# SOLUBLE FORMS OF AN ANTI-*XYLELLA* ANTIBODY AND STRAINS OF *ALCALIGENES XYLOSOXIDANS DENITRIFICANS* CAPABLE OF SECRETING THEM

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## ABSTRACT
Several methods were used to create soluble forms of a single chain antibody (scFv S1) that binds to the surface of the grape strains of *Xylella fastidiosa* (*Xf*). S1 fused to a *pelB* leader and secreted from *E. coli*. These forms were not secreted correctly and could not bind *Xf* in an ELISA. Maltose binding protein fusions of S1 were soluble and could be used to detect *Xf* in an ELISA. We also successfully secreted S1 from *Alcaligenes xylosoxidans denitrificans* (*Axd*) using a leader sequence that directed S1 to the periplasmic space. Strains of *Axd* that secrete anti-*Xylella* factors are being developed for use in a strategy to prevent the spread of *Xf*.

## 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 (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 (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 patients (Beninati et al., 2000). Paratransgenesis 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, 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 several years we developed the technology to stably modify *Axd* by inserting genes into its chromosome, have developed methods to suppress horizontal gene transfer, and have isolated a single chain antibody (scFv) that recognized 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.

We report here the evaluation of various S1 constructs for solubility and the construction of a prototype *Axd* strain capable of secreting S1.

## OBJECTIVES
1. To create soluble and functional forms of the S1 single chain antibody.
2. To construct strains of Axd capable of secreting scFvs.

## RESULTS
### Objective 1: Soluble forms of the S1 scF.
We expressed a soluble form of the S1 scFv in two ways. S1 was expressed from a construct carrying a *pelB* leader sequence which targets the protein to the periplasm of the cell, from which it can "leak" out into the growth medium and be collected. Several strains of *E. coli* were used for this test. We also fused the S1 sequence to *E. coli* maltose binding protein and purified the fusion protein using affinity chromatography. S1 proteins expressed in these two ways were assayed in a western blot to see if they could be detected at all and were also used in an ELISA to determine whether or not they could still bind to the surface of *Xf*. The results of these assays are shown in Table 1.

**Table 1.** Constructs, expression, and ELISA details for soluble S1 anti-*Xylella* scFv.

| **Protein expression Species / Strain** | **S1 construct** | **Detectable in Western?** | **Detectable in *Xylella* ELISA?** |
|---|---|---|---|
| *E. coli* Top10F' | pAM5: Plac-driven expression of S1. | Yes-Strong expression[1] | No |
| *E. coli* HB2151 | pAM5: Plac-driven expression of S1. | Yes- Weak expression | No |
| *E. coli* TB1 | pAM5: Plac-driven expression of S1. | Yes- Weak expression | No |
| *E. coli* Top10F' | pAM62: Plac-driven expression of MBP[2] - full-length S1. | Yes (purified protein) | Yes |
| *E. coli* Top10F' | pAM63: Plac-driven expression of MBP-S1 lacing periplasmic S1 targeting sequence. | Yes (purified protein) | Yes |

[1]. The non-pMAL constructs used in this table are meant to secrete a soluble scFv into the supernatant of the culture by leakage from the periplasmic space.
[2]. MBP = maltose binding protein.

As can be seen in Table 1, there was a strong strain-specific effect on the amount of expression of S1. Furthermore, in each case, the proteins secreted from *E. coli* were of the wrong size (see below) and did not bind to the surface of *Xylella* in an ELISA. On the other hand, purified maltose binding protein-S1 fusion proteins were easily detected on westerns and retained the S1 binding activity which is present when S1 is fused to the gIII protein of M13 phage and is present as part of a viral particle.

**Objective 2: Secretion of an S1 scFv construct from Axd**

Secretion from Gram negative bacteria 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 six identified types of secretion systems. Unfortunately, these systems are unpredictable when expressed heterologously. In other words, there is no "one-size-fits-all" system that can be used in all species. Periplasmic targeting, however, can be achieved easily by using leader sequences that are functional in a broad range of organisms. We used the *pelB* leader from *Erwinia caratovora* to target S1 to the periplasm of *E. coli* and *Axd*. Constructs were made as single-copy insertions into the chromosome delivered on a *mariner* transposon. Cultures of these strains were allowed to grow overnight for *E. coli* and for two days for *Axd*. Western analysis was used to detect the S1 in the medium. The results are shown in Figure 1.

