EXPLOITING XYLELLA FASTIDIOSA PROTEINS FOR PIERCE'S DISEASE CONTROL

Project Leaders and Cooperators

Project Leader
George Bruening
gebruening@ucdavis.edu
Department of Plant Pathology
University of California
Davis, CA 95616

Co-Project Leader
Edwin Civerolo
eciverolo@fresno.ars.usda.gov
USDA, ARS
San Joaquin Valley Agricultural Sciences Center
Parlier, CA 93648

Cooperators:

Paul Feldstein
pafeldstein@ucdavis.edu
Dept. of Plant Pathology
Univ. Calif.
Davis, CA 95616

Abhaya M. Dandekar
amdandekar@ucdavis.edu
Department of Pomology
Univ. Calif.
Davis, CA 95616

Goutam Gupta,
gxg@lanl.gov
MS M888, Biol. Division
Los Alamos Nat. Lab.
Los Alamos, NM 87544

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Objectives and Activities

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 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 or their ability to induce symptoms. We present circumstantial evidence that the Xf protein translation elongation factor-temperature unstable (EF-Tu) may be involved in symptom induction.

Objectives

1. Discover or develop peptides and proteins with high affinity for the *Xylella fastidiosa* (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 or symptom development 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

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 rather than a peptide, should prove to be more potent in bioassays. In this reporting period, we have employed a bacteriophage M13-based single chain antibody (scFv) library as developed by Tomlinson (de Wildt et al., 2000) with Xf cells as the panning target. In the second part of the activities section, below, 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 or Xf induction of symptoms.

Selection of Xf cell-binding single-chain antibody

Both the Xf-cell-binding peptides selected previously and the system employed here are based on bacteriophage M13 and substitution into the P3 adhesin protein of M13. The P3 protein recognizes and binds to the bacterial cell receptor to initiate infection. The P3 protein is able to retain this function even with substantial insertions into the amino-end region of the protein. Although a conventional antibodies have multiple chains and therefore would be unsuitable for insertion into P3, single-chain antibodies have been developed which link the heavy chain and light chain combining sites together into a functional single-chain antibody. We applied conventional panning procedures with Xf cells as the target using bacteriophage M13 particles carrying single chain antibodies (scFv) derived from the Tomlinson I and J libraries. The I and J libraries differ with regard to the diversity of sequences available, with the J library being more complex. It is not unusual for either the I or the J library to be more effective in panning procedures against a particular target (de Wildt et al., 2000). Three rounds of selection were performed for each of the libraries. At the end of the third round, 24 individual selected library members (molecular clones) were chosen and grown as individuals. These were tested for their ability to "agglutinate" Xf cells using fluorescent antibody against the bacteriophage particles as in our previous work with the Ph.D.-12 peptide library (Bruening et al., 2006). Only clones from the Tomlinson I library were positive in this assay. 10 positive clones were selected, the scFv DNA was amplified using PCR, and the sequences were determined. All 10 individuals had the same sequence (Table 1) implying a potentially strong scFv binding. Table 1 compares the sequence we obtained with one obtained by (de Wildt et al., 2000) using bovine serum albumin (BSA) as the target during the panning procedure.

Table 1. Sequence of a single chain antibody selected for its affinity to Xf cells

Panning	CDRH2*	CDRH3	CDRL2	CDRL3
Against Xf cells	DITNTGNTTSYADSVKG**	AGTTFDY	AASSLQS	QQSYSTPNT
Against BSA	TISTSGGYTYYADSVKG**	GGSSFDY	TASALQS	QQTYSYPST

*CDR: complementarity determining region, on which the selection is expected to act; H: heavy chain sequence; L: light chain sequence

**underlined sequences are not varied, but rather are fixed, in the Tomlinson I library

The unusual physical state of translation elongation factor-temperature unstable (EF-Tu) in Xf cells.

