ABSTRACT

Innovating Recombinant Production of Short Peptide Targeted Antimicrobial Peptides Utilizing Clean Purification in Plant and *E. coli* Bioreactors

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Antibiotic-resistant pathogens continue to become a pressing issue globally and current broad spectrum therapies fail to meet this challenge. Therapeutics equipped with targeting moieties would alleviate this issue without facilitating the rise of opportunistic pathogens, and employing small antimicrobial peptides (AMPs) would avoid resistant strains. Fusion of the two could be produced recombinantly at commercial levels in bioreactors such as plants with a purification tag, but issues with phytotoxicity and unexplained low recombinant AMP yield from plant tissue are major limiting factors.

This dissertation addresses the issues of the plant expression platform for AMPs as well as properly assessing the selectivity of a previously discovered 12mer targeting domain (A12C) specific to *Staphylococci*. To understand why AMPs are poorly expressed in plant expressions systems, meta-analysis of peptide databases was performed and revealed plant-derived AMPs are less cationic in net charge compared to AMPs from organisms like animals and fungi. Using the elastin-like polypeptide as a tag for increased recombinant production and clean temperature shift purification, a survey of

AMPs falling under the cysteine-stabilized motif ranging in net charge were produced in *Nicotiana benthamiana*. It was observed that only the anionic fusions were expressed, and at record levels as high as 563 μg/gram fresh leaf weight with retained antimicrobial activity only pre-protease cleavage. This phenomenon of activity maintained while still fused was studied in *E. coli*, and it was found the size of the ELP fusion partner dictates antimicrobial potential.

For quicker turnover to facilitate targeting studies, cationic AMPs plectasin and eurocin were expressed in *E. coli* with the SUMO solubility tag. The targeting domain was assessed for antimicrobial selectivity against a panel of gram positive bacterium. Unexpectedly, there was no enhancement of activity against target bacterium, but a significant decrease in antimicrobial activity against non-target genus.

These studies elucidate that the peptide net charge dictates recombinant AMP expression in plants, and a genus-specific targeting domain can be derived from as small peptide sparing commensal non-target bacteria. Together, these discoveries provide the foundation for the inexpensive production of targeted AMPs in plant bioreactors or in transgenic seed for poultry or livestock.

Innovating Recombinant Production of Short Peptide Targeted Antimicrobial Peptides Utilizing Clean Purification in Plants and *E.coli* Bioreactors

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

Introduction

The dawn of the 'post-antibiotic era' has been on a steady rise for the past half century (Davies and Davies, 2010). Since the widescale application of broad spectrum antibiotics, resistant pathogenic strains of microbes have become increasingly prevalent and lethal, as conventional antibiotics lose efficacy and unintentionally affect commensal bacterial populations (Aminov, 2010). Alternatives in antibodies, probiotics, and bacteriophages both wild-type and engineered have all been considered as possible answers to these resistant pathogens (Czaplewski *et al.*, 2016). Investigations into antimicrobial peptides (AMPs) have revealed the small peptides potential as safe alternatives to conventional antibiotics, due to their mechanism of action targeting essential microbial membrane and intracellular components too essential for the bacteria to adapt, resulting in little to no resistance developing against them (Maro´ti *et al.*, 2011).

AMPs are amphiphilic peptides with a wide range of structures and folding patterns with varying stability in different environmental conditions. Found in nature across bacterial, plant, animal, and fungal taxa, these peptides naturally exist in low concentrations and thus must avoid direct purification from host organisms to achieve any commercial success (Hancock and Sahl, 2006). Alternative methods of production include either chemical synthesis (Li *et al.*, 2010) or recombinant expression in bacteria, yeast or plant bioreactors (Parachin *et al.*, 2012). Chemical synthesis is a more precise method assembling the peptides a single amino acid at a time, but suffer from high costs

when pursuing large scale production and complex post-translational modifications, like forming disulfide bonds amongst cystine residues and glycosylation (Münzker *et al.,* 2017). Expression of recombinant AMP DNA in heterologous host cells can bypass these hurdles for high level production when paired with a fusion chaperon and affinity tag for proper protein folding and purification, respectively (Chatterjee *et. Al.,* 2006). In searching for a medicinally applicable AMP that could realistically replace or assist antibiotic treatment, enhancing the stability of the peptide, establishing a reliable production and purification method, and identifying a specific molecular targeting system are needed.

