ROLES OF *XYLELLA FASTIDIOSA* PROTEINS IN VIRULENCE

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ABSTRACT
This work derives from a preliminary experiment by Civerolo and Bruening in which *Chenopodium quinoa* (Cq) was found to develop a localized chlorosis 24-48 hr after leaves were infiltrated with a suspension of live or heat-killed *Xylella fastidiosa* (*Xf*) cells. Excised electrophoresis gel regions were assayed for chlorosis-inducing activity, which was associated with a protein band with mobility corresponding to an estimated mass of 40K. Mass spectrometry of material the gel band revealed peptides corresponding to about 40% of the peptides predicted for the mopB gene. *Xf* mopB is an ompA protein. Members of the ompA group are located in the outer membrane of Gram-negative bacteria. The mopB translation product, signal peptide, and mature mopB protein were identified. In the present period, we showed that fluorescent anti-mopB IgG binds to intact *Xf* cells grown in liquid culture, confirming the location of mopB on the *Xf* cell surface. Previously we found that *Xf* cells extracted with SDS under specific conditions are depleted in most non-mopB proteins. This observation was extended by solubilizing mopB from the SDS-extracted cells at a slightly elevated pH, at 30°C in the presence of SDS and sodium perchlorate. Only traces of other proteins contaminated the mopB preparation. We postulate that mopB, as the major outer membrane protein of *Xf*, may participate in an interaction between *Xf* and the xylem sites at which colonization of the plant by *Xf* is initiated. Understanding a mopB-xylem interaction could direct strategies for interfering with *Xf* infection of grape and other *Xf* hosts. We demonstrated that thin balsa wood squares are able to absorb mopB from a mixture of mopB with other proteins, in the presence of non-ionic detergents known to be effective in solubilizing integral membrane proteins. Cellulose, in the form of washed filter paper, was similarly selective. These results support, but do not prove, our hypothesis about a role for mopB in *Xf* infection, possibly acting to bind *Xf* to the xylem interior. It may be possible to exploit mopB as a target for controlling Pierce's disease.

INTRODUCTION
The bacterium, *Xylella fastidiosa* (*Xf*), is the causative agent of Pierce's disease of grape. We reported previously the observation that untreated or heat-killed suspensions of 10^6 to 10^8 *Xf* cells/ml, pressure infiltrated into leaves of *Chenopodium quinoa* (Cq), induced a chlorosis in 24-48hr that conformed to the infiltrated area. The chlorosis reaction was the basis semi-quantitative assay for potency of *Xf*-derived preparations. The chlorosis-inducing activity survived treatment with the protein-denaturing detergent sodium dodecyl sulfate (SDS). Nevertheless, proteases inactivated the chlorosis-inducing activity. Extraction of *Xf* suspensions with SDS under mild conditions (30˚C, pH approx. 8.5 buffer) removed many *Xf* proteins (analysis by gel electrophoresis, SDS-PAGE) from the insoluble fraction, but the chlorosis-inducing activity remained insoluble. Guided by stained bands in an SDS-PAGE gel, unstained gel segments were extracted, concentrated and tested by infiltration of Cq leaves. The bulk of the chlorosis-inducing activity was associated with material with mobility corresponding to molecular weight of about 40K. Analysis of tryptic digests by mass spectrometry revealed peptides derived from the *Xf* outer membrane protein mopB. The abundance of *Xf* mopB suggests that it is the major outer membrane protein of the bacterium. Although a minor contaminant of the mopB preparations could be responsible for the Cq chlorosis, most likely the chlorosis inducing agent is mopB itself. We identified the likely start of translation for the mopB gene, MKKKILT…(corresponding to a 40.7K translation product), recognized a candidate 22 amino acid residue signal peptide, and determined that the pyroglutaminyl-terminated peptide pyro-QEFDDR in tryptic digests mapped to the mopB gene sequence (Simpson, Reinach et al. 2000). Results from other experiments suggest that the pyroglutaminyl residue is the natural amino-terminal end of mature *Xf* mopB protein, predicted molecular weight 38.5, and is <u>not</u> created as an artifact of our analysis by cyclization of an amino-terminal glutamine residue.

Xf mopB was further purified by solubilizing the insoluble fraction (from 30˚C SDS extraction of *Xf* cells) with hot SDS solution and chromatographing on 6% agarose beads. Pooled fractions were concentrated and used to raise polyclonal antibody. Attempts at cloning *Xf* mopB in *E. coli*, using constructions that encompassed the entire *Xf* mopB gene, including its putative promoter, were not successful. However, an inducible bacteriophage T7 RNA polymerase and T7 promoter system was adapted to create *E. coli* cultures that, when induced with IPTG, generated, at low levels, a protein with the mobility and immunological properties of mopB (work of Paul Feldstein). *Xf* mopB accumulation may sicken *E. coli*, accounting for the low level accumulation and requiring another approach for mopB production in *E. coli*.

