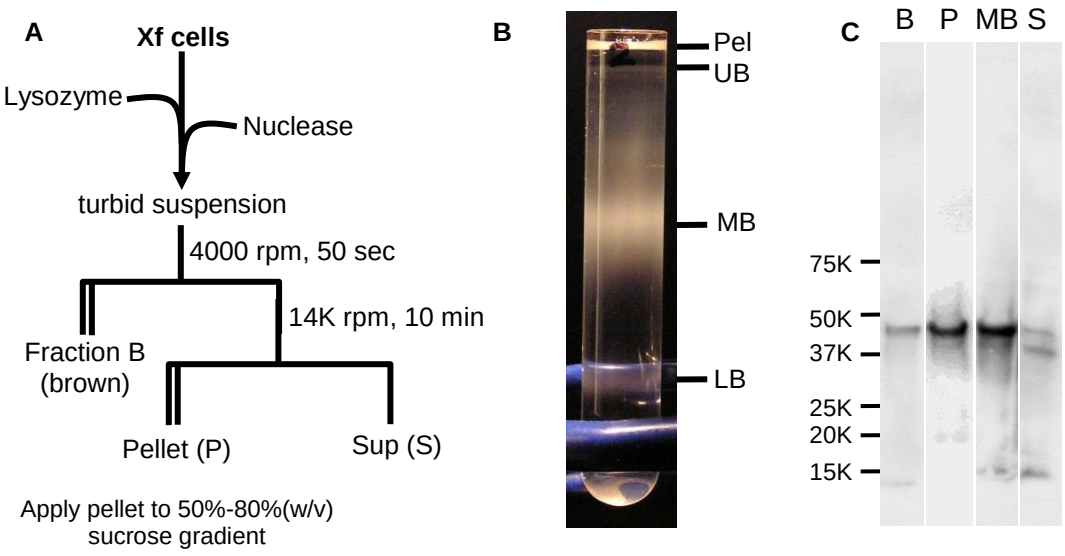


Figure 4. Xf EF-Tu is tightly associated with an insoluble fraction released from Xf cells by treatment with lysozyme. **A.** Xf cells were suspended in a mild detergent solution ("BugBuster®," EMD Biosciences), and the suspension was incubated at room temperature with lysozyme and then with a general nuclease (Benzonase, EMD Biosciences), which greatly reduced the viscosity of the suspension. Differential centrifugation produced a small brown precipitate (B), a white, fluffy precipitate (P), and a supernatant fraction (S). **B.** The P fraction was applied to a 50%-to-80%(w/v) sucrose gradient in 20 mM Tris-HCl, pH 8. Centrifugation was for 4 hr at 35,000 rpm in a Beckman SW-41Ti rotor at 4˚C. For photography, the tube was mounted in a blue clamp, visible near the bottom of the image. In addition to the main band (MB) of white, turbid material, material also accumulated at the bottom of the tube, in a pellicle (Pel) floating on the gradient, and in two other minor bands (UB and LB). **C.** Fractions from centrifugation and sucrose gradient centrifugation were heated in SDS-urea solution and were analyzed by SDS-PAGE on an 8-16% gradient gel. The bulk of the material reacting with peptide antibody to Xf EF-Tu in an immunoblot was associated with the MB fraction, which had a buoyant density of about 1.24 g/mL.

To mimic 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. An *E. coli* strain was designed and constructed, based on the phagemid pCANTAB, to express a fusion of the bacteriophage fd outer membrane protein PIII with Xf EF-Tu forming the amino end of the fusion. PIII is an adhesin responsible for initiating attachment of the bacteriophage fd particle to the bacterial F-pilus as an essential first step in bacteriophage infection. Prior to extrusion of the new bacteriophage particles from the infected cell, or when PIII is expressed in transformed but uninfected cells, PIII resides in the cell outer membrane, with its carboxyl end portion in the membrane and amino end portion on the outside of the membrane. Therefore, our expectation is that a fusion protein of the form EF-Tu-PIII will be targeted to the outer membrane. An immunoblot of an extract of lysozyme- and nuclease-treated *E. coli* EF-Tu-PIII transformed cells revealed a signal at the expected mobility for the fusion protein but no similar signal for extracts of *E. coli* cells transformed with PIII alone (Fig. 5B).

