

## DESIGN OF CHIMERIC ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF *XYLELLA*

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### ABSTRACT

*Xylella fastidiosa* (*Xf*), is a gram-negative xylem-limited bacterium and causative agent of Pierce's disease (PD) in California grapevines. During very early stages of *Xf* infection, specific carbohydrates/lipids/proteins on the outer membrane of *Xf* interact with plant cells and are important for virulence (3). Design of a protein inhibitor that interrupts this step of the plant-*Xf* interaction will be useful in anti-microbial therapy and controlling PD. Traditionally, antibiotics are prescribed as a preferred therapy; however, a pathogen often develops antibiotic resistance and escapes their anti-microbial action (4). In this UC/LANL project, we propose a novel protein-based therapy that circumvents the shortcomings of an antibiotic. We have designed a chimeric anti-microbial protein with two functional domains. One domain (called the surface recognition domain or SRD) will specifically target the bacterium outer-membrane whereas the other will lyse the membrane and kill *Xf*. In this chimera, Elastase is the SRD that recognizes mopB, the newly discovered *Xf* outer membrane protein (5). The second domain is Cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have successfully tested each of these components individually and demonstrated that they each (Elastase and Cecropin B) display activity against *Xf*, which is increased when both proteins are combined. We have tested Elastase against purified mopB and intact *Xf* cells and found that mopB is degraded in both cases, suggesting that it is potentially a target for Elastase. The HNE-GSTA-Cecropin B chimera gene has been synthesized and is currently being cloned into vectors for overexpression in insect and grapevine cells in order to test its activity against *Xf in vitro*. We have also initiated transgenic grapevine cultures expressing a pear polygalacturonase inhibiting protein that is secreted into the medium using a CELLline 350 bioreactor. In the future, we plan to use this system to test secretion and anti-*Xf* of the chimeric protein.

### INTRODUCTION

Globally, one-fifth of potential crop yields are lost due to plant diseases primarily of bacterial origin. *Xylella fastidiosa* (*Xf*) is a devastating bacterial pathogen that causes PD in grapevines, citrus variegated chlorosis (CVC) in citrus, and leaf scorch disease in numerous other agriculturally significant plants including almonds in California (<http://danr.ucop.edu/news/speeches>). Since the glassy-winged sharpshooter (an insect vector) efficiently transmits PD, a great deal of effort has been focused on using insecticides to localize and eliminate the spread of this disease. However, the availability of the whole genome sequences of PD and CVC strains of *Xf* offer new avenues to directly target and inactivate the pathogen. In this project, we propose a structure-based approach to develop chimeric anti-microbial proteins for rapid destruction of *Xf*. The strategy is based upon the fundamental principle of innate immunity that plants recognize and clear pathogens in rapid manner (1-2). Pathogen clearance by innate immunity occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction by anti-microbial processes. Different sets of plant factors are involved in different steps of innate immunity. Our strategy of combining a pathogen recognition element and a pathogen killing element in the chimeric molecule is a novel concept and has several short and long term impacts.

## OBJECTIVES

- Objective 1: a) Utilize literature data and computer modeling to identify an SRD that specifically targets mopB (Elastase)  
b) Utilize literature data and computer modeling to identify a useful Cecropin (i.e., Cecropin B)  
c) *In vitro* testing of anti-*Xylella* activity of the mopB-specific SRD (Elastase) and *Xylella*-specific Cecropin B and demonstration of synergistic killing effect due to the combined use of Elastase and Cecropin B.
- Objective 2: a) Design and construction of synthetic gene encoding Elastase-Linker-Cecropin B Chimeric protein  
b) Expression Elastase-Linker-Cecropin B in insect and plant cells and testing activity *in vitro*.
- Objective 3: a) Expression in transgenic plants  
b) Testing for anti-*Xylella* activity *in planta* and testing for graft transmissibility.