OBJECTIVES

1. Identify specific *Xylella fastidiosa* (*Xf*) protein(s) and determine their roles in virulence, particularly major outer membrane protein mopB
2. Develop strategies for interfering with *Xf* infection of grape and/or with development of Pierce's disease

RESULTS AND CONCLUSIONS

Xf mopB appears to be accessible on the outside of intact Xf cells

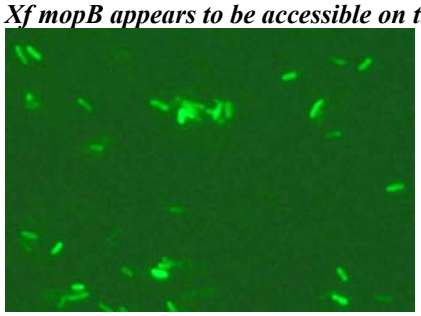


Figure 1. Fluorescent immuno-staining of *Xf* cells IgG from polyclonal antiserum against mopB and IgG prepared from the corresponding pre-immune serum each were labeled with Alexa 488 (fluorescein succinimidyl ester). *Xf* cells grown in liquid culture were exposed to the labeled IgG, diluted with SCP, washed twice with SCP, and examined in an epifluorescence microscope. Labeled *Xf* cells were observed (image at left) for the anti-mopB IgG, whereas the field for cells exposed to pre-immune IgG was dark (not shown). These results are consistent with mopB being accessible on the outside of the *Xf* cells. We have found *Xf* cells grown on agar plates (PD3 medium) to be a more suitable source for purification of mopB than cells grown in liquid PD3 culture. However, our attempts to label even very extensively washed cells from solid medium with fluorescent IgG against mopB were not successful (photomicrographs by Steve A. Wilson). It is possible that *Xf* cells grown on solid medium are encapsulated sufficiently to prevent access of the labeled IgG. We also observed that *Xf* cells from solid medium adhered readily to plastic surfaces, whereas *Xf* cells from liquid culture did not.

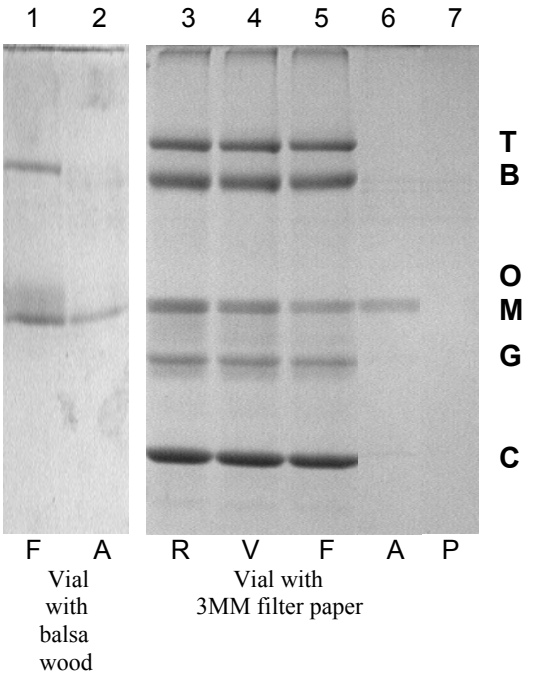
Purification of Xf mopB in soluble form

The methods we previously developed allowed us to purify *Xf* mopB in a soluble form, but only after heating, and presumably denaturing, mopB in SDS solution. Membrane-bound proteins such as mopB are notoriously difficult to coax into a soluble, purified form under mild conditions. Our starting material for trials of solubilization procedures was the insoluble fraction, enriched in mopB, that was obtained as described above by extraction of *Xf* cells with SDS at pH 8.5 and 30°C. We found, by testing a variety of conditions, that increasing the pH to 8.8 and including $NaClO_4$ in the extraction medium efficiently solubilized mopB at 30°C, whereas most of the other remaining *Xf* proteins remained in the insoluble fraction. Centrifugal filtration allowed us to remove excess SDS and $NaClO_4$, creating a very finely divided suspension that appears to dissolve in certain non-ionic detergent solutions. Analysis by SDS-PAGE suggests that the mopB is as pure as mopB recovered after solubilization in hot SDS and chromatography on 6% agaraose beads, but, of course, more likely to be biologically active (work of Jamal Buzayan).