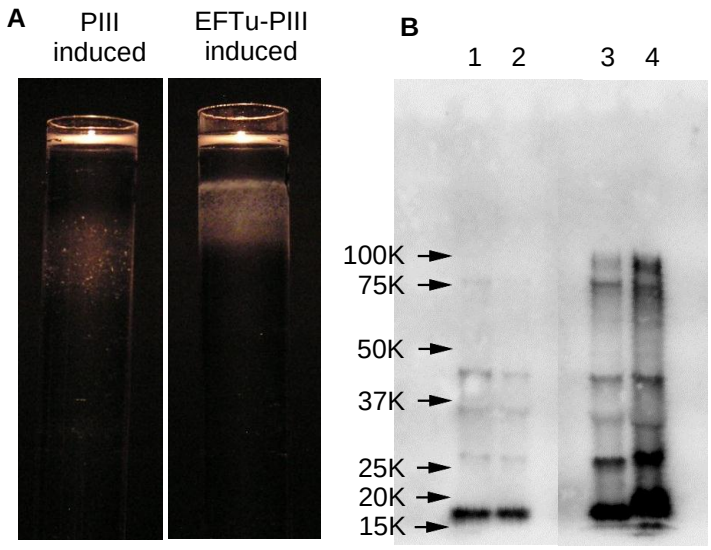


Figure 5. Expression of a fusion protein incorporating Xf EF-Tu in *E. coli* altered the insoluble residual found after digestion with lysozyme. **A.** A 40 mg/mL (wet weight) suspension of *E. coli* cells that had been induced with IPTG was exposed to 30 U/µL recombinant lysozyme in BugBuster® detergent for 15 min and then to 0.05U/µL nuclease (EMD benzonase) for 10 min at room temperature. The resulting white precipitate was applied to the top of preformed 45%w/v – 75%w/v sucrose gradients. Gradients were centrifuged at 35,000 rpm for 4 hr at 4˚C. **B.** An immunoblot was prepared using anti-Xf EF-Tu peptide antibody. Lysozyme digestion product for lanes 1 and 2 were from PIII-transformed cells and for lanes 3 and 4 were from PIII-EF-Tu-transformed E. coli cells. Lanes 1 and 3 received digestion product, whereas lanes 2 and 4 were loaded with material recovered from the sucrose gradients. The uppermost band in lanes 3 and 4 has a mobility expected for a Xf EF-Tu-PIII fusion protein. Arrows indicate the locations of reference proteins by their molecular weight.

Alteration of E. coli cell morphology by expression of Xf EF-Tu

E. coli transformed for PIII expression increased in culture at a rate that was comparable to that for the corresponding untransformed strain. However, the *E. coli* strain bearing the EF-Tu-PIII construction grew slowly. We examined cells from the two cultures in the light microscope after staining with safranin (Fig. 6); cells from the two cultures were of very different appearance. Compared to the PIII-expressing cells, the presumed EF-Tu-PIII-expressing cells (Fig. 6B) were both longer, greater in diameter, and much less intensely stained with safranin. The results reported here are consistent with a radical alteration in the cell wall of *E. coli* cells due to Xf EF-Tu that was targeted to the cell wall.

The altered growth rate and cytology of *E. coli* cells expressing a EF-Tu-PIII fusion have a biochemical correlate. Digestion with lysozyme and nuclease, and recovery of insoluble material by centrifugation, was performed for *E. coli* expressing PIII or EF-Tu-PIII (Fig. 5A), as had been done for Xf cells (Fig. 4A). Sucrose gradient centrifugation revealed a band for the EF-Tu-PIII digestion product that was not seen for the PIII digestion product (Fig. 5A). The results, in Figs. 3-6, suggest that Xf EF-Tu can cause a marked alteration in the outer membrane of *E. coli* cells and therefore presumably has a strong influence on the structure of the Xf cell outer membrane.

Does EF-Tu of Xf contribute to the symptoms seen on Xf-infected grapevines? It is well established that local concentrations of Xf cells and symptom severity on leaves are not correlated (Gambetta et al., 2007). Therefore, symptom development could be the result of factor(s) secreted by Xf cells and accumulating at the leaf margin, for example. Difficulties in pressure infiltrating grapevine leaves have prevented us from directly testing the effects of Xf EF-Tu preparations in leaf tissue. Other methods of Xf EF-Tu delivery are in progress, as are explorations of plant reactions to this protein.