The sturdiest of AMPs are typically stabilized with disulfide-bonds amongst cystine amino acid residues and possess a beta-sheet secondary structure (Yount and Yeaman, 2004). These peptides vary in net charge and antimicrobial potency but are consistent in hydrophobicity and are best suited for volatile environments. For consistency in proper AMP folding, host cell protection from microbial activity and ease of downstream purification, the fusion partners small ubiquitin-like modifier (SUMO) equipped with the hexahistidine (6His) affinity tag or elastin-like polypeptides (ELP) were selected for AMP fusion (Lin *et al.,* 2017; Floss *et al.,* 2009). The solubility-enhancing fusion partner SUMO is commonly used for increasing the rate of successful folding of a passenger peptide, and the 6His tag binding to metal matrices allows for specific purification when run through a Nickle column. ELP is a variable repeat pentamer sequence of VPGVG aggregation and purification tag that utilizes a temperature-dependent inverse transition cycling (ITC) for non-chromatographic purification (Conley *et al.,* 2009). In regards to guiding the antimicrobial activity of the

AMP, the coding sequence of a relatively small bacteriophage coat protein specific to *Staphylococcus aureus*, A12C, was employed.

Targeting studies have previously used antibody fragments (Peschen *et al.*, 2004) or bacterial pheromones as targeting moieties (He *et al.*, 2009), but those proteins are typically large in relation to an AMP and may impair antimicrobial activity due to steric hinderance. The A12C peptide was previously tested to be selective towards *Staphylococcus* (Yacoby *et al.*, 2006), thus its 12 amino acid coding sequence was cloned directly to the N-terminus of a chosen AMP open reading frame as a molecular targeting system.

Initial tests explored whether the cystine-stable AMPs could be recombinantly produced in high concentrations, starting with recombinant expression in the plant platform via *Nicotiana benthamiana* leaves. Literature has reported low yields of recombinant AMP expression in plant tissue relative to other modes of production (Zeitler *et al.*, 2013; Parachin *et al.*, 2012). Therefore, a study in what physiochemical properties of AMPs in particular may limit yield was investigated using the ELP fusion expression method. Similar cystine-stabilized AMPs were also fused to the His-tagged SUMO expression vector and tested in the standard *E.coli* expression system to assess the antimicrobial selectivity of an AMP fusion to a short peptide targeting system. Lastly, the ELP/AMP fusion proteins expressed earlier in plants were further tested in *E.coli* to confirm the proper fusion orientation and antimicrobial efficacy of a purified pre-cleavage ELP fusion, and also elucidate what factors may contribute to the loss of antimicrobial activity of this fusion post ELP/AMP separation.

Attributions

This dissertation includes manuscripts with co-authorships as well as first authorships. CHAPTER ONE and CHAPTER TWO are manuscripts are currently under revision and review.

For CHAPTER TWO, experimental design was carried out by Christopher Kearney, Mishu Islam and Meron Ghidey. Maintaining plants and experimental procedures were carried out by Grace Pruett, Mishu Islam and Meron Ghidey. Manuscript writing carried out by Christopher Kearney, Mishu Islam and Meron Ghidey.

For CHAPTER THREE, experimental design was carried out by Christopher Kearney, Ankan Choudhury, Mishu Islam and Meron Ghidey. Experimental procedures were executed by Ankan Choudhury, Mishu Islam and Meron Ghidey. Manuscript writing by Christopher Kearney, Ankan Choudhury, Mishu Islam and Meron Ghidey.

For CHAPTER FOUR, experimental design was carried out by Christopher Kearney, Ankan Choudhury and Meron Ghidey. Experimental procedures were executed by Ankan Choudhury and Meron Ghidey. Manuscript writing by Christopher Kearney, Ankan Choudhury and Meron Ghidey.

