DEVELOPMENT OF PEPTIDE ANTIBIOTIC-BASED CONTROL STRATEGIES FOR *XYLELLA FASTIDIOSA*

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ABSTRACT
Peptide antibiotics are short (generally less than 70 amino acid residue-long), pore forming peptides encoded by single genes. Because peptide antibiotics are 'gene-based' they can be produced directly at the target location where they are needed (e.g., grape stock). In this project, we are testing the hypothesis that peptide antibiotics such as cecropins A, B, and/or P1 can be used as an effective means to control or reduce the spread of *Xylella fastidiosa*-induced disease. During the reporting period, we have established the optimal growth and assay conditions for the *X. fastidiosa* bacterium. Under these optimal conditions, we found that cecropin A can effectively inhibit *X. fastidiosa* growth over a two-week period (initial concentration of 0.05μM). Longer-term growth inhibition was seen only when higher concentrations of cecropin A were used suggesting that the cecropin A is being degraded under the conditions of our assay. On the basis of the effectiveness of cecropin A against *X. fastidiosa*, a synthetic plant codon (i.e., *Arabidopsis thaliana*) optimized, cecropin A gene was synthesized. The product of this synthetic cecropin A gene was expressed using the baculovirus expression vector system (BEVS) in insect cells. In insect cells roughly 90 mg/liter of culture of biologically active cecropin A was produced by the recombinant baculovirus. Following confirmation of biological activity of the insect cell produced cecropin A, the synthetic cecropin A gene was inserted into the pCAMBIA1305 series of plasmid vectors in order to express the cecropin A in transgenic *A. thaliana* and eventually grape stock. Four different recombinant pCAMBIA1305 vectors were generated (carrying either the pro or mature cecropin A gene fused to either an authentic insect- or plant- (rice glycine-rich protein) derived signal peptide sequence). We are currently in the process of generating transgenic *A. thaliana* using these pCAMBIA vectors. We believe that continuous expression (although potentially at relatively low levels) of cecropin A will be effective for reducing or inhibiting the growth of *X. fastidiosa* within the plant.

INTRODUCTION
Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop the growth of bacteria. Antibiotic inhibition of *X. fastidiosa* (at least 17 isolates tested) has been analyzed for six different antibiotics (ampicillin, kanamycin, neomycin, penicillin, streptomycin, and tetracycline) [1, 2]. These studies demonstrate that antibiotic treatment is potentially an effective method for the control of *X. fastidiosa*. Under field conditions, however, barriers between the antibiotic and bacterium, and degradation effects will require significantly higher application doses than those found effective in the laboratory. Such doses may be impractical especially for broad-spectrum antibiotics due to secondary effects (e.g., toxicity against mammalian red blood cells) and the risk of increasing resistance. Thus, although traditional antibiotics such as tetracycline are highly active, an effective delivery system to bring them in contact with *X. fastidiosa* in the plant or insect vector is not available.

Recently, a great deal of scientific effort is being put into the study of a second type of antimicrobial agent called peptide antibiotics. Peptide antibiotics have been identified from a wide range of organisms including bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals. In general, peptide antibiotics are small (less than 50 amino acids), have a net positive charge, and are composed of 50% or more of hydrophobic amino acids [3, 4]. One class of peptide antibiotic is composed of so-called ribosomally synthesized peptides [5]. These peptides are encoded by single genes and synthesized by a protein complex (ribosome) that is found in all cells and processed following synthesis via common pathways [3, 6]. In other words, unlike traditional antibiotics, peptide antibiotics have the potential to be easily produced by common protein expression systems or in transgenic organisms (e.g., plants). Furthermore, because peptide antibiotics 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.

Some of the best-characterized peptide antibiotics are the cecropins. Cecropins were the first peptide antibiotics to be identified in an animal, the giant silkmoth *Hyalophora cecropia* [7, 8]. At least ten different cecropins have been isolated from lepidopteran (moths and butterflies) and dipteran (flies) insects [9, 10]. Cecropins are composed of a single chain of 35-39 common L-amino acids and do not contain disulfide bonds [10]. Cecropins are active against many Gram(-) bacteria and

some Gram(+) bacteria, but are inactive against eukaryotic cells at concentrations that are antimicrobial [4, 9, 11] and possibly at concentrations up to 300 times higher [8]. *X. fastidiosa* is a Gram(-) bacterium [12]. In Gram(-) bacteria, the antibacterial activities of cecropins A, B, and P1 are up to ten-times greater than tetracycline [9, 13]. Cecropins have a unique combination of characteristics (specificity, gene basis, small size, potency against Gram(-) bacteria, etc.) that may make them potentially ideal substances for the control of *X. fastidiosa* in GWSS.