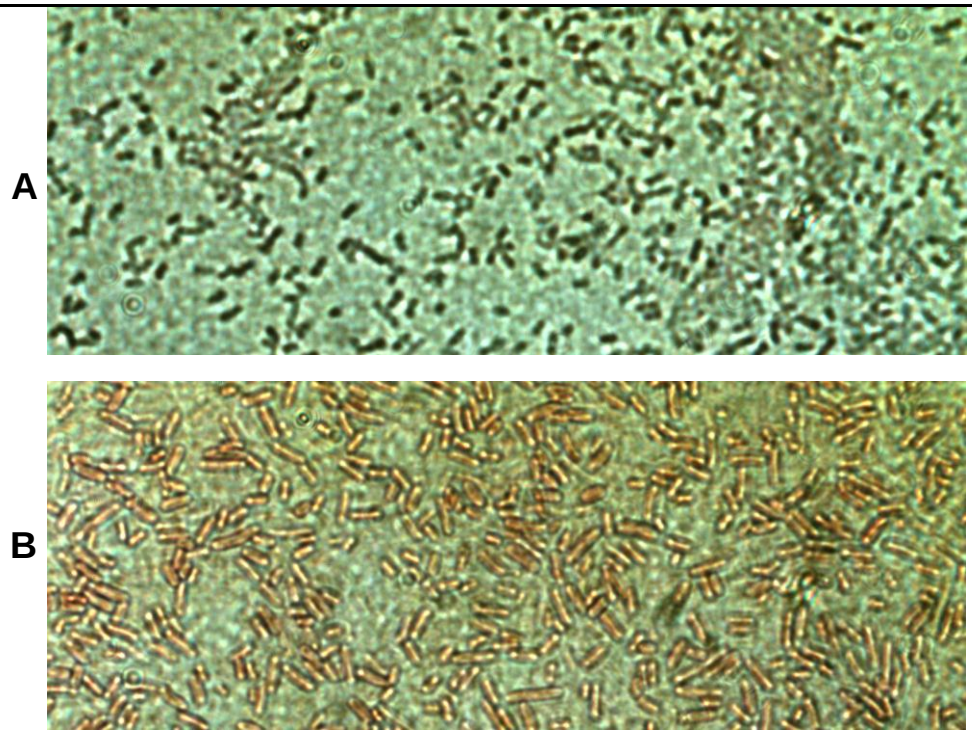


Figure 6. An outer-membrane-targeted Xf EF-Tu fusion protein appears to alter the cell morphology of *E. coli*. The suspensions of *E. coli* cells for both panels were stained with the same preparation of the Gram counter-stain safranin. **A**. A *RecA*-minus *E. coli* strain was transformed to express the bacteriophage M13 adhesin protein PIII, which accumulates in the cell outer membrane when expressed alone and in the absence of active bacteriophage M13 infection. **B**. the same RecA-minus strain was transformed for expression of a fusion protein, EF-Tu-PIII. Compared to the PIII-protein-expressing cells of panel A, the EF-Tu-PIII-expressing cells grew much more slowly, were greater in both length and diameter, and were greatly reduced in staining by safranin.

Is Xf EF-Tu a MAMP or an inducer of symptoms in Xf-infected grapevines, or both?

The above results suggest that Xf EF-Tu is present in Xf in an immobilized or insoluble form not expected for a protein that is engaged in protein synthesis. What function might this altered form of Xf EF-Tu have? The chlorotic response of *C. quinoa* leaves to infiltrated Xf EF-Tu (Bruening et al., 2007) demonstrates plant recognition of this protein. Whether a Xf factor such as EF-Tu is involved in symptom induction or in defense response in grapevine, the relevant events must include specific recognition of the factor by grapevine, which should be reflected in altered transcription. Previous work from the laboratory of Prof. Douglas Cook identified grapevine promoters whose transcription was increased specifically in Xf-infected plants (Cook et al., 2005; da Silva et al., 2005). Transgenic Thompson Seedless grapes containing constructs with one of these promoters, promoter 9353, coupled to a green fluorescent protein (GFP) sequence, were prepared in the laboratory of Prof. David Gilchrist (Gilchrist et al., 2007). Plants of the transformed line have been demonstrated to accumulate GFP in the pith region of petioles after inoculation of Xf but not after inoculation of another xylem-invading bacterium, *Xanthomonas campestris* (Gilchrist et al., 2008).

No GFP signal was observed for transgenic grapevine petioles injected with water (Fig. 7a), and little or no signal for injected bovine serum albumin (BSA) in water (Fig. 7b). However, the pith region of 9353-

promoter-GFP-transformed grapevine generated an apparent GFP signal (Fig. 7c) after injection of purified *E. coli* EF-Tu protein in water.