CHAPTER TWO

Making Plants into Cost-Effective Bioreactors for Highly Active Antimicrobial Peptides

Abstract

As antibiotic-resistant bacterial pathogens become an ever-increasing concern, antimicrobial peptides (AMPs) have grown increasingly attractive as alternatives. Potentially, plants could be used as cost-effective AMP bioreactors; however, reported heterologous AMP expression is much lower in plants compared to *E. coli* expression systems and often results in plant cytotoxicity, even for AMPs fused to carrier proteins. It's unknown if there were a physical factor that made heterologous AMPs difficult to express in plants. Using a meta-analysis of protein databases, it was determined that native plant AMPs were significantly less cationic than AMPs native to other taxa. To apply this finding to plant expression, this study tested the transient expression of 10 different heterologous AMPs, ranging in charge from +7 to -5, in the tobacco, *Nicotiana benthamiana*. Initially several carrier proteins were tested and AMPs expressed only with elastin-like polypeptide (ELP). Conveniently, ELP fusion allows for a simple, cost-effective temperature shift purification. Using the ELP system, all five anionic AMPs expressed well, with two at unusually high levels (375 and 563 µg/gfw). Furthermore, antimicrobial activity against *Staphylococcus epidermidis* was an order of magnitude stronger (average MIC = 0.26 µM) than that typically seen for AMPs expressed in *E. coli* expression systems, and this antimicrobial activity was associated with the uncleaved fusion peptide. In summary, this study described a means of expressing AMP fusions in

plants in high yield, purified with a simple temperature-shift protocol, resulting in a fusion peptide with high antimicrobial activity, without the need for a peptide cleavage step.

Introduction

The use of traditional antibiotics to control bacterial infections is threatened due to two undermining factors. First, drug discovery for new antimicrobial agents has been on the decline for the past three decades. The major classes of antibiotics have already been discovered and commercial incentives to develop new antibiotics have decreased [1,2,3]. Second, the overuse of antibiotics has led to pathogenic and commensal bacteria incorporating and retaining genes for detoxification or export of antibiotics, inevitably resulting in resistance to all new antibiotics introduced [4,5,6,7].

Both of these undermining factors are addressed by antimicrobial peptides (AMPs). First, the resources available to develop new AMP drugs is vast and recombinant peptide variants can be quickly generated, unlike the slow discovery and development cycle for antibiotics. AMPs are abundant across the taxa, being found in vertebrates, insects, fungi and plants. Thousands of AMPs have been isolated and tested experimentally [8] and many more can be discovered using algorithms to scan genome data bases [9]. Second, though resistance to AMPs has been shown to develop in bacteria [10], the multiple antimicrobial activities and low affinity targets typical of AMPs have been thought to make them more difficult targets for resistance development by pathogenic bacteria [11]. From an environmental perspective, AMPs are not long-lasting in waste water, whereas low concentrations of antibiotics can induce resistance in soil and water-borne microbial communities [12].

AMPs are not capable of completely replacing antibiotics, but could serve as replacements for some applications if they were produced at low cost. Though AMPs have been used clinically [13], AMPs have a special potential for large-scale applications. Examples might include their use as a food preservative, as a topical disinfectant, or as a feed supplement for livestock or poultry. These sorts of applications would be dependent upon developing scalable and simple protocols for both production and purification.

Currently, there remain some roadblocks to developing these simplified protocols for large scale production. *E. coli* expression systems have been extensively demonstrated to effectively produce AMPs, but the AMP must be fused to a carrier protein in order to protect the bacterium from antimicrobial activity [14]. Various fusion partners have been used, such as SUMO [15,16], GST [17] and TRX [18], but these must be removed post-production to restore antimicrobial activity to the AMP, adding an extra cost to production. A variety of ingenious methods have been proposed to perform the cleavage event without the use of proteases post-production [18,19], but, with one exception [20], AMPs that retain antimicrobial activity while still bound to the fusion partner have not been produced in bacteria. Plant expression of AMPs is an attractive alternative, since they are not themselves targeted by AMPs and have potential as highly scalable protein production systems. However, the yields so far reported for plant expression of AMPs [21,22,23] have been much lower than those reported for *E. coli* expression systems [24]. Even if production levels were competitive with *E. coli* systems, downstream processing contributes the bulk of production costs [25], and this must be addressed especially for low-cost/large-scale applications.

