NATIVE SECRETION SYSTEMS FOR THE GRAPEVINE ENDOPHYTE *PANTOEA AGGLOMERANS* USEFUL FOR THE DELIVERY OF ANTI-*XYLELLA* EFFECTOR PROTEINS

Project Leader:
David Lampe
Department of Biological Sciences
Duquesne University
Pittsburgh, PA 19219

Cooperators:
Angray Kang
Molec. & Applied Biosciences
University of Westminster
London, UK W1B 2UW

Thomas A. Miller
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported are from work conducted April 2007 to October 2007.

ABSTRACT
Symbiotic control is a strategy to deliver anti-pathogen effectors to plants or their insect vectors by modifying naturally occurring symbiotic bacteria. In order to deliver these effectors, secretion systems must be developed, especially for Gram – negative bacteria that contain an inner and outer cell membrane. We report here the development of secretion systems based on secreted proteins isolated from the grapevine endophyte, *Pantoea agglomerans* (*P. agglomerans*).

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is the principle vector of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapes. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission or by interfering with the replication of *Xf* in the plant may stop the spread of PD. These endpoints can be accomplished by genetically modifying bacteria that live in the sharpshooter, the plant, or both in a method called symbiotic control. Symbiotic control seeks to modify the phenotype of an organism indirectly by modifying its symbiotic bacteria.

Symbiotic control approaches to disrupt pathogen infection of humans are being developed by several groups. These include interference with the ability of triatomid bugs to transmit pathogens causing Chagas' disease (Beard et al., 2001), interference with HIV attachment to its target cells in the reproductive tracts of humans (Chang et al., 2003; Rao et al., 2005), and the elimination of persistent *Candida* infections from biofilms in chronically infected human patients (Beninati et al., 2000). Symbiotic control has also been applied to deliver cytokines mammalian guts to relieve colitis (Steidler et al., 2000; Steidler, 2001). Thus, the method has wide applicability.

One way to deliver anti-*Xf* protein factors from symbiotic bacteria is by secretion. Secreted anti-*Xf* factors might circulate throughout the plant, reaching foci of infection across physical xylem boundaries. Secretion from Gram-negative bacteria, however, is complicated by the fact that these species have two membranes that a protein must cross before appearing outside the cell. Gram negatives contain at least 6 identified types of secretion systems. Unfortunately, many of these systems are unpredictable when expressed heterologously.

We report here the evaluation of three proteins secreted from the grapevine bacterial symbiont *P. agglomerans* for use as secretion partners of anti-*Xf* protein effectors.

OBJECTIVES
1. To create a system to secrete anti-*Xf* effector proteins from the grapevine symbiont, *P. agglomerans* based on one or more of its native secreted proteins.

RESULTS
While we have been successful in secreting a wide variety of proteins using the *Escherichia coli* (*E. coli*) hemolysin system, not all proteins secreted in this way have proved to be functional. We set out to develop a secretion system specifically for *P.* (=*Enterobacter*) *agglomerans*. To do this we first identified several major secreted proteins of *P. agglomerans*. We collected spent medium from log-phase cultures of *P. agglomerans* and subjected them to 2D electrophoresis. We were able to separate over 20 spots and picked each one for identification via MALDI-TOF using a facility at Harvard Medical School (Figure 1). We reasoned that since *P. agglomerans* was sufficiently similar to *E. coli* some of its proteins should be able to be identified in this way, which normally requires a sequenced genome. Of the 20 spots we picked, only three returned identities in which we were confident. Fortunately, these were among the most abundant secreted proteins. These were: *fliC* (flagellin, Figure 1); *flgL* (flagellar hook protein); and, *ssb* (single-stranded DNA-binding protein).

We designed degenerate PCR primers based on the peptide sequences obtained from MALDI-TOF and also designed another set of degenerate primers aimed at isolating the flanking sequences of the genes by an "arbitrary" PCR method. To date, we have assembled all of the sequence of *fliC* and have partial sequences for *flgL* and *ssbI*. The *fliC* gene is particularly interesting to us since it has been used as the basis of a secretion system in *E. coli* already and it is the single most abundant proteins secreted from *P. agglomerans*.