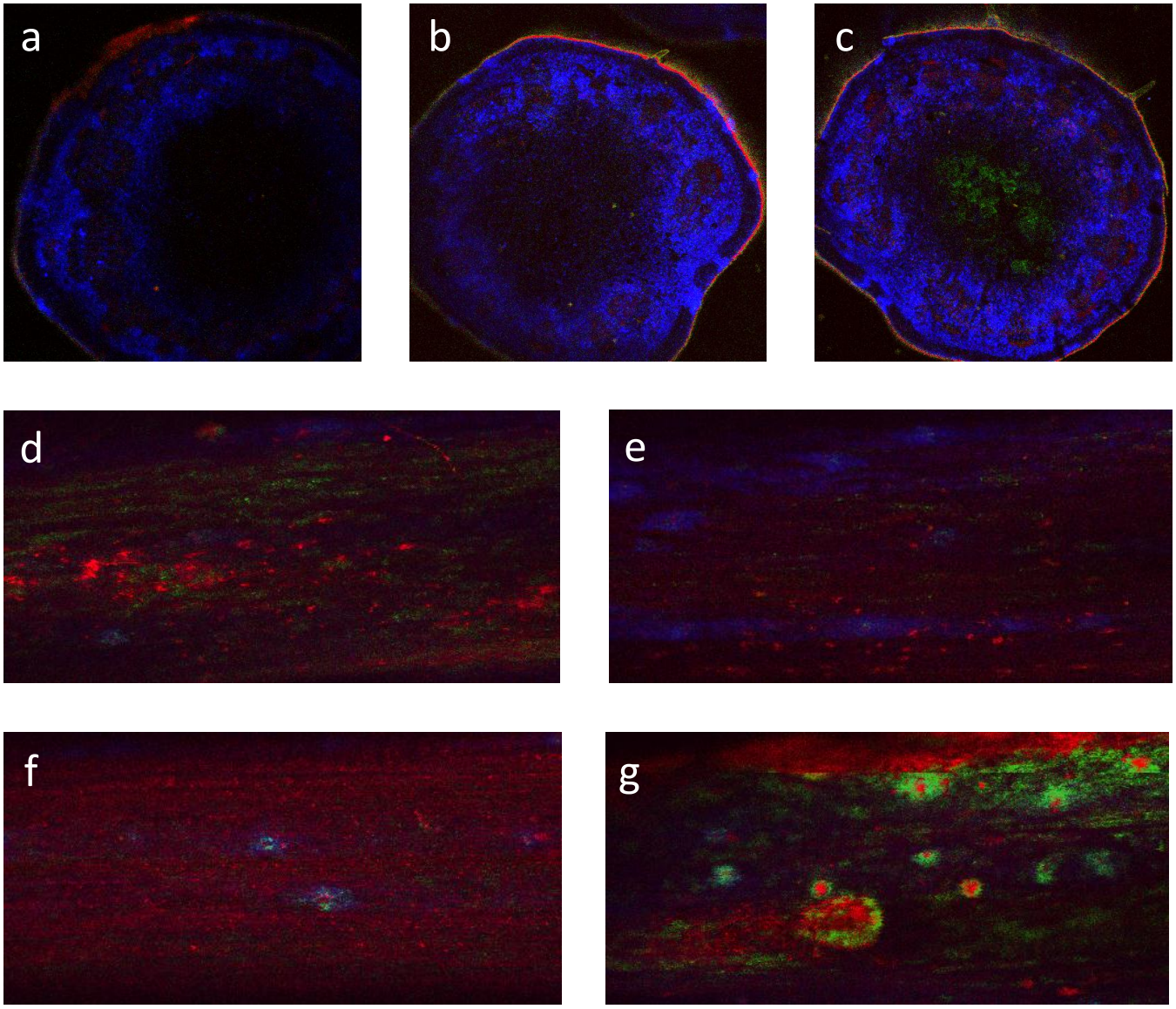


Figure 7. A Xf-specific grapevine promoter is activated by purified *E. coli* EF-Tu and by *E. coli* cells expressing Xf EF-Tu-PIII. All images are from petioles of transgenic Thompson seedless grape plants bear constructions for green fluorescent protein (GFP) expression under the control of the *Xf*-infection inducible 9353 promoter (da Silva et al., 2005; Gilchrist et al., 2007). For (a-c), each petiole was injected twice, ~0.5 cm apart and near the stem with 20 µL of liquid. Petiole (a) received water, (b) received bovine serum albumin (BSA), and (c) received chromatographically purified E. coli EF-Tu, both proteins at about ~0.9 µM in water. At 9 days after injection, leaves were removed and cross-sections were prepared at a site ~1 cm towards the lamina from the injection site. For (d-g), transgenic grape petioles were inoculated with *E. coli* cells bearing either PIII (d, e) or EFTu-PIII (e,g), all at 2 x 10^8 cells per mL. An aliquot of cells was supplied with the gratuitous inducer IPTG (f,g) for 2 hr before storing the cells overnight at 4^{o}C and inoculation. At 13 days post inoculation, transverse sections of petiole were prepared using material a few mm from the inoculation site. Confocal microscope images were captured by Dr. James Lincoln.

Unlike *E. coli* EF-Tu, the insolubility of Xf EF-Tu has prevented us from obtaining this protein in purified, soluble form for injection. Therefore, *E. coli* bearing the Xf EF-Tu-PIII construction was

inoculated to petioles of 9353-GFP grapevine. Accumulation of GFP was observed by confocal microscopy (Fig. 7g) in the transgenic grapevine petioles after inoculation with *E. coli* cells of the strain transformed for expression (Fig. 5B, lane 4) of the EF-Tu-PIII fusion protein, provided the cells were induced by exposure to IPTG (Fig. 7g compared to Fig. 7e). This image does not show GFP accumulation in pith cells, unlike the result for inoculated intact Xf cells (Gilchrist et al., 2008). Significant GFP signals were not observed for the *E. coli* cells transformed for PIII protein expression (Fig. 7d,f). In the Fig. 7 experiment, *E. coli* cells had been stored at 4^{o}C overnight before infiltration. Cold storage may lead to substantial bacterial death (data not shown), particularly for cells made more fragile by expression of EF-Tu-PIII. Therefore, based on these results, we are not able to connect the observed induction of GFP synthesis to live cells actively accumulating EF-Tu-PIII or even to intact, EF-Tu-PIII-containing cells, because *E. coli* EF-Tu released from ruptured cells could have been the inducer of promoter 9353-controlled GFP expression. In a subsequent experiment, the transformed *E. coli* cells were held at room temperature, and GFP accumulation in pith cells was observed (not shown). The results presented here suggest that EF-Tu protein from Xf or *E. coli* cells may act as an elicitor that is recognized by grapevine. Whether this recognition has a role in symptom development or defense against Xf infection was not settled in this research but is the topic of a subsequent project.