In our previous reports we presented evidence indicating that EF-Tu is present in Xf cells in two forms. Preparations of the Xf major outer membrane protein MopB contained small amounts of EF-Tu migrating during SDS-PAGE as expected for a 43K protein. Material recovered from the 43K-size gel band induced chlorosis in pressure-infiltrated *Chenopodium quinoa* (Cq) leaves. MopB itself did not appear to induce chlorosis. Although trace amounts of "soluble" EF-Tu are present in extracts of Xf cells, the bulk of the EF-Tu has unusual properties. An analysis was performed by (i)direct hot-SDS extraction of *E. coli* or Xf cells, (ii)sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the extract, and (iii)immunoblotting with anti-*Escherichia coli* EF-Tu antibody. For the *E. coli* extract, a single band was observed with a mobility corresponding to the expected molecular weight of 43K. For the Xf extract, the signal was confined to the well of the gel (Bruening et al., 2007) or, in a few cases, a smear from the

well into the gel. Analysis of an extract of an *E. coli* strain that had been transformed for expression of a Xf EF-Tu fusion to the M13 adhesin and outer membrane protein P3 (EF-Tu-P3) revealed two signals, one, apparently for *E. coli* EF-Tu, at the expected position in the gel, and the other in the gel well.

These results suggest that the bulk of the Xf EF-Tu occurs in an aggregated or conjugated form that is not dissociated by heating in slightly alkaline, concentrated urea-SDS solution. That is, possibly Xf EF-Tu is involved in a covalent aggregate. Given the ability of Cq to recognize Xf EF-Tu and the apparent unusual physical state of Xf EF-Tu, we decided to investigate Xf EF-Tu further.

As indicated, for the MopB-preparation-derived EF-Tu, chlorosis is induced in pressure-infiltrated Cq leaves that is comparable to the chlorosis induced by pressure infiltrating Xf cells. EF-Tu, along with flagellin, chitin, certain lipopolysaccharides, and a few other molecules that are highly conserved in microbes, are designated as "microbe-associated molecular patterns" or MAMPs. MAMPs induce defense responses in a variety of plants (Jones & Dangl, 2006). MAMPs appear to be proteins or other macromolecules that are highly conserved within a pathogen type, providing a "pattern" that is a reliable indicator to the plant of the presence of a pathogen of that type. EF-Tu is the most abundant protein of rapidly growing *E. coli* cells. 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 plant defense gene mRNAs (Kunze et al., 2004). The elicitor activity of *E. coli* EF-Tu was traced to the amino-end-N-acetylated peptide of the protein and has been considered to be specific for brassicas (Kunze et al., 2004) (Cq is not a brassica). 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 for >100 eubacterial sequences (Kunze et al., 2004). 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. Other indications of multiple roles for the EF-Tu of Gram-negative bacteria occur in observations of *Mycoplasma pneumoniae* and *Lactobacillus johnsonii*. Both 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 cells were exposed to various concentrations of recombinant lysozyme (RLysozyme, Novogen). At 160 mg (wet weight) of Xf cells per mL and 2 U/µL RLysozyme in pH 8 buffer, the cell suspension was rapidly reduced in turbidity and increased in viscosity. Subsequent nuclease treatment reduced the viscosity. The result was a white, fluffy suspension, which was recovered as a precipitate and as a band in centrifuged sucrose gradients as indicated in Fig. 1AB. A similar treatment of wildtype *E. coli* cells resulted in complete dissolution of the cells, with no remaining precipitate. As suggested by the results in Fig. 1C, the lysozyme treatment released Xf EF-Tu in an insoluble form. However, hot-SDS extraction of the insoluble material released EF-

Tu as a molecule migrating with the expected mobility during SDS-PAGE, which hot-SDS extraction of Xf cells not treated with lysozyme did not do. Possibly Xf EF-Tu is covalently attached to a cell wall component, explaining its behavior in hot-SDS extracts of whole Xf cells. Even after lysozyme treatment releases Xf EF-Tu from its apparent covalent attachment to a cell wall component, Xf EF-Tu binds tightly to the lysozyme-released insoluble material, as evidence by its association with that material in the main band of a sucrose gradient (Fig. 1B). That is, the insoluble material was layered on the top of the sucrose gradient, and in the process of migrating to its equilibrium position, that material would have been subject to the equivalent of multiple washes without elution of the EF-Tu.