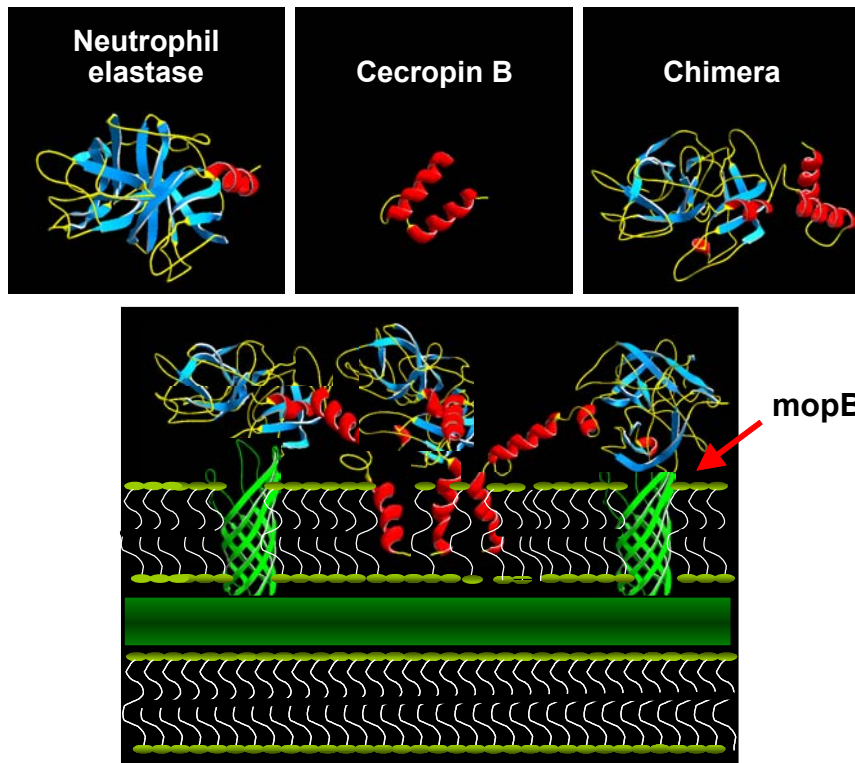
## RESULTS AND CONCLUSION

Human Neutrophil Elastase (HNE) (6) was chosen as our first SRD. Neutrophils contain a variety of proteins that enable the cells to migrate toward and eliminate microbial pathogens (7). Until 1991, no specific antibacterial activity had been ascribed to HNE (8). However recent research has established that HNE is the only human neutrophil protein, which is capable of individually killing *Borrelia burgdorferi*, the causative agent of Lyme disease (9, 10). Furthermore, it is known that HNE can augment the cidal properties of other active proteins (11). Sequence-structure analysis of mopB revealed that it contained an specific cleavage site for HNE that is exposed on the surface. We have studied the efficacy of HNE in combination with the antibacterial peptide Cecropin B, that inserts preferentially into the lipid bilayer of gram-negative bacteria, in killing *Xf*. Measuring the number of colony forming units remaining after the bacterium was exposed to HNE, Cecropin B and the combination of both, we found that HNE greatly stimulates the lysis induced by Cecropin B. In addition, we found that Mop B was partially digested by HNE after incubating either purified Mop B or *Xf* cells with HNE for an hour. Based on these preliminary results, we have designed a chimeric protein of Cecropin B and HNE; in order to stabilize the Cecropin B peptide and enhance the overall affinity of the ligands for the bacterial surface. The covalent attachment of Cecropin B to HNE is proposed to increase the stability of the peptide by lowering the conformational entropy of its unfolded state and to increase the overall affinity for the bacterial surface by minimizing the degrees of motion at the binding site, thereby increasing binding between the ligands and the surface.

Our strategy began with the generation of a 3-D model of the chimera. The modeling was based on published protein data bank (PDB) structures of HNE and nuclear magnetic resonance structures of peptides homologous to Cecropin B. A short G-S-T-A peptide linker was inserted between the C-terminus of HNE and the N-terminus of Cecropin B to allow both functional domains to make contact with the bacterial surface simultaneously without steric interference. Energy minimization and molecular dynamics analysis using the AMBER 7.0 force field indicated that the chimera forms a stable structure. The HNE-GSTA-Cecropin B chimera gene was synthesized and is currently being cloned into a baculovirus vector for overexpression in insect cells. The chimera will be purified from insect cells and tested for its activity against *Xf in vitro*. The chimera will be also cloned into a plant vector for transformation of grape embryogenic callus growing in a CELLline 350 bioreactor where they will be analyzed for the production and anti-*Xf* activity of the secreted protein. We will choose the most promising embryogenic lines for plant regeneration. The plant expression vector will have necessary regulatory sequences to facilitate transcription and extracellular delivery of the protein product. Currently we are investigating grapevine embryogenic callus for the extracellular production of the pear polygalacturonase inhibiting protein (pPGIP). This protein has been found in the xylem exudate of transgenic grapes expressing the pPGIP gene and will be used to modify delivery of the chimeric protein to grapevine xylem tissues.

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IVGRRARPHAWPFMVSLQLRGGHFCGATLIAPNFVMSAAHCVANVNVRAVRVVLGAHNLSRREPTR
QVFAVQRIFEDGYDPVNLNDIVILQLNGSATINANVQVAQLPAQGRRLGNGVQCLAMGWLLGRNRG
IASVLQELNVTVTSLCRRSNVCTLVGRGRQAGVCFGDSGSPLVCNGLIHGIASFVRGGCASGLYPDAFAP
VAQFVNWIDSIIQGSTAKWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL
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**Figure 1. HNE-cecropin B chimeric amino acid sequence.** HNE is attached to cecropin B (shown in bold) by the GSTA linker, which is underlined.



**Figure 2. Design and mechanism of chimeric protein targeted to *X. fastidiosa*.** The top panel shows the two domains of the chimera in separate planes: neutrophil elastase (1HNE from PDB) is on the left. A homology model of cecropin B is shown in the middle. The right plane shows the energy minimized model of the elastase-cecropin B chimera. The bottom panel is a schematic of the hypothetical mechanism of the chimeric protein. Elastase binds to and cleaves a specific loop on the *X. fastidiosa* outer membrane protein mopB. This action brings cecropin B in close contact with the membrane, where it associates with other cecropin molecules and disrupts the membrane by forming a pore, thereby disabling the bacterium.

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