Peptides with affinity for Xf cells

Prior results from our research identified MopB as a, or possibly the, major outer membrane protein of Xf. Based on similarities of Xf MopB to *E. coli* outer membrane protein OmpA, we cast the amino-end half of MopB into a conformation very similar to that of the amino-end half of OmpA, based on the crystallographic structure of OmpA (Pautsch and Schulz, 1998) and computer predictions of folding for OmpA and MopB. The OmpA half molecule is known to be as capable as the full length molecule of embedding itself into the outer membrane. The OmpA half molecule forms 8 transmembrane β-strands connected by 3 short periplasmic turns and 4 relatively large surface-exposed hydrophilic loops (Koebnik, 1999a; Koebnik, 1999b; Pautsch and Schulz, 1998; Singh et al., 2003). Intact Xf cells stain with fluorescent anti-MopB antibody, as expected. The abundance of MopB in Xf cell extracts, the known packing density of OmpA in a crystal, and the Xf cell dimensions allowed us to estimate that MopB probably accounts for at least 10% of the Xf cell exterior. Thus, MopB is a highly suitable target for inactivation of Xf cells. Objective 1 calls for the discovery or creation of low molecular weight protein(s) capable of binding tightly to the Xf surface protein MopB (or, by extension, to any other surface feature of Xf cells). Objective 2 concerns assessing the biological effects, if any, of such binding proteins on Xf cells.

We developed both peptides and proteins that bind to the Xf cell surface. For some of these peptides, the Xf cell target of binding and the stoichiometry of binding have been tentative identified. Some Xf-cell binding peptides distinguished Xf cells according to the culture conditions under which they were raised and mutations in specific surface proteins.

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 (Fig. 8) (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 into male *E.*

coli. M13 progeny were partially purified before initiating the next round of selection (Barbas et al., 2001; Smith and Scott, 1993).

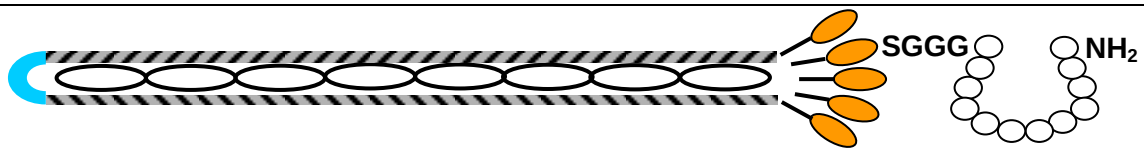
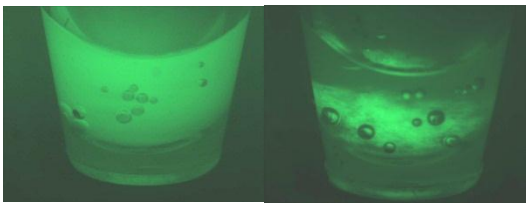


Figure 8. Diagram of a bacteriophage M13 random peptide library member. The random peptide 12-mer (open ovals) is incorporated at the amino end of the absorption protein (designated PIII or P3) of the M13 particle by a flexible tri-glycine-serine sequence and is encoded in the bacteriophage DNA. For 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 cells recovered from from two different culture methods proceeded differently. For Xf cells scraped from the surface of nutrient agar (followed by washing), the titer increased about 10^2 at each cycle, and 14 of the 20 clones obtained after 4 cycles were positive for binding to nutrient agar-derived Xf cells. For the planktonic cells (from liquid culture), 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. Fig. 9 presents results of an assay for Xf-cell-binding by selected M13 clones, and Table 1 presents results for several M13 clones.

Figure 9. Xf cells binding of 12-mer peptide-bearing M13. Each M13 clone preparation (~10^{11} particles, 2.7µg) was incubated with Temecula Xf cells derived from plate cultures, and the cells were washed three times to remove unbound M13 particles. Fluorescently labeled anti-M13 major coat protein IgG was added. Left panel: no reaction detected because the fluorescent antibody remained in solution (M13 clone 4N2). Right panel: binding was detected as evidenced by agglutination (M13 clone 4N1).



None of the nine M13 clones selected with nutrient agar-derived Xf cells bound 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), 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 significantly different**. Given the low copy number of HxfA and HxfB proteins in Xf, it is likely that the changes in specificity of the Xf-cell-binding peptides and proteins are due to some rearrangement of the cell surface resulting from the deletion of one or the other of the two hemagglutinin-like proteins, rather than to direct binding to them.