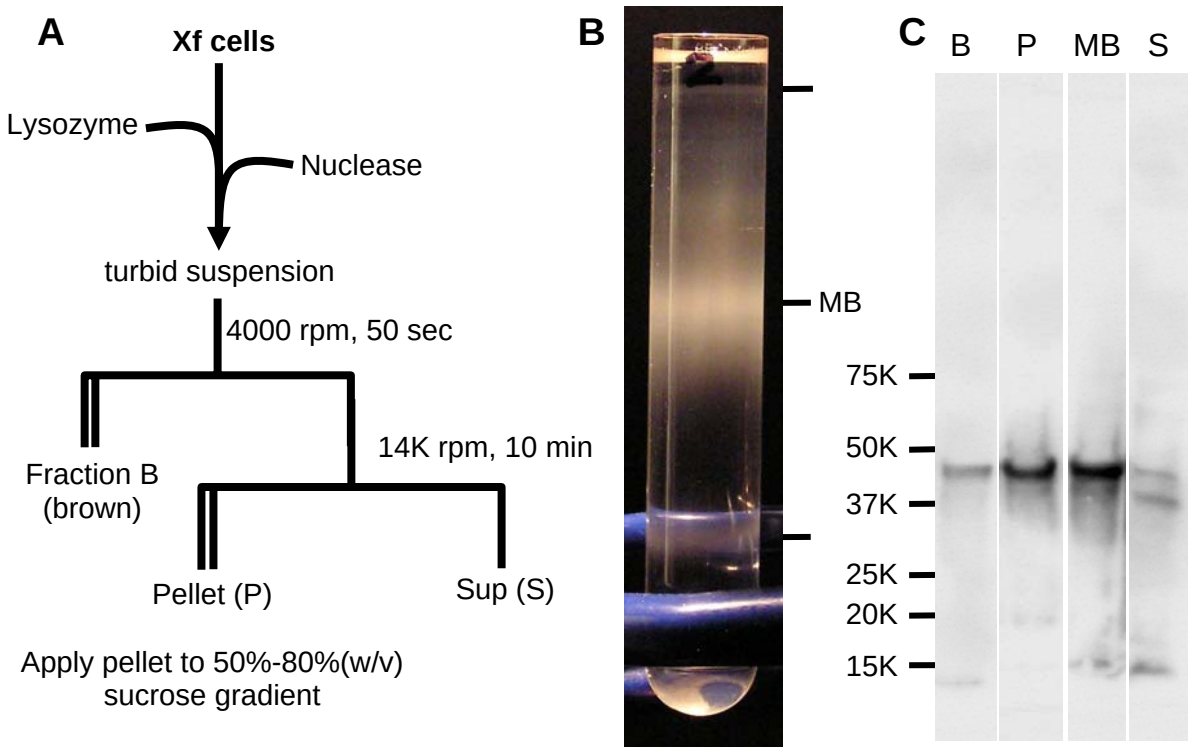

Figure 1. Xf EF-Tu is tightly associated with an insoluble fraction released from Xf cells by treatment with lysozyme. **A.** Xf cells were suspended in a mild detergent solution ("BugBuster," Novozyme), and the suspension was incubated at room temperature with lysozyme and then with a general nuclease (Benzonase, Novozyme), 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. 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 floating on the gradient, and in two other small bands marked by lines beside the image. **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.

A phenotype for Xf EF-Tu

As indicated above, an E. coli strain was designed and constructed to express a fusion of the bacteriophage M13 outer membrane protein P3 with Xf EF-Tu, Xf EF-Tu forming the amino end of the fusion. P3 is an adhesin responsible for initiating attachment of the bacteriophage M13 particle to the bacterial F-pilus. Prior to extrusion of the bacteriophage particle from the infected cell, or when P3 is expressed in transformed but uninfected cells, P3 resides in the cell outer membrane. Therefore, our expectation is that the fusion protein EF-Tu-P3 will be targeted to the outer membrane. An immunoblot of an extract of lysozyme- and nuclease-treated *E. coli* EF-Tu-P3 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 P3 alone. Omitting the lysozyme treatment resulted in no signal or a signal in the gel well only (data not shown). Therefore, the behavior of Xf EF-Tu in Xf and a Xf EF-Tu-containing fusion protein in *E. coli* were similar. *E. coli* transformed for P3 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-P3 construction grew slowly. We examined cells from the two cultures in the light microscope (Fig. 2); they were of very different appearance. The presumed EF-Tu-P3-expressing cells were larger in both length and diameter than the P3-expressing cells. The results reported here are consistent with incorporation of Xf EF-Tu, but not E. coli EF-Tu, into the *E. coli* cell wall.

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 intensities on leaves do not correlate (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 under consideration. We are working on identifying the sub-cellular location of Xf Ef-Tu, on obtaining more highly purified Xf-EF-Tu and on examining Xf-EF-Tu for various biological properties and its putative covalent connection to the Xf cell wall.

