SYMBIOTIC CONTROL OF PIERCE'S DISEASE: CONSTRUCTION OF TRANSGENIC STRAINS OF *ALCALIGENES XYLOSOXIDANS DENITRIFICANS* EXPRESSING SURFACE ANTI-*XYLELLA* FACTORS AS MICROBIAL PESTICIDES FOR PIERCE'S DISEASE CONTROL

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

Project Director:
Thomas A. Miller
Dept. of Entomology
University of California
Riverside, CA 92521

Cooperators:
Carol Lauzon
Dept. of Biological Sciences
California State University
Hayward, CA 94542

Don Cooksey
Deparment of Plant Pathology
University of California
Riverside, CA 92521

Blake Bextine
Dept. of Entomology
University of California
Riverside, CA 92521

Steven Lindow
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Consultant:
Frank Richards
Yale University
New Haven, CT 06520

Reporting period: The results reported here are from research conducted from April 2003 to October 2004.

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 (paratransgenesis) is a promising method of pathogen control. Paratransgenesis seeks to modify the phenotype of an organism indirectly by modifying its symbiotic bacteria to confer vector-incompetence

Paratransgenic 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 [1], interference with HIV attachment to its target cells in the reproductive tracts of humans [2], and the elimination of persistent *Candida* infections from biofilms in chronically infected patients [3]. Paratransgenesis has also been applied to deliver cytokines mammalian guts to relieve colitis [4; 5]. Thus, the method has wide applicability.

Alcaligenes xylosoxidans denitrificans (Axd) is Gram negative, beta proteobacterial species that can colonize the GWSS foregut and cibarium, as well as various plant tissues, including xylem. It is non-pathogenic in insects, plants and healthy humans. Given these characteristics, *Axd* has become the focus of our paratransgenesis efforts to control PD in grapes. Over the past two years we developed the technology to stably modify *Axd* by inserting genes into its chromosome and also isolated as single chain antibody that recognized an epitope on the surface of the Pierce's Disease strain of *Xf*. [6].

We report here the construction of strains of *Axd* that express an anti-*Xylella* single chain antibody (scFv) on the outer surface of *Axd* as fusions to three different heterologous outer membrane proteins. In each case, strains of varying fitness were recovered as measured by growth rate as compared to wild-type strains.

OBJECTIVES

1. Construct anti-*Xylella* scFv-membrane protein fusions;
2. Construct strains of *Axd* that express the scFv-membrane protein fusions in the outer membrane;
3. Construct transgenic *Axd* strains of varying fitness.

RESULTS

A. Membrane Protein-scFv Gene Fusions

We fused an anti-*Xylella* scFv gene to three different outer membrane protein genes in order to display the scFv on the outer membrane of *Axd*. These were a lipoprotein-outer membrane protein A (*lpp-OmpA*) fusion from *E. coli* [7]; the ice nucleation protein Z (*inaZ*) from *Pseudomonas syringae* (a gift of Steven Lindow); and an internally-deleted form of *inaZ* that eliminates the internal ice nucleation repeat sequence but retains the N and C terminus of the protein necessary to export and

anchor it in the outer membrane (short-*inaZ*)[8]. Each of these was placed on a *Himar1 mariner* transposon and random chromosomal insertions were obtained for each generating multiple strains[9] (see Table 1).

B. Expression of scFv Fusions on the Surface of Axd.

We determined the degree of surface expression of the scFv fusions on *Axd* by two methods. The first was a "spun cell ELISA"[7]. This method uses a suspension of cells that express a target epitope as the substrate for an ELISA. Detection of the scFv was accomplished before and after induction of the *lac* promoter by either reaction with Protein L-conjugated HRP (which detects scFv light chains) or with a HPR conjugated antibody that reacts with the haemagglutinin epitope tag on the scFv. Results of spun cell ELISAs on different strains are shown in Table 1. Strains varied considerably in their scFv surface expression levels, presumably due to the site of insertion. Most strains of short-*inaZ* fusions, for example were poor expressers when induced and strain AL8.2 only showed appreciable levels of surface expression when uninduced.

Table 1. Characteristics of transgenic *A. xylosoxidans* strains expressing an anti-*Xylella* single chain antibody as an outer membrane protein fusion.