In a few experiments, we investigated possible targets of the binding proteins and the extent of binding. 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 (Fig. 9). 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. In other experiments, polyclonal rabbit serum to Xf MopB also did not interfere with the agglutination reaction, suggesting that MopB is not the target of the M13 peptides. Other potential targets for the M13 clones are two hemagglutinin-like proteins of Xf, HxfA and HxfB (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).

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?	HxfA-minus Xf plate cells?[c]	HxfB-minus Xf plate cells?[c]
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 use in 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 PIII (Fig. 1), although in two instances the sequence was found to be altered to GRGS
c: HxfA, HxfB, 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

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 Fig. 9 assay) was fewer than 100.

A protein with affinity for Xf-cell-derived materials

Compared to the binding of peptides to the Xf cell surface, proteins should be capable of tighter binding because the larger size of a protein should allow more contacts with the cell surface. We obtained a library of single chain (scFv) antibodies, expressed on bacteriophage M13 particles, from the University of Cambridge, UK. Liquid cultures of the rapidly growing and minimally aggregating Xf strain HXfA-, which has a transposon insertion in the HXfA gene (Guilhabert and Kirkpatrick, 2005), were centrifuged to collect the cells. This cell preparation was used to "pan" the M13-scFv library through three cycles. Ten of the 24 selected M13-scFv clones were sequenced, and all had the same deduced amino acid sequence. Therefore, all subsequent analyses were applied to just one selected scFv antibody, designated A2scFv. To identify the epitope bound by the A2scFv, Xf proteins were resolved by electrophoresis and were subjected to immunoblot detection using the A2scFv-PIII fusion protein-carrying bacteriophage particle as the "primary antibody" and anti-M13 major coat protein as the target of the secondary antibody conjugate. A single band was observed with mobility corresponding to slightly less than an apparent molecular weight of 47K (results not shown). This band has an electrophoretic mobility similar to two

other Xf proteins which we have investigated: Xf EF-Tu (formula weight 42.9K) and Xf mopB (mobility corresponding to molecular weight 45K). To test whether the A2scFv is recognizing either of these Xf proteins, we attempted to interfere with A2scFv binding by pre-incubating two immunoblots with peptide polyclonal antibody against Xf EF-Tu or polyclonal antibody against Xf MopB. No apparent interference with the binding of the A2scFv-carrying bacteriophage M13 particles was observed (data not shown). One consideration in interpreting these results is that anti-EF-Tu is a peptide antibody and therefore may not block binding by the selected scFv to other parts of EF-Tu.

The A2scFv single-chain antibody was purified under non-denaturing conditions from solution using its encoded hexahistidine sequence and was labeled with Alexa-488 (fluorescein). The Alexa-488 labeled A2 scFv was incubated with wild-type Xf strain Temecula 1 cell preparations in phosphate-buffered saline-Triton X100 (PBS-T) (Fig. 10), and the cell preparations were washed with PBS-T before observing fluorescence. The fluorescence was observed not in association with the cells, as might be expected, but with somewhat larger, amorphous structures that apparently had been collected with the cells during centrifugation or formed during centrifugation. Similar results were observed with the Temecula Xf strains bearing mutations in hemaglutinin-like protein genes, HxfA- and HxfB- (Guilhabert and Kirkpatrick, 2005). These experiments do not eliminate the possibility of weak binding of the scFv to intact Xf cells, since weak binding would not have been detected after the washing procedures followed here. At this point, both the protein target and the larger-than-cells target of the A2 scFv monoclonal antibody remain unknown.

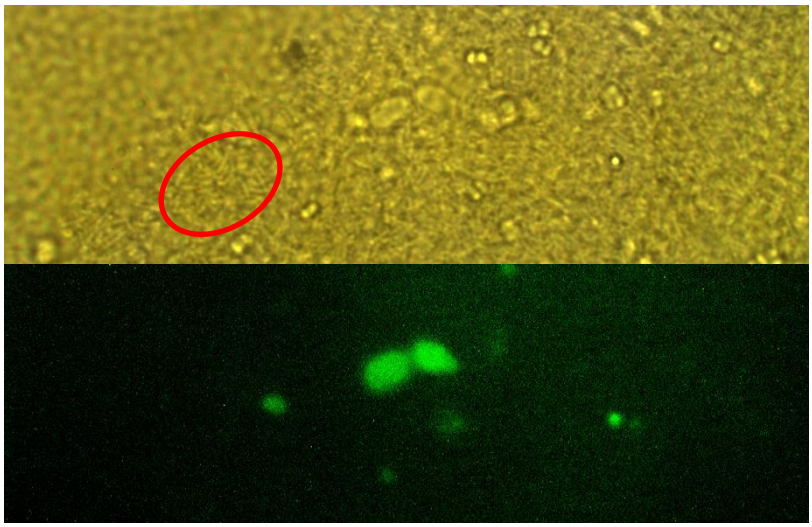


Figure 10. A Temecula 1 strain Xf cell suspension, prepared by centrifugation, was observed under white light (upper panel) and 488nm light using an epifluorescence microscope. Alexa-488 (fluorescein) labeled A2scFv monoclonal antibody was added to the cell suspension, which subsequently was washed 3 times with phosphate-buffered saline containing 0.1% Triton X-100 (PBS-T). The red oval identifies an in-focus field of Xf cells.