Summary of Research Accomplishments

Objective 1: obtained a single-chain antibody that binds to the Xf cell surface

Objective 2: identified Xf EF-Tu as a possible target for interfering with Xf cell function or Xf-induced symptom development; characterized unusual behavior of Xf EF-Tu that suggests a significant role for this protein in the Xf infection cycle

Publications and Reports

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

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

Presentations on Research

Paul A. Feldstein, George Bruening, Marta Francis, and Edwin L. Civerolo (2007) Biological and biochemical effects of peptides selected for their affinity to the *Xylella fastidiosa* cell surface. Annual Meeting of the American Phytopathological Society, San Diego, CA, 28 July-1 August, 2007.

George Bruening, Paul Feldstein, Edwin L. Civerolo (2007). Some characteristics of the *Xylella fastidiosa* translation elongation factor EF-Tu. *Pierce's Disease Research Symposium*, San Diego, CA, 12-14 December, 2007.

Research Relevance Statement

The research project described here is aimed at altering grapevine plants to resistance against, or tolerance to, *Xylella fastidiosa*, the causative agent of Pierce's disease.

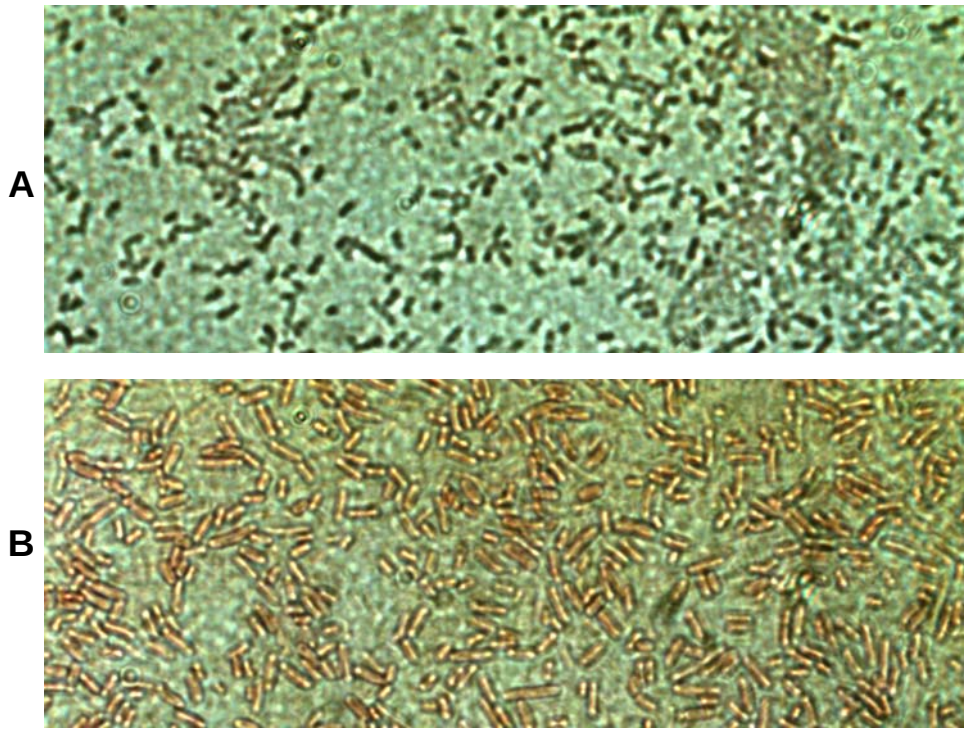

Figure 2. An outer-membrane-targeted Xf EF-Tu fusion protein appears to alter the cell morphology of *E. coli*. The suspensions of *E. coli* cells for both panels were stained with the same preparation of the Gram counter-stain safranin. **A**. A *RecA*-minus *E. coli* strain was transformed to express the bacteriophage M13 adhesin protein P3, 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-P3. Compared to the P3-protein-expressing cells of panel A, the EF-Tu-P3-expressing cells grew much more slowly and were greater in both length and diameter and were more readily visualized after staining with safranin.

Lay Summary of Current Four Month's Results

The goal of this research is to create grapevine lines that are able to resist infection by the causative agent of Pierce's disease, the bacterium Xylella fastidiosa, or to interfere with symptom development that accompanies infection. A protein was selected that binds to the surface of X. fastidiosa cells and could be incorporated with other protein elements to make an

anti-bacterial protein. A protein of the bacterium was identified that may influence symptom development.

Status of Funds

Funds will remain at the end of the contract. A no-cost extension of the contract has been requested.

Summary and Status of Intellectual Property

Not applicable

References

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