Strain	scFv fusion	Surface expression[1]	Relative Fitness[2]	Insert location[3]
AL7.2	*P. syringae inaZ*	++	G	- major facilitator superfamily transporter
AL7.5	""	+++	G	- inorganic pyrophosphatase
AL7.7	""	++	S	-fructose transport system repressor
AL7.10	""	+	G	ND
AL8.2	*P. syringae* short *inaZ*	+++ (uninduced only)	G/P	-probable transporter
AL8.3	""	BK	P	ND
AL9.1	*E. coli lpp-ompA*	+	S	ND
AL9.4	""	+++	S/G	ND
AL9.5	""	+	S	ND

[1] These values are relative to background as measured in a spun cell ELISA: BK = background levels; + noticeable expression, ++ strong expression; +++ very strong expression.
[2] Fitness values are measured as growth rates in liquid culture relative to that of wild-type *A. xylosoxidans*. S (= strong, essentially wild type); G (= good, but slower than wild type); P (= poor)
[3] Most likely identity of genes where transgenes were inserted. These were obtained using tblastx with flanking insertion sequences against the microbial nucleotide database from Genbank. ND= not determined.

The second method used to determine whether expression was occurring in the outer membrane of *Alcaligenes* was a test for ice nucleation. Wild-type *Axd* cannot nucleate ice (unpublished observations). We tested whether or not AL7 and AL8 strains could nucleate ice. All of the AL7 strains could nucleate ice while neither of the AL8 strains did so. This is consistent with surface expression of the full-length *P. syringae* ice nucleation protein on the surface of the AL7 strains. AL8 strains express a form of inaZ that has the internal repeat region removed. This is the region that is responsible for ice nucleation in these proteins.

C. Fitness of transgenic Axd strains

Our strains are built via transposon insertion and so should vary in fitness depending on the site of insertion in the chromosome. We measured the fitness of each strain compared to wild type by measuring their growth rates in log phase in liquid culture. These relative fitness values are shown in table 1 along with the most likely site of insertion of the transposon used to make the strain. We determined the site of insertion by sequencing outward from the transposable element inverted terminal repeats into the flanking genomic DNA and then using tblastx against the microbial genomic database in Genbank. There are no *Axd* sequences in Genbank, so the matches we obtained were typically to species in the genus *Pseudomonas*, another basal beta proteobacterial group.

Strains were highly variable in their fitnesses. Some strain fitnesses were indistinguishable from wild type (e.g., AL7.7 and AL9.5), while others were obviously affected in their growth rates (e.g., AL8.3). There was no obvious correlation between fitness and ability to surface express the scFv fusions. Indeed, one of our best expressing strains was only a modest grower (AL7.5) while other strains grew well and expressed the transgene poorly (e.g., AL9.5). The ability to isolate strains that

vary in fitness is an important aspect of paratransgenesis since we are interested in providing *Axd* reagents that vary in their level of persistence.

D. Determining the target of the anti-Xylella scFv

We attempted to determine the target of the anti-Xylella scFv we isolated previously. We used a combination of 1-D and 2-D SDS-PAGE gels and western blotting to determine a size range for the target protein.

CONCLUSIONS

We have created multiple transgenic strains of the plant and insect symbiotic bacterium, *Alcaligenes xylosoxidans* (*denitrificans*) that carry a surface expressed anti-*Xylella* antibody. These strains carry chromosomal insertions of the genes for the scFv and we were able to recover strains that varied in fitness and in their expression level for the scFv on their outer membranes. These initial strains are currently being tested for their ability to interfere with the transmission of *X. fastidiosa* by sharpshooters.

The future goals of this project are to isolate new anti-*Xylella* factors that can be expressed on the surface of *Axd*, to incorporate genetic systems aimed at preventing horizontal gene transfer of the transgenes, and to improve expression levels of the transgenes on the surface of the cell. All of these features are aimed at developing strains of *Axd* that can interrupt the spread of *Xylella* from the glassy-winged sharpshooter to uninfected grapevines.

REFERENCES.

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

Chang, T. L., Chang, C. H., Simpson, D. A., Xu, Q., Martin, P. K., Lagenaur, L. A.*, 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 U S A* 100, 11672-7.

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

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W.*, et al.* (2000). Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. *Science* 289, 1352-5.

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

Bextine, B., Lauzon, C., Potter, S., Lampe, D. & Miller, T. A. (2004). Delivery of a genetically marked Alcaligenes sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. *Curr Microbiol* 48, 327-31.

Benhar, I., Azriel, R., Nahary, L., Shaky, S., Berdichevsky, Y., Tamarkin, A.*, et al.* (2000). Highly efficient selection of phage antibodies mediated by display of antigen as Lpp-OmpA' fusions on live bacteria. *J Mol Biol* 301, 893-904.

Lee, S. Y., Choi, J. H. & Xu, Z. (2003). Microbial cell-surface display. *Trends Biotechnol* 21, 45-52.

Rubin, E. J., Akerley, B. J., Novik, V. N., Lampe, D. J., Husson, R. N. & Mekalanos, J. J. (1999). *In vivo* transposition of *mariner*-based elements in enteric bacteria and mycobacteria. *Proc Natl Acad Sci U S A* 96, 1645-1650.

FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service and USDA Cooperative State Research, Education, and Extension Service BRAG (start date September 15, 2004).