This study has expressed AMPs in a plant expression system and have addressed the two roadblocks mentioned above, achieving high expression of AMPs in plants and avoiding the carrier protein cleavage step, using a simple purification protocol. It was found that peptide charge of the AMP fusion was correlated with yield, as all of the anionic AMP fusions tested were expressed in plants while none of the cationic peptides produced any detectable AMP fusion protein. To reduce downstream processing costs an elastin-like polypeptide (ELP) carrier protein was used [26], which confers to the fusion protein insolubility at 37˚C, at which most protein contaminants are soluble, and solubility at 4˚C. Centrifugation at 37˚C pellets the fusion protein, which is then resuspended at 4˚C. The activity of these ELP AMP fusions were, in fact, at least 10x stronger than that typically reported for cleaved AMPs produced in *E. coli* expression studies [27,18] or from AMPs synthetically produced [28,29,10]. This system fully leverages the potential unique advantages of plant production of AMPs as compared to other modes of production. The described method may thus serve as an antibiotic replacement platform for applications requiring large-scale, low-cost protocols.

Materials and Methods

Computation of Hydrophobic Ratio and Net Charge Distributions

AMP databases (PMID: 26602694, PMID: 18957441) were examined to determine correlations between taxonomic distribution and two protein structural factors, hydrophobicity and net charge. First, candidate peptide sequences were collected. To reduce other factors in the comparisons, the only AMPs examined were those having the most commonly occurring AMP structure, namely the sequential tri-disulfide peptide

(STP) structure defined by a C1-C4, C2-C5, C3-C6 cystine bonding pattern [30,8]. To collect STP-AMPs, AMPs ranging from 30 to 50 amino acids were manually processed through the PredSTP tool [30] and the resulting peptide sequences were collected. The CD-HIT [PMID: 23060610] program was used to remove redundant sequences by setting a cutoff of sequence identity at 80%. The remaining sequences were grouped into the plant or non-plant origins using the original metadata.

After the candidate peptide sequences were collected, the hydrophobic ratio and the net charge of each sequence was calculated applying the identical formula used in the ADP3 server (PMID: 18957441). For the hydrophobicity calculation, A, I, L, M, W, V, C and F were considered hydrophobic amino acids, as shown below, with *n* being the number of occurrences of each corresponding amino acid in the peptide and *L* being the total number of amino acids in the peptide.

$$Hydrophobic\ ratio = \frac{\sum(nA+nI+nL+nM+nW+nV+nC+nF)}{\mathrm{L}} \times 100 \quad \text{.....(Equation 1)}$$

For the net charge calculations, the difference between the counts of negative (D + E) and positive (R + K) amino acids was defined as net charge for each peptide:

$$Net\ charge = (nR + nK) - (nD + nE) \quad \text{.....(Equation 2)}$$

ANOVA tests were performed using R version 3.4.0 to observe any significant difference in the mean of hydrophobic ratio or net-charge in the STP-AMP sequences from plant or non-plant origins.

ELP Vector

All subsequent work in the comparative expression of AMPs of different net charge was carried out using the ELP carrier protein. The ELP used in this study comprised 28 units of VPGVP pentapeptide repeats fused to the protein of interest [34]. The pCaMterX/ELP vector was modified to include unique restriction sites to allow insertion of AMP open reading frames (ORFs) with the excision of the eGFP ORF native to the original vector (Figure 1). Additionally, a TEV protease cleavage site (ENLYFQ) was inserted at the C-terminus of the AMP. The final construct allows for insertion/replacement at three sites for marker genes, AMPs and purifications tag (Figure 1).