SR-1 tobacco as a grapevine surrogate in testing for anti-Xf activity of proteins

Results in this section support satisfying Objectives 2 and 4. We wished to have a plant, for testing the effects of various proteins and peptides, that is experimentally more easily manipulated than grapevine. To facilitate our goal of creating grape rootstock that can confer resistance to Xf on its grafted scion, we tested several tobacco (*Nicotiana tabacum*) lines. Line SR-1 was readily infected by needle inoculation into the petiole axil or stem. Xf was recovered from the petiole above inoculation points, whereas no bacteria were recovered from water-inoculated controls. Symptoms developed (Fig. 11A) and Xf accumulated, as indicated by ELISA, quantitative PCR, and clogging of xylem vesicles (Fig. 11B), providing unequivocal evidence of infection.

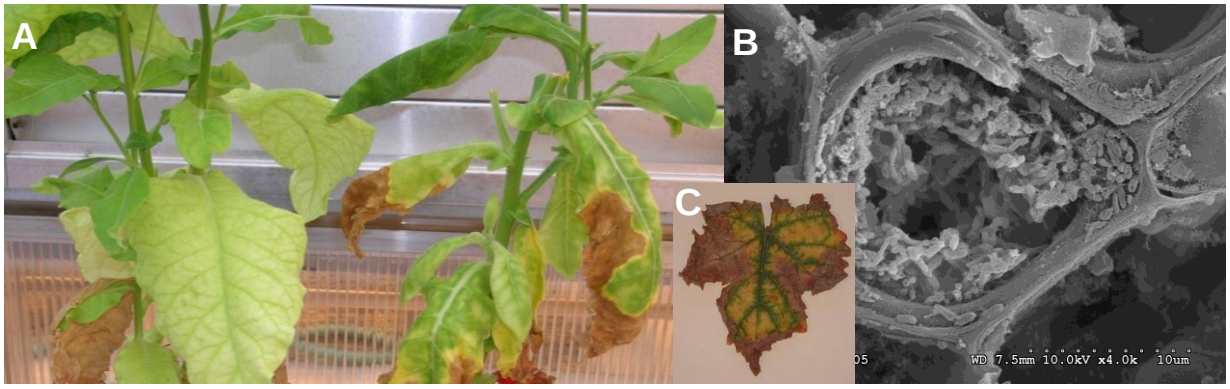


Figure 11. SR-1 tobacco as a host for Xf. **A**. Water-infiltrated (left) and Xf-inoculated plants were photographed 3 months after inoculation at the 6 leaf stage. Although leaves of control plants developed senescence, none developed the downward curvature, cupping and tip- and margin-necrosis with chlorotic halo that are characteristic of the Xf-inoculated plants. 4/4 leaves from two control plants were negative for Xf by ELISA and PCR. Extracts of 7/7 leaves from three Xf-inoculated plants generated ELISA signals averaging 4x the control level; quantitative PCR signals exceeded the threshold product accumulation at 19-31 cycles. **B**. Electron microscopy of SR-1 petiole sections at 10-12 nodes above the inoculated leaf revealing bacterial cells occluding a xylem element. C. Sap from Temecula-1 Xf-inoculated, symptomatic SR-1 tobacco was inoculated to grapevine cuttings, resulting in typical PD symptoms and accumulation of Xf.

Others have succeeded in infecting *N. tabacum* strains with Xf (Alves et al., 2003; Lopes et al., 2000). The symptoms we observe appear to be more dramatic than those reported by others. As we have reported (Francis et al., 2005), Xf isolated from SR-1 tobacco caused typical PD symptoms following artificial inoculation to grapevines (Fig. 11C). It is fortunate that line SR-1 proved to be experimentally useful in Xf research, since SR-1 is routinely transformed and regenerated at the UC Davis College of Agricultural and Environmental Sciences Plant Transformation Facility.

The usual approach for the testing of selected, target-binding random peptides would be to incorporate them into a scaffold protein for testing against the target. We expected to have a large number of selected peptides and therefore needed 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). Therefore, it seemed reasonable to test whether the M13 PIII protein itself could act as a suitable scaffold.

Xf cells were mixed with each of the six M13 clones (4N1, 4N2, 4N3, 4T2, 4T3, 5-19) 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 (Fig. 12). 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 in the section "Peptides with affinity for Xf cells and Xf-cell-derived materials," above.

