PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: TOXIC PEPTIDES AGAINST *XYLELLA*

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ABSTRACT
The use of symbiotic bacteria in insects to disrupt pathogen transmission is a new approach to disease control. *Alcaligenes xylosoxidans denitrificans* bacterium was isolated from the mouthparts of wild glassy-winged sharpshooter and was chosen to be the first candidate for delivery products that inhibit *X. fastidiosa.* To find an appropriate agent for control of Pierce's disease, 90 antimicrobial peptides (AMPs) derived from a combinatorial peptide library (in addition to 59 screened previously from different sources) were tested for activity on 11 *X. fastidiosa* and 3 *Alcaligenes* strains. Forty four peptides showed potent antimicrobial activity against all strains studied. Six antimicrobial peptides (in addition to 4 found last year) were selected with toxicity to *X. fastidiosa* but not against *Alcaligenes* as a candidates for engineering of the sharpshooter's symbiont. More detailed studies of minimum inhibitory concentrations of these peptides were conducted. The Glutathione s-transferase gene fusion and *trc* expression systems are being developed to express individual AMPs *in vitro.*

INTRODUCTION
Xylella fastidiosa causes of Pierce's disease (PD), an important disease of grapevines in the United States. Because of the mobility and vector capacity of glassy-winged sharpshooter (GWSS), PD has become a great concern to grape production in California. One promising method for long-term *X. fastidiosa* control is limiting pathogen spread by rendering GWSS vector-incompetent. Paratransgenesis (Beard et al. 2001), which is the genetic alteration of bacteria carried by insect is currently being developed to deliver pathogen toxic substances that would inhibit *X. fastidiosa* and reduce disease transmission.

Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop growth of bacteria. A second type of antibiotics called antimicrobial peptides (AMPs) are produced by organisms including bacteria, plants, insects, birds, amphibians, and mammals (Cammue et al. 1992, Casteells et al. 1993, Nayler et al. 1989, Schroder 1999). These compounds interact directly with target bacterial membranes, but can do so with a receptor-like specificity, and can act via both membrane ion pore formation and by preventing cell wall formation (Maloy and Kari 1995). Because AMPs are "gene-based", they can be produced directly at the location where they are needed and their synthesis can potentially be regulated by using appropriate gene promoters. For example, the antimicrobial peptide MSI-99, an analog of Magainin 2, was expressed via the chloroplast genome to provide inhibition of growth against *Pseudomonas syringae* pv *tabaci*, a major plant pathogen (DeGray 2001). A combinatorial libraries represent a vast new source of molecular diversity for the identification of potential lead antimicrobial and antifungal compounds (Blonde and Lohner 2000, Jing et al. 2003). A combinatorial peptides are significantly shorter than other AMPs isolated from various biological sources. An amphipathic structure may allow this peptide to penetrate deeper into the interfacial region of membranes, leading to local membrane destabilization (Jing et al. 2003).

Use of symbiotic bacteria to deliver gene-based product is a new strategy of disease control. We demonstrated previously the expression of *Bacillus thuringiensis* toxin Cyt1A in the symbiotic bacterium *Enterobacter gergoviae* isolated from the gut of the pink bollworm (Kuzina et al. 2002). Bextine et al. (2004) used the expression of a red fluorescent protein (dsRed) by *Alcaligenes* (*Axd*) to study the colonization of the cibarial region of the GWSS. Genetically transformed symbiotic bacteria have been used to control the pathogen that caused Chagas disease (Beard et al. 1992, Beard et al. 2001, Durvasula et al. 1997).

OBJECTIVES
The overall goal of this project is to genetically transform symbiotic bacterium of the glassy-winged sharpshooter to produce toxic substances that would inhibit or kill *X. fastidiosa* and reduce disease transmission.
1. Identify toxic peptides effective against *X. fastidiosa* but non-toxic to *Alcaligenes*, selected symbiotic bacterium.
2. Design and construct genes encoding indolicidin and other peptides.
3. Develop a transformation system for expression of indolocidin .
4. Construct a transport cassette for secretion of indolicidin into *Alcaligenes.*

