USE OF THE *E. COLI* α-HEMOLYSIN SECRETION SYSTEM IN BACTERIA DESIGNED FOR SYMBIOTIC CONTROL OF PIERCE'S DISEASE IN GRAPEVINES AND SHARPSHOOTERS

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Reporting Period: The results reported are from work conducted from April 2006 to October 2006.

ABSTRACT
Strains of *Alcaligenes xylosoxidans denitrificans* (*Axd*) that secrete anti-*Xylella* factors are being developed for use in a strategy to prevent the spread of *Xf* or possibly to cure infected grapevines. We built constructs that fused the last 60 amino acids of the autotransporter α-hemolysin from *E. coli* to test proteins. These were two different forms of anti-BSA single chain antibodies (scFvs) which are surrogates for anti-*Xylella* effector proteins. These proteins were efficiently secreted from *E. coli* when co-expressed with the proteins HlyB and HlyD. HlyB, HlyD, and TolC together form the membrane structure used by α-hemolysin to cross both the inner and outer membrane of the cell. We report here on efforts to move this system into *Axd*, a species not closely-related to *E. coli*, but that can survive in both grapevine and sharpshooters.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is the principal 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 interfereing 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.

Alcaligenes xylosoxidans denitrificans (Axd) is Gram negative pseudomonad-like species that can colonize the GWSS foregut and cibarium, as well as various plant tissues, including grape xylem. It is non-pathogenic in insects, plants and healthy humans. Given these characteristics, *Axd* has become the focus of our symbiotic control efforts to control PD in grapes. Over the past several years we developed the technology to stably modify *Axd* by inserting genes into its chromosome via transposition, have developed methods to suppress horizontal gene transfer, and have isolated a single chain antibody that recognizes an epitope on the surface of the PD strain of *Xf*. (Bextine et al., 2004). We are currently engaged in combining these systems in order to produce strains of *Axd* that are suitable for environmental release in a practical strategy symbiotic control strategy for PD.

One way to deliver anti-*Xylella* protein factors from *Axd* in grapevine is by secretion. Secreted anti-*Xylella* 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. One system that seems to have wide applicability is the a-hemolysin autotransporter from *E. coli* (Fernandez et al., 2000). This protein is secreted in a single energy-dependent step across both membranes of Gram negative bacteria when the other components of the system are also present (the proteins HlyB, HlyD, and TolC). Fusion of the last 60 amino acids of the protein is sufficient to target any N-terminal passenger protein for secretion.

We report here the evaluation of the *E. coli* α-hemolysin system for use in *Axd* to secrete soluble anti-*Xylella* protein effectors in grapevine and insects.

OBJECTIVES
1. Test the *E. coli* α-hemolysin secretion system in *E. coli* and *Axd*.

RESULTS

Secretion of scFv-hemolysin fusions from *E. coli*. We used a two plasmid system to secrete two test constructs from *E. coli*. One plasmid carried the *hlyB* and *hlyD* genes and were encoded on a low copy number plasmid based on pSC101. The others carried either an antiBSA scFv gene fused to a sequence encoding the last 60 amino acids of HlyA or an antiBSA scFv capable of dimerizing to form a diabody that was similarly fused to HlyA (Fernandez et al., 2000; Fraile et al., 2004). These plasmids were grown in the same strain overnight, the supernatant harvested, and small samples of the supernatant loaded on SDS-PAGE gels. A subsequent western blot used an antibody directed at an epitope on the scFv constructs. These results are shown in Figure 1. Neither scFv constructs alone (scFv-HlyA) nor the HlyB-D plasmids produced any detectable secreted protein. When both plasmids were grown together, single chain antibody proteins could be detected easily in the supernatants of their respective cultures.

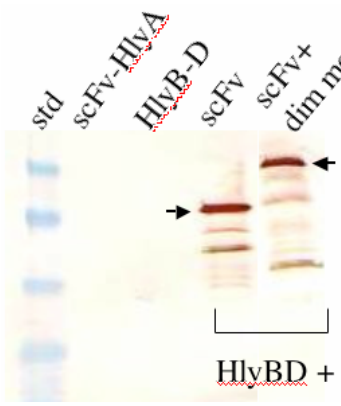

Figure 1. Supernatants from *E. coli* cultures expressing secretable forms

Progress toward secretion of a single chain antibody constructs from *Axd*. We added origins of transfer (*oriT*) to each of the plasmids in the *a*-hemolysin secretion system described above and attempted to move these plasmids into *Axd* via mating from *E. coli*. We recovered no exconjugants whatsoever. Control matings from one *E. coli* strain to another yielded abundant exconjugants. From these data we conclude that the origins of replication of the secreting plasmids do not function in *Axd*. Efforts at constructing and testing the secretion system on broad host range plasmids like pRO1600 are underway (Shanks et al., 2006).