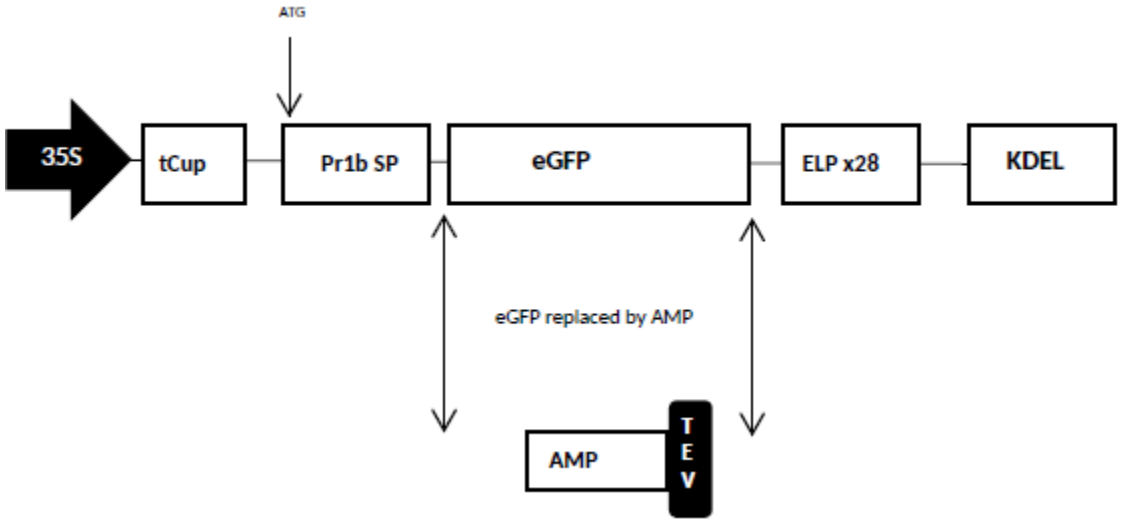

Figure 1 STP-AMP/Elastin-like polypeptide fusion sequence expressed via agroinoculation in *Nicotiana benthamiana*. 35S, CaMV 35S dual enhancer promoter; tCUP, translational enhancer; Pr1b SP, tobacco secretory signal peptide; KDEL, ER retention signal; TEV, tobacco etch virus protease recognition site (ENLYFQ).

Agroinoculation, Protein Purification and Analysis

Agrobacterium tumefaciens strain GV3101 was electroporated with the AMP/ELP binary vector and agroinoculation proceeded as described [32], including the silencing suppressor, p19. All experiments were done in triplicate. As negative and positive controls, uninfected leaves and leaves infected with both the original eGFP/ELP as well as an ELP without a fusion partner constructs were collected and processed through the same ELP extraction and purification processing as the AMP/ELP samples.

AMP/ELP purification was performed as previously described [35]. Specifically, plant leaves collected at 3-4 days post-inoculation were frozen in liquid nitrogen and ground with a pre-chilled mortar and pestle, then homogenized in three volumes (v/w) of ice cold 1X PBS. Extract was centrifuged in 4°C at 20,000 x g for 15 minutes. For the temperature-dependent inverse transition cycling, the supernatant above was warmed in a 37°C water bath with NaCl added to a concentration of 3 M. After 15-45 minutes of incubation extract was centrifuged at 37°C for 20,000 x g for 15 minutes. Supernatant was discarded, and the pellet was resuspended in ice cold 1X PBS at 1/10th the volume and centrifuged at 4°C at 20,000 x g for 15 minutes. The resulting supernatant was the uncleaved protein product used for microbial inhibition studies.

To test the effect of cleavage on the fusion protein's toxicity, AMP/ELP protein from the resuspended pellet was cleaved with TEV protease at a mass ratio of 4:1 in TEV protease buffer (50 mM tris HCl (pH 8.0), 0.5 mM EDTA, 1 mM DTT). The cleavage products (AMP and ELP) were not separately isolated and were analyzed as a mixed solution.

Protein extracts were analyzed by SDS-PAGE and mass spectrometry. Recombinant AMP protein yield was assessed by densitometry of SDS-PAGE band images measured against a BSA standard using NIH ImageJ. Mass spectrometry was used to confirm the presence of intact AMP and carrier peptide after TEV protease treatment and to confirm the identity of AMP-ELP fusion peptide from extracts not treated with TEV protease. Specifically, AMP/ELP fusion protein was first extracted from leaves using two cycles of the temperature shift protocol described above and TEV protease was used to cleave the fusion peptide into AMP and ELP. Cleaved or uncleaved fusion peptide was digested with trypsin and analyzed using LC-ESI-MS (Synapt G2-S, Waters) at the Baylor University Mass Spectrophotometry Center, followed by data analysis using MassLynx (v4.1). The results can be found in Supplementary Figures 5-40.