OBJECTIVES

I. Identify peptide antibiotics (cecropins) that are effective against *Xylella fastidiosa*
   i. Determine the antibiotic sensitivity of *X. fastidiosa* to chemically synthesized cecropins
   ii. Produce recombinant cecropins using baculovirus expression vectors
   iii. Determine the toxicity of cecropins against GWSS cells grown in culture
II. Analyze the effectiveness of cecropins produced in transgenic *Arabidopsis*
   i. Generate transgenic *Arabidopsis* expressing cecropin that is active against *X. fastidiosa*
   ii. Determine the localization, yield, activity, and stability of plant-expressed cecropin
   iii. Analyze the effect of cecropin expression on the transgenic *Arabidopsis*
   iv. Analyze the effectiveness of plant-expressed cecropin for the control of *X. fastidiosa* transmission

RESULTS AND CONCLUSIONS

In order to establish the optimal conditions for the growth, storage, and assay of *X. fastidiosa* (Temecula strain) in our laboratory, we tested three different media (PD3, PW, and GYE; see [14]) and various inoculation routines. In general, our procedures were modified from protocols established in the Bruce Kirkpatrick laboratory at U.C. Davis. Optimal conditions for the generation of bacterial (*X. fastidiosa*) lawns for agar disc diffusion assays were also determined. Of the three media that were tested, PD3 gave the fastest growth of *X. fastidiosa* in liquid medium (roughly 20-and 135-fold increases in the OD_{600} at 7 and 14 days post inoculation, respectively) and on agar plates (formation of a lawn by 7-10 days post seeding). In order to generate a lawn, 150 µL of a 14 day-old culture (OD_{600}=0.48-0.5) was spread onto a 10 cm-diameter plate containing PD3 agar medium.

Using the optimal growth conditions with PD3 medium, we examined the minimal inhibitory concentration (MIC assay) at which cecropins A, B, and P1 (commercially purified peptides) were effective in inhibiting the growth of *X. fastidiosa*. We found that cecropins A, B, and P1 were effective at partially inhibiting the growth of *X. fastidiosa* at concentrations that were equal to or greater than 0.05, 0.25, and 0.5 µM, respectively, at two weeks post inoculation (Table 1). In general, cecropin A was the most effective against *X. fastidiosa*. The effectiveness of the cecropins as well as kanamycin was reduced by three weeks post inoculation. This was speculated to be the result of antibiotic degradation.

Once the sensitivity of *X. fastidiosa* to the various cecropins was established, a codon-optimized (for *A. thaliana*) cecropin A gene (pro gene including the insect-derived signal peptide sequence) was synthesized using commercially synthesized oligomers. A comparison of the *A. thaliana*-optimized (upper) and authentic (lower) cecropin A gene sequences is as follows:

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ATGAACTTCTCTAGAATCTTCTTCTTCGTGTTCGCTTGCCTCACTGCTCTCGCTATGGTGAACGCTGCTCCTGAGCCTAAGTGGAAGCTCTTCAAGAAGA 100
ATGAACTTTTCGAGGATCTTTTTCTTCGTCTTCGCTTGCCTGACGGCTCTAGCAATGGTCAATGCGGCGCCGGAACCTAAATGGAAGTTATTCAAGAAGA 100
******** ** ** ***** ******** *********** ** ***** ** ***** ** ** ** ** ** ***** ****** * **********