CONCLUSIONS

The *E. coli* hemolysin secretion system is a robust and powerful way to secrete various proteins in a single step across both membranes of Gram negative bacterial cells. In the presence of HlyB and HlyD proteins, fusions of two different scFv to the last 60 amino acids of HlyA were secreted into the growth medium of the E. coli cells carrying them. Attempts to use these fusions in *Axd* failed because the origins of replication (ColE3 and pSC101) were not functional in this species. Moving the secretion system onto plasmids that do replicate in *Axd* (like pRO1600 and its derivatives) should result in secretion from this species as well. It should then be possible to secrete anti-*Xyllela* effector proteins within grape plants that can circulate within the xylem of the plant.

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FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service.

GENOTYPIC CHARACTERIZATION OF *ALCALIGENES XYLOSOXIDANS* SUBSP. *DENITRIFICANS* (*AXD* HC01) AND FOUR RELATED STRAINS

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Reporting period: The results reported here are from work conducted September 2005 to September 2006.

ABSTRACT
In symbiont therapy, an insect's natural symbionts are genetically modified to prevent the transmission of a pathogen. This strategy is currently under investigation as a way to control the spread of Pierce's disease (PD) of grapevine. PD is caused by the bacterium *Xylella fastidiosa* (*Xf*), which is transmitted by the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*). The five GWSS symbionts used in this research were identified through biochemical testing as *Alcaligenes xylosoxidans denitrificans* (*Axd*) Hc01, *Axd*1, *Axd*2, *Axd*3, and *Axd*4. The genetic relatedness of these bacteria, as well as their relationships to other bacterial species was analyzed using two highly conserved prokaryotic genes, the 16S rDNA sequence and the gyrase B sequence. These sequences were used to construct phylogenetic trees using the neighbor-joining method. Analysis of the 16S tree indicated that all of these bacteria were closely related to members of the genus *Pseudomonas*. The phylogenetic trees that were constructed using the gyrase B gene also supported the conclusion that these bacteria are closely related to members of the genus *Pseudomonas*. Further testing using the 16S-23S intergenic spacer region one is currently underway.

INTRODUCTION
One new potential management strategy for Pierce's disease (PD) of grapevine is the use of symbiont therapy. Symbiont therapy exploits the interactions among a pathogen-transmitting organism, its bacterial symbionts, and the pathogenic organism itself (Beard 2002). First, a bacterial symbiont that occupies the same niche as the pathogen must be identified. These symbionts are genetically modified to produce a molecule that hinders the spread of the pathogen in question. The genetically modified bacteria are re-introduced into the vector so that they can reduce its ability to transmit the pathogen in question. For this approach to be successful, the bacterial symbiont must be easily cultured and manipulated *in vitro*, and the genetic modification cannot alter their value to the host organism or their ability to occupy their niche. In addition, the bacterial symbionts cannot be pathogenic to either their host or to non-target organisms before or after the genetic modification (Durvasula 2003). Symbiont therapy has been investigated as a way to control the spread of Chagas disease (Beard 2002; Durvasula 2003), murine colitis (Steidler 2000), and HIV (Chang 2003).

For symbiont therapy to be effective in limiting the spread of PD, a culturable symbiont that inhabits the pre-cibarium and cibarium of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis)* is required, since these areas are colonized by *Xf*. Three bacterial species that meet these requirements are *Chryseomonas* spp, *Ralstonia* spp, and *Alcaligenes* spp (Bextine 2004). The *Alcaligenes* species were of particular interest because they were frequently isolated from wild GWSS (Kuzina 2004) and because they could also successfully colonize the xylem of various plants, including citrus (Araujo 2002; Bextine 2005). Five *Alcaligenes* species were isolated from the mouthparts of GWSS and identified as *Alcaligenes xylosoxidans* subspecies *denitrificans* (*Axd*) using standard morphological and biochemical tests. Four of these species were designated as *Axd*1, *Axd*2, *Axd*3, and *Axd*4. The other *Alcaligenes* species that was found in GWSS was designated as *Axd* Hc01 and selected for further study (Bextine 2004). However, the classification of *Axd* Hc01 remains unsettled.

OBJECTIVE
1. If *Axd* Hc01 is to be used as part of a symbiont therapy program, the issues surrounding its identity must be resolved. One way to help clarify its identity and relationship to other identified *Axd* strains is to construct phylogenetic trees based on the sequences of universally present, highly conserved prokaryotic genes (Laguerre 1994). The goal of this research is to help identify *Axd* Hc01 by placing it in phylogenetic trees based on 16S, gyrase B, and 16S-23S intergenic spacer region sequences.