Antibacterial Assay of Recombinant AMPs

Purified AMPs, individual ELP control, and AMP/ELP fusion peptides were tested for their antimicrobial activity using a Minimum Inhibitory Concentration (MIC) assay. Specifically, 10 mL of *Staphylococcus epidermidis* was grown overnight in a shake culture at 150 rpm at 37°C. Turbidity was assessed with McFarland standard tubes and the culture was diluted to 0.5 OD_{600}. The peptide was first added to the first well of a 96-well microtiter plate and serial 1:2 dilutions of the peptide were made across the plate using fresh LB medium. Then, 100 µl of *S. epidermidis* culture was added to each well containing the peptide dilutions and the culture was allowed to grow in the well at 37°C without shaking. To measure bacterial growth, resazurin was added to 0.00015% and

plates were allowed to grow an additional 30- 120 minutes until dye color changed to indicate bacterial growth or inactivity. All MIC experiments were run in triplicate.

Results

STP-AMPs Native to Plants are less Cationic than those from Non-Plant Sources

A publicly available AMP database was used to access AMP sequences and metadata, but first filters were applied to narrow the pool to those peptides of greatest practical value for heterologous expression in plants. Since this study is interested in peptides possessing the highly stable sequential tri-disulfide peptide (STP) structure, the PredSTP algorithm [30] was employed to narrow the pool of AMPs gathered from the AMP database to only STPs. The pool was further narrowed to only peptides 30-50 amino acids in length and eliminated redundant sequences (80% sequence similarity cutoff), resulting in a final data set of 96 STP-AMPs of plant origin and 58 STP-AMPs of non-plant origin (Supplemental File 1).

Once the plant and non-plant STP-AMPs groups were collected, hydrophobicity and net charge was compared amongst them. There was no significant difference between the two groups in hydrophobicity (Figure 2). However, peptides of plant origin were found to be significantly less cationic than peptides of non-plant origin (Figure 2). A p-value of 4.47e-05 was determined by ANOVA for this comparison, with mean net charges of +1.77 versus +3.46 for STP- AMPs of plant vs. non-plant origin, respectively. Therefore, an unfavorably positive net charge may have been responsible for the poor expression of non-plant AMPs expressed in plant expression systems as reported in the literature to date.

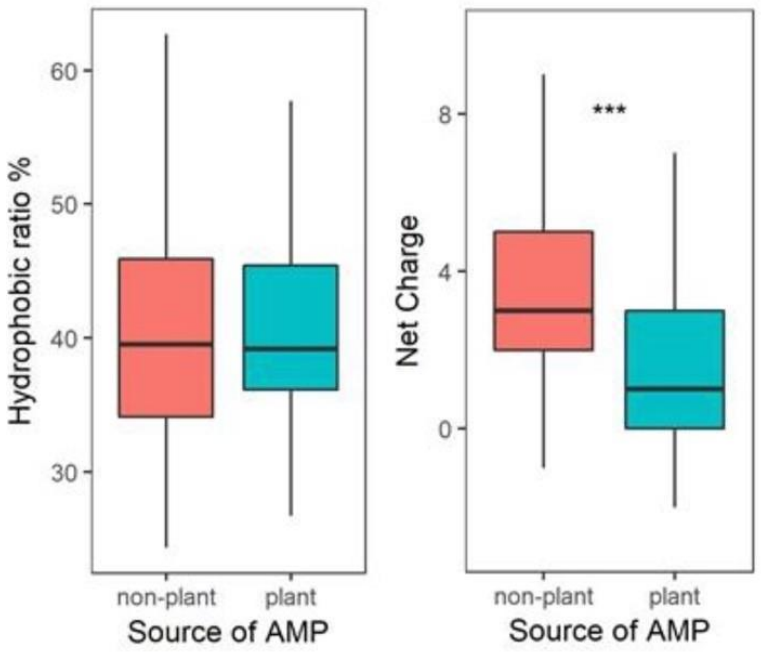

Figure 2 Meta-analysis of AMPs from Antimicrobial Peptide Database 2. Hydrophobicity and net charge were calculated and compared for STP-AMPs native to plant versus non-plant sources.