As noted above, *E. coli* expressed S1 incorrectly when fused with a *pelB* leader for periplasmic expression. Material of ca. 40kD, from the media and the pellet could be detected in the western. Furthermore, higher molecular weight material of ca. 70 kD could be detected only in the pellet. Media in which S1-*Axd* strains were grown, however, secreted a protein of the correct size for monomeric S1 (ca. 26 kD). In overloaded lanes of pellet (= cell lysate) material, a product of 70 kD could also be detected as it was in *E. coli*. These results indicate that periplasmic targeting and "leakage" from the periplasmic space can be used as a kind of secretion system in *Axd*. The difference in expression and secretion of S1 in two different Gram-negative bacterial species clearly indicates that species-specific factors are at work and need to be carefully evaluated when constructing strains for use in symbiotic control.

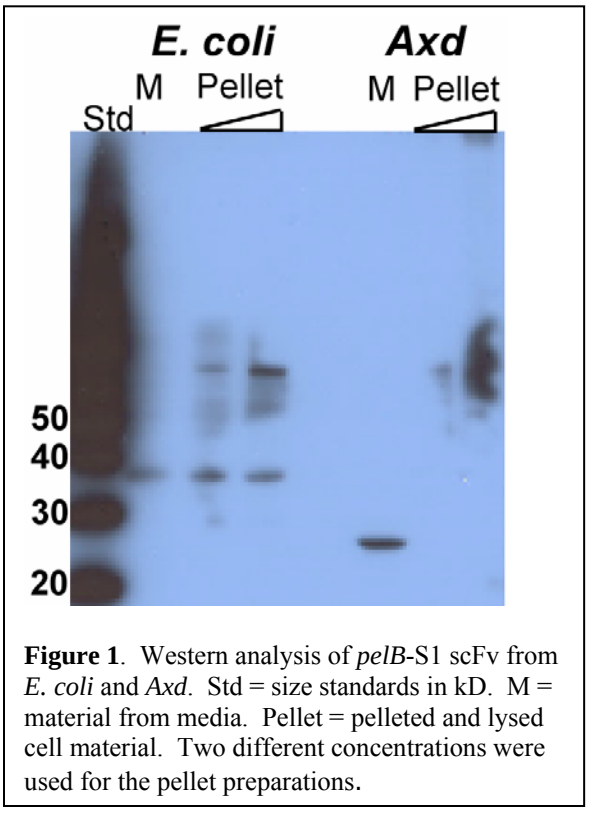


**Figure 1**. Western analysis of *pelB*-S1 scFv from *E. coli* and *Axd*. Std = size standards in kD. M = material from media. Pellet = pelleted and lysed cell material. Two different concentrations were used for the pellet preparations.

**CONCLUSIONS**

Paratransgenesis to control PD in grapevine requires that several conditions be met. First, a suitable microorganism must be found. Second, anti-*Xylella* factors must be isolated. Third, strains of the microorganism must be constructed that can deliver the anti-*Xylella* factor appropriately and in an environmentally-safe way. We previously isolated an anti-*Xylella* single chain antibody by panning a scFv phage library. The phage antibody reacts strongly only with grape strains of *Xf*. We converted the scFv from phage-form to soluble form by targeting the scFv to the periplasmic space without the phage gIII protein. In *E. coli*, this results in an incorrectly produced protein that lacks the ability to bind *Xf*. Expression of S1 as a maltose binding protein fusion results in a soluble protein that retains *Xf* binding ability. We thus conclude that it is possible to convert the phage antibody to a soluble form; however we will need to evaluate different constructs for proper binding and secretion behavior. Finally, we were able to successfully secrete S1 from transgenic strains of *Axd* by using the very simple method of targeting the protein to

the periplasmic space. Protein collected from the medium after two days was of the correct size. We conclude that secreting strains of *Axd* are possible routes to the creation of usable bacterial strains targeting *Xf* in a paratransgenesis approach to controlling PD.

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