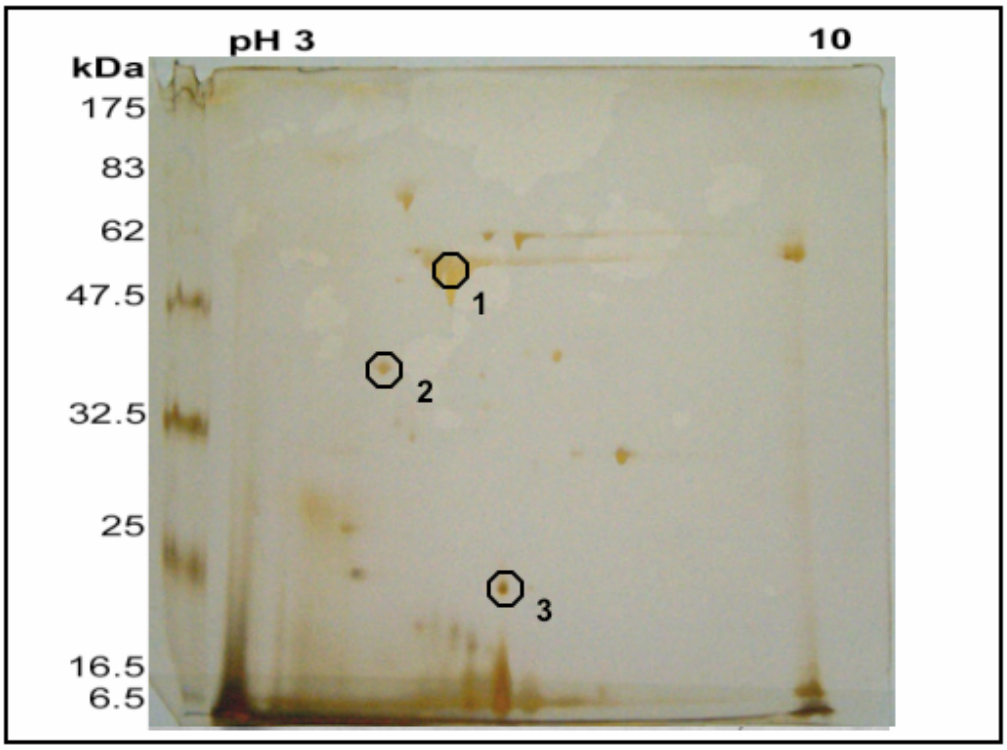

Figure 1. 2D-PAGE of *P. agglomerans* secreted protein profile in LB broth. A silver-stained gel of proteins isolated from late log phase cultures of *P. agglomerans*. The identity of circled spots was determined by MALDI-TOF analysis and peptide sequence comparison to *E. coli*. The identities of the proteins were as follows: 1. flagellin (*fliC*); 2. flagellar hook-associated protein (*flgl*); 2. single-stranded DNA binding protein (*ssb*).

We have begun to construct a secretion system for *P. agglomerans* based on the *fliC* gene. We designed several gene fusions between *fliC* and a single chain antibody (scFv) specific to bovine serum albumin (BSA). The fusion protein is relatively large and can be easily assayed for activity directly from spent media to determine if the protein is folding correctly. Similar experiments using the homologous gene in *E. coli* showed that only a portion of *fliC* is necessary to obtain secretion, perhaps as little as the 5'untranslated region (Majander et al., 2005). We designed constructs using the full-length *fliC* gene fused to the scFv and several C-terminal deletions of *fliC* down to as little as the 5'UTR. These will be tested for secretion by collecting the medium of log-phase cultures of *P. agglomerans* carrying the different fusion constructs. The smallest sequence of *fliC* that can successfully mediate secretion of the scFv will be used to test secretion of known anti-*Xf* effector proteins.

CONCLUSIONS

We were able to identify several abundant proteins secreted by the grapevine symbiont, *P. agglomerans*. These are under development for use in the secretion of anti- *Xf* effector proteins into grapevine xylem after colonization by the symbiont. The most promising of them is flagellin, which in E. coli can secrete proteins using only the 5' UTR of the *fliC* gene. Similar experiments will test this capacity of the *P. agglomerans fliC* gene.

REFERENCES

Beard, C. B., E. M. Dotson, P. M. Pennington, S. Eichler, C. Cordon-Rosales & R. V. Durvasula, 2001. Bacterial symbiosis and paratransgenic control of vector-borne Chagas' disease. Int J Parasitol 31(5-6): 621-7.

Beninati, C., M. R. Oggioni, M. Boccanera, M. R. Spinosa, T. Maggi, S. Conti, W. Magliani, F. De Bernardis, et al., 2000. Therapy of mucosal candidiasis by expression of an anti-idiotype in human commensal bacteria. Nat Biotechnol 18(10): 1060-4.

Chang, T. L., C. H. Chang, D. A. Simpson, Q. Xu, P. K. Martin, L. A. Lagenaur, G. K. Schoolnik, D. D. Ho, et al., 2003. Inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two-domain CD4. Proc Natl Acad Sci USA 100(20): 11672-7.

Majander, K., L. Anton, J. Antikainen, H. Lang, M. Brummer, T. K. Korhonen & B. Westerlund-Wikstrom, 2005. Extracellular secretion of polypeptides using a modified *Escherichia coli* flagellar secretion apparatus. Nat Biotechnol 23(4): 475-81.

Rao, S., S. Hu, L. McHugh, K. Lueders, K. Henry, Q. Zhao, R. A. Fekete, S. Kar, et al., 2005. Toward a live microbial microbicide for HIV: commensal bacteria secreting an HIV fusion inhibitor peptide. Proc Natl Acad Sci USA 102(34): 11993-8.

Steidler, L., 2001. *Lactococcus lactis*, A Tool for the Delivery of Therapeutic Proteins Treatment of IBD. Scientific World Journal 1(5): 216-7.

Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers & E. Remaut, 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science 289(5483): 1352-5.

FUNDING AGENCIES

Funding for the project was provided by the USDA Animal and Plant Health Inspection Service, and a Ruth L. Kirschstein National Institutes of Health Senior Fellowship to DJL.