TCGAGAAAGTGGGTCAGAACATCAGAGATGGAATCATCAAGGCTGGACCAGCTGTGGCTGTGGTGGGACAGGCTACACAGATCGCTAAGGGTTGA 195
TTGAGAAAGTCGGTCAGAACATTCGAGATGGCATCATCAAAGCTGGCCCAGCCGTCGCTGTTGTAGGCCAGGCAACACAGATTGCTAAGGGTTAA 195
**  ******* ******** ***** ***** ** ***** ** ** ***** ******* ******** *********** ********** *
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Of the 195 nucleotides that encode the pro gene, 33 nucleotides were mutated for optimal expression in *A. thaliana* (and putatively in grape stock). The synthesized gene was directionally cloned into the baculovirus transfer vector pAcUW21 at the *Bgl*II and *Eco*RI sites. Subsequently, the recombinant transfer vector was used to generate a recombinant baculovirus (vAcCecA) expressing the cecropin A gene using standard procedures. Expression of biologically active cecropin A was confirmed by minimal inhibitory concentration assays using *E. coli* by comparison of vAcCecA- or wildtype AcMNPV-infected insect Sf-21 cell culture supernatants or cell extracts (Table 2). These experiments confirmed that the synthetic gene encoded a functional peptide and that this peptide was correctly processed in insect-derived cells. vAcCecA expressed high levels (roughly 90 mg/liter of insect cell culture (2×10^6 cells/mL)) of cecropin A.

Following confirmation that the synthetic gene produces biologically active cecropin A, the synthetic gene was inserted into the pCAMBIA1305 series of plasmid vectors in order to express the cecropin A in transgenic *A. thaliana* (and eventually grape stock). Four different recombinant pCAMBIA1305 vectors were generated by PCR-amplification as follows:

1. pro cecropin A sequence with authentic insect signal peptide sequence
2. pro cecropin A sequence with rice glycine rich protein and authentic insect signal peptide sequences
3. mature cecropin A sequence with rice glycine rich protein signal peptide sequence

4. mature cecropin A with no signal peptide sequence

The authenticity of the PCR-amplified sequences was confirmed by nucleotide sequencing in both directions and the constructs are currently being used to generate transgenic *A. thaliana* by standard procedures.

Table 1. Effect of cecropins and kanamycin against the growth of *X. fastidiosa*

	Concentration (μM)	Increase in bacterial concentration in comparison to cultures lacking antibiotic		
		Week 1 (% ± s.d.)	Week 2 (% ± s.d.)	Week 3 (% ± s.d.)
cecropin A	0.5	69 ± 3	47 ± 47	64 ± 42
	0.25	72 ± 10	80 ± 21	117 ± 5
	0.1	103 ± 13	68 ± 2	87 ± 25
	0.05	110 ± 46	50 ± 1	91 ± 22
cecropin B	0.5	69 ± nd	114 ± 6	87 ± 45
	0.25	63 ± 31	75 ± nd	110 ± 15
	0.1	72 ± 101	128 ± 63	90 ± nd
	0.05	93 ± 17	101 ± 18	74 ± 10
cecropin P1	0.5	98 ± 18	70 ± 40	70 ± 62
	0.25	82 ± 18	98 ± nd	120 ± 17
	0.1	111 ± 52	93 ± 24	72 ± 24
	0.05	93 ± 10	99 ± 22	73 ± 18
kanamycin	2	11 ± 3	9 ± 8	16 ± 2
	1	19 ± 8	32 ±39	33 ± 22
	0.5	42 ±16	77 ± 9	103 ± 16
	0.25	60 ± 13	72 ± 17	105 ± 12

nd = not determined

Table 2. Effect of recombinant cecropin A on the growth of *E. coli*

Source of recombinant cecropin A	Inoculum dose (bacteria/mL)	Inhibition (%)
Sf21 cell pellet (1 x 10^5 cells)	1.1 x 10^3	3.1 ± 13.2
Sf21 cell supernatant (undiluted)	1.1 x 10^3	99.7 ± 0.1
Sf21 cell supernatant (undiluted)	1.0 x 10^4	57.9 ± 1.6
Sf21 cell supernatant (undiluted)	8.5 x 10^4	51.6 ± 0.2
Sf21 cell supernatant (undiluted)	7.3 x 10^5	13.1 ± 0.1
Sf21 cell supernatant (1:5 diluted)	7.0 x 10^5	11.1 ± 0.2
Sf21 cell supernatant (1:10 diluted)	7.0 x 10^5	2.5 ± 0.1

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