RESULTS

During the reporting period, we have screened an additional 90 antimicrobial peptides derived from a combinatorial library for activity on 11 *X. fastidiosa* and 3 *Alcaligenes* strains. *Axd* was isolated from the mouthpart of wild captured GWSS by Carol Lauzon. We found that 44 AMPs showed potent antimicrobial toxicity against all strains studied. Six AMPs were found with activity toward *X. fastidiosa* and non-toxic to *Alcaligenes*. These 6 peptides (along with 4 these screened last year) were more extensive examined for effective inhibitory concentration to *Xylella* and toxicity to *Alcaligenes* and *E. coli* as a target organism (Table 1). Blake Bextine studied the ability of GWSS to transmit *X. fastidiosa* to naive grapevine seedlings by oral delivery one of several antimicrobial peptide - indolicidin at 2 concentration: 100 μg/ml and 500 μg/ml. *X. fastidiosa* transmission rates were reduced from 50% in the control group, to 35% with the 100 μg/ml concentration and 7% with the 500 μg/ml concentration when GWSS were exposed to indolicidin prior to inoculation access. Therefore, indolicidin was chosen to be the first candidate for the development of gene-cassette. Artificial gene(s) to code indolicidin were designed and constructed for expression in *E. coli*. cDNA-encoding this peptide was amplified by PCR with incorporation of a *Sal*1 restriction site and/or *BamH*1 and *EcoR*1 restriction sites. We are using the Glutathione s-transferase gene fusion system (GST) (Pharmacia Biotech. Inc) and *trc* expression system (Invitrogene Co.) to express individual peptides. The GST gene fusion system is an integrated system for the expression, purification and detection of fusion proteins produced in *E. coli*. A pTrcHisTOPO expression kit provides a highly efficient, rapid cloning strategy for direct insertion of *Taq* polymerase-amplified PCR product into a plasmid vector for expression in *E. coli*. No ligase, post-PCR procedures, or PCR primers containing specific sequences were required. We transformed competent cells of *E. coli* DH5λ and TOPO by pGEX and pTrcHisTOPO vectors containing indolicidin gene. Several transformants were selected using LB medium containing ampicillin at 50 μg/ml (Sigma) and currently are being examined for production of indolicidin with and without IPTG.

Table 1. Toxicity of antimicrobial peptides to X. fastidiosa, Alcaligenes, and E. coli strains

Peptide	Range of MICs (μg/ml) to *X. fastidiosa*[a]	*Alcaligenes sp.*[b]	*E. coli*[c]	Source
1. Indolicidin	16-64	-	-	APS[d]
2. PA2	32-128	-	-	NCSU[e]
3. PA6	32-64	-	-	NCSU
4. PA7	32-64	-	-	NCSU
5. DCR1	16-32	-	-	TPIMS[f]
6. DCR2	8-16	-	-	TPIMS
7. DCR3	32-64	-	-	TPIMS
8. DCR4	16-32	-	-	TPIMS
9. DCR5	16-32	-	-	TPIMS
10.DCR6	8-16	-	-	TPIMS

[a] *– MICs of the antimicrobial peptides to eleven* X. fastidiosa *strains studied*
[b] *– Activity of AMPS to* Alcaligenes xylosoxidans denitrificans *134, 135, and 136 is negative*
[c] *– Activity of AMPs to* E. coli *DH5λ and TOPO is negative*
[d] *– American Peptide Company, Sunnyvale, CA*
[e] *– North Carolina State University, Raleigh, NC*
[f] *– Torrey Pines Institute for Molecular Studies, San Diego, CA*

CONCLUSIONS

The 10 antimicrobial peptides were found with toxicity to 11 *X. fastidiosa* strains isolated from grape, oleander and almond, but not against the glassy-winged sharpshooter gut bacterium *Alcaligenes xylosoxidans denitrificans*. We consider these AMPs as a candidates for use as reagents in delivery vehicle for paratransgenesis: Indolicidin, a 13-residue peptide-amide, isolated from the cytoplasmic granules of bovine neutrophils (Selsted 1992); 3 pescidins, isolated from the mast cells of aquacultured fish (Silphaduang and Noga 2001); and 6 peptides derived from a combinatorial peptide library (Blonde and Lohner 2000) (Table 1). *Alcaligenes* will be engineered to produce a peptide(s) toxic substance that would inhibit *X. fastidiosa* and reduce disease transmission. To develop a transformation system to express peptide(s) in *E. coli* first, we are using the Glutathione s-transferase gene fusion and *trc* expression systems. We got several ampicillin resistant transformants which are being studied for production of indolicidin. Artificial genes of other peptides are being designed for expression and secretion by *E. coli* and *Alcaligenes* as well.

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