Preliminary Vector Work

In preliminary experiments, two carrier proteins were tested as fusion partners for AMP transient expression i**n** *N. benthamiana* via leaf agroinoculation. This work was performed before the meta-analysis of peptide charge described above. First, AMP was fused to the C-terminus of Jun a 3, a protein that expresses strongly and accumulates well in the apoplast of tobacco [31]. The Jun a 3 fusion was expressed using the plant viral vector FECT [32]. The AMPs tested were C16G2(+9), tachystatin B-1 (+7), protegrin (+6) and circulin-A(+2). No AMP expression was detected by SDS-PAGE/Coomassie Blue analysis (data not shown). Second, AMP was fused between eGFP and hydrophobin in the plant expression vector pCaMterX [33]. The AMPs tested were C16G2(+9), tachystatin B-1 (+7), sarcotoxin (+5), circulin-A(+2) and laterosporulin (-1). No AMP expression was detected by SDS-PAGE/Coomassie Blue analysis (data not shown).

However, some GFP fluorescence was noted in plants inoculated with the anionic laterosporulin construct. In addition, the anionic insecticidal STP, Hv1a (-1), used as a positive control, expressed well in both of these systems. These were the first experimental data suggesting that peptide net charge may be a factor in the successful plant expression of AMPs.

Only Anionic AMPs were Expressed in Transiently Transgenic Plants

From these findings, the hypothesis that the expression of AMPs in plant expression systems may be improved by using AMPs which were anionic, neutral, or only slightly cationic was formed. This was tested experimentally by expressing in the tobacco *Nicotiana benthamiana* a set of 10 AMPs ranging in net charge from highly cationic (+7) to highly anionic (-5). To eliminate the variables of peptide size, peptide structure and plant vs. non-plant origin, we selected only AMPs of 30-50 amino acids in length, possessing a core STP structure, and being of non-plant origin (Table 1).

When this range of 10 AMP/ELP fusions were expressed in *N. benthamiana* leaves, peptide net charge was seen linked to both yield and plant symptoms. Plants inoculated with cationic peptides showed a strong tendency to develop necrosis in the agroinoculated leaves and this effect was more severe the more cationic the peptide (Figure 3, top row). The neutral AMP, eurocin, and all anionic AMPs (bottom row) induced no leaf necrosis when agroinoculated as AMP/ELP fusion peptides. In line with these symptom observations, no expression of AMP/ELP was detected by SDS-PAGE with any of the cationic AMPs, nor with the neutral AMP, eurocin.

In contrast, every anionic AMP tested expressed as an AMP/ELP fusion to levels detectable by SDS-PAGE as a simple extract (Figure 4). In SDS-PAGE analysis

(Figure 4), extracts representing only one temperature shift cycle, with no further purification, were loaded onto the gel in order to demonstrate the purity of this relatively crude extract. The results also demonstrate the reliable yield obtained, as bands were clearly detectable for all anionic AMP/ELP peptides with standard Coomassie Blue staining even without any further concentration steps or nickel columns. The lowest levels of expression of anionic AMP/ELP fusions yielded 20 µg/gram fresh weight, which is comparable to the highest levels reported for plant expression of AMPs [43]. Furthermore, for two of the anionic AMP/ELP fusions, it was noted there was over 10x greater expression with average yields of 375 and 563 mg/gram fresh weight for laterosporulin-1 and ADP2-3, respectively (Figure 3). This corresponds to 96 and 124 µg/gram fresh weight for each of the individual AMPs without the ELP carrier, values calculated by the percent mass of AMP amongst the entire ELP/AMP fusion based on predicted individual mass according to amino acid sequence.

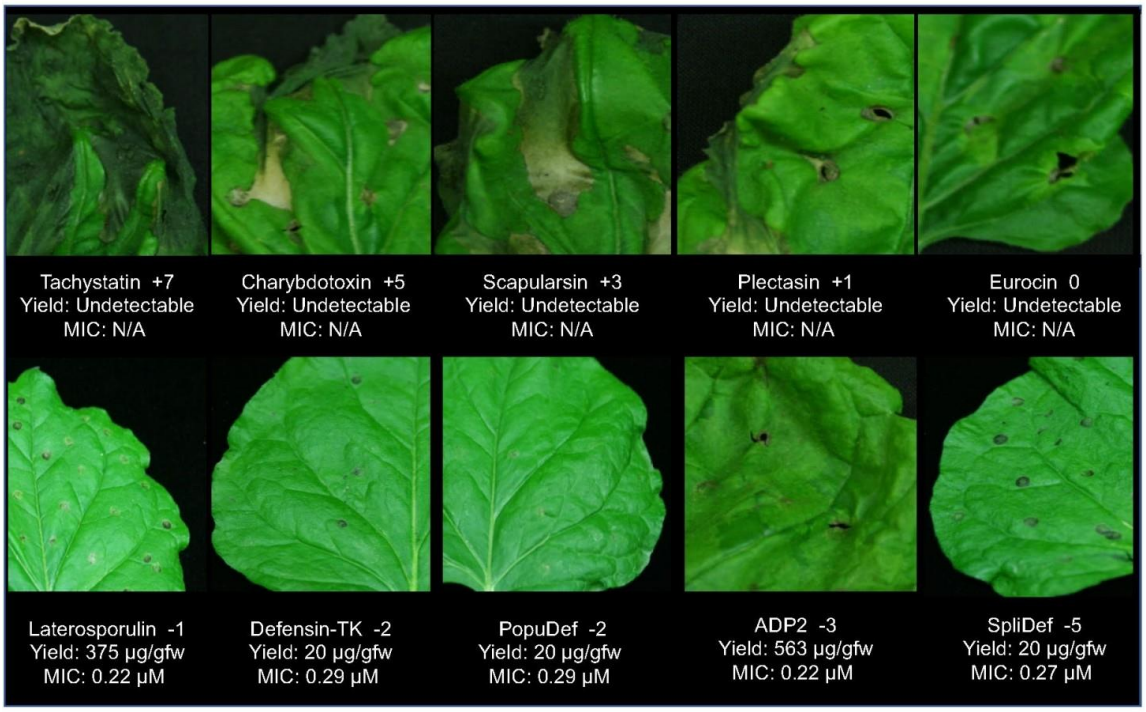

Figure 3 Expression of AMPs as ELP fusions in *Nicotiana benthamiana* via agroinoculation. Numbers following AMP name indicate net peptide charge, with most cationic at top left corner and most anionic at bottom right. Yield is expressed as µg peptide/gram fresh weight of plant tissue. Minimum inhibitory concentration (MIC) is against *Staphylococcus epidermidis*. The circular necrotic spots seen in all leaves results from mechanical injury at agroinoculation injection points.

Table 1. STP-AMPs cloned as ELP fusions, with the six cysteines participating in disulfide bonding underlined.

AMP	Amino Acid Sequence	Net Charge	Source
Tachystatin B1	YVS**C**LFRGAR**C**RVYSGR S**CC**FGYY**C**RRDFPGSIFG	+7	Horseshoe crab
Charybdotoxin	EFTNVS**C**TTSKE**C**WSV **C** QRLHNTSRGK**C**MN	+5	Scorpion
Scapularisin-6	GFG**C**PFDQGA**C**HRH**C**QSI GRRGGY**C**AGFIKQT**C**T**C**	+3	Tick/Arachnid
Plectasin	GFG**C**NGPWDEDDMQ**C** HNH**C**KSIKGYKGGY**C**A	+1	Fungus
Eurocin	GFG**C**PGDAYQ**C**SEH**C**R ALGGGRTGGY**C**AGPWY	0	Fungus
Laterosporulin	A**C**Q**C**PDAISGWTHTDYQ **C**HGLENKMYRHVYAI**C**	-1	Bacterium
PopuDef	GASPALWG**C**DSFLGY**C**RI A**C**FAHEASVGQKD**C**AE	-2	Amphibian
Defensin-TK	SPAIWG**C**DSFLGY**C**RLA**C** FAHEASVGQKE**C