The observed interference with Xf infectivity supports the feasibility of the overall approach that was taken in this project. However, it is important to note that Fig. 12 presents results from a single experiment and, though the results are statistically significant, the experiment must be repeated to validate the results. The observed effect is small and presumably would require more effective peptides and/or

incorporation of binding peptides into bactericidal constructions to create a more powerful anti-Xf technology.

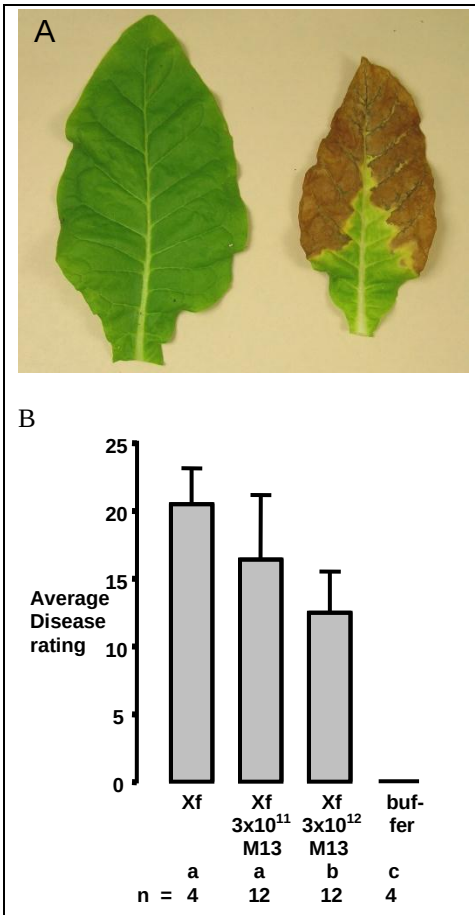


Figure 12. Biological activity of M13 clones selected for their affinity to the surface of Xf cells. Six M13 clones were tested for their ability to interfere with Xf infection of SR-1 tobacco, using two plants per clone. The SR-1 plants were cut back to three leaves per plant. Temecula 1 Xf cell suspension 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; for no observed symptoms characteristic of Xf infection (leaf on the left, panel A), the score was 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).

Intellectual Property Issues

We are not aware of any intellectual property issues associated with this project.

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Publications resulting from work

Bruening, G., and Civerolo, E. L. 2006. Exploiting *Xylella fastidiosa* proteins for Pierce's disease control. In *Pierce's Disease Research Symposium*, pp. 221-224. San Diego, CA: California Department of Food and Agriculture, Sacramento, CA, T. Esser, editor.

Bruening, G., Civerolo, E. L., Feldstein, P. A. & Francis, M. 2006. Exploiting *Xylella fastidiosa* proteins for Pierce's disease control. In *Pierce's Disease Research Symposium*, pp. 215-218. San Diego, CA: California Department of Food and Agriculture, Sacramento, CA, T. Esser, editor.

Bruening, G., E. L. Civerolo, and P. A. Feldstein. 2007. Exploiting *Xylella fastidiosa* proteins for Pierce's disease control, pp. 173-176, Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA, T. Esser, editor, San Diego, CA.

Kunkel, M., Vuyisich, M., Gnanakaran, G., Bruening, G. E., Dandekar, A. M., Civerolo, E., Marchalonis, J. J. & Gupta, G. 2007. Rapid clearance of bacteria and their toxins: Development of therapeutic proteins. *Critical Reviews in Immunology* 27, 233-245.

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Francis, M., Civerolo, E. L. & Bruening, G. 2008. Improved Bioassay of *Xylella fastidiosa* Using Nicotiana tabacum Cultivar SR1. *Plant Disease* 92, 14-20.

The first draft is completed on a paper that has authors from the Dandekar, Gupta, Bruening and Civerolo laboratories and that documents progress against what are stated as Objectives 3 and 4 of this project, with MopB as the Xf cell-surface target.

Contributions to solving the PD problem of California

Research on this project took a path somewhat different from the path we expected, a path that is both interesting and potentially practical. When this project began, our best candidate for the Xf protein that induces chlorosis in *C. quinoa* was MopB. Results from assays with recombinant MopB and partially purified Xf EF-Tu are consistent with EF-Tu being the chlorosis-inducing agent, not MopB. Xf EF-Tu proved to have unusual properties, compared to the EF-Tu proteins of most eubacteria, being largely confined to insoluble fractions of Xf cell extracts. Results obtained subsequent to this project show (i) by electron microscopy that EF-Tu is located primarily at or near the Xf cell surface and (ii) by expression in plants and secretion into intercellular spaces that EF-Tu can induce scorching-like symptoms in both tobacco and grapevine without the presence of Xf cells or any other Xf protein. A widely accepted notion about Pierce's disease is that the associated scorch symptoms result from blockage of xylem elements with biofilm-protected Xf cells. Since Xf EF-Tu alone seems to be able to induce scorch symptoms, the plant's reaction to Xf EF-Tu may be the principle determinant of Pierce's disease symptom induction. Our results suggest that interference with grapevine perception of Xf EF-Tu could prevent or decrease PD symptom development.