A functional assay for Xf mopB and its implications

The Introduction, above, reports our observation of chlorosis-inducing activity of *Xf* mopB and our characterization of the mopB protein in the context of that activity. Little was revealed about functions of mopB in the context of Pierce's disease. Part of the work conducted in the past year was devoted to identifying possible mopB functions in the initiation of infections. The *Pseudomonas fluorescens* ompA-group, major out membrane protein OprF and the *Xf* mopB protein, unlike most other ompA proteins, have a proline-rich region preceding the carboxyl end region of similarity generally shared by ompA proteins. *P. fluorescens* competes against certain root-pathogenic fungi because of its ability to colonize root surfaces. De Mot and Vanderleyden (1991) purified OprF and demonstrated that OprF binds tightly to roots and probably is responsible for some aspect of the root-adhesion capabilities of *P. fluorescens*. This supposition also is consistent with mutational studies (Deflaun, Marshall et al. 1994). Therefore, we postulated that mopB may contribute to *Xf* virulence by adhering to xylem element interior surfaces. We selected balsa wood as a model for grape xylem because balsa wood is highly porous and readily available. The figure below reveals specific absorption of mopB to balsa wood in the presence of two other proteins, bovine serum albumin and ovalbumin. Absorption was not observed for mopB exposed to SDS at an elevated temperature, validating the new mopB purification method, based on solubilization in SDS and $NaClO_4$, as an improved approach to obtain functional mopB. We believe that absorption to balsa wood, which is composed largely of xylem, constitutes a functional assay for mopB that may have implications for strategies designed to interfere with *Xf* infection.

Figure 2. Binding of *Xf* mopB to balsa wood and to filter paper. Binding of *Xf* mopB to balsa wood and to filter paper. Lanes 1 and 2: Partially purified *Xf* mopB (migration position indicated by "M" on the right) was mixed with bovine serum albumin (BSA, "B") and ovalbumin ("O"). 1 mL of the mixture was exposed to a 2.5 x 2.5cm piece of 1.3mm thick balsa wood for 90min at room temperature with orbital shaking at 100rpm in SCP (succinate, citrate, phosphate) buffer containing 2mg/mL NP-40 non-ionic detergent. The liquid was combined with two 0.5mL rinses of the balsa wood (free fraction "F" lane 1). The balsa wood was eluted at 65°C with pH9 Tris-buffered 8mg/mL SDS, 10mg/mL mercaptoethanol (absorbed fraction "A" lane 2). The F and A fractions were concentrated by centrifugation over a 30K cut-off filter. Results show that part of the mopB protein but little or none of the BSA and ovalbumin (broad band) was absorbed by balsa wood. Lanes 3 through 7: Conditions were as for lanes 1 and 2 except that the reaction volume was 0.25mL, the sample contained transferring (T), BSA (B), glyceraldehydes phosphate dehydrogenase (G) and carboxyanhydrase (C) in addition to mopB (M), and the absorbent was two 8mm disks of Whatman 3MM paper. Lanes 5 and 6 analyze the F and A fractions and show selective absorption of mopB from the protein mixture. Lane 3 received the sample only; lane 4 corresponds to lane 5, except no filter paper was placed in the vial. Lane 7 corresponds to lane 6 except that the filter paper received no sample.



Cellulose is a principal component of xylem. The figure, just above, reveals that cellulose, in the form of washed Whatman 3MM filter paper, also selectively binds mopB in the presence of other proteins. The mopB band is diminished in intensity in lane 5 compared to lane 4, and the "missing" mopB was recovered in lane 6 as material eluted from the paper with hot, alkaline SDS solution.

Subsequently, we have shown that *Xf* cells bind tightly to 3MM filter paper, and that the proteins eluted from the paper by hot SDS include a protein with the mobility of mopB. Although additional experiments will be needed to demonstrate that the binding by cellulose of *Xf* cells is the result of mopB binding to cellulose, our results suggest that mopB may be an essential factor in the attachment of *Xf* cells to cellulose in the interior of xylem elements.

The proposed role of mopB in binding of *Xf* cells to the interior of xylem elements suggests potential transgenic and chemical strategies for interfering with *Xf* infection of grape. Transgenic approaches would involve a grape transgene, encoding a mopB-binding protein, a mopB-inactivating protein, or a hybrid protein capable to binding to mopB and inactivating *Xf* bacterial cells (this last approach being taken by Dandekar and Gupta) expressed in rootstock or scion and targeted to xylem. The expressed transgene protein presumably could overcome the small dose of GWSS-injected *Xf* inoculum and prevent or reduce the incidence of Pierce's disease development. Similarly, a chemical that is capable of being taken up by the grapevine and that interfered with mopB binding to cellulose could be applied as a prophylactic when survey information indicated invasion into the vineyard area of *Xf*-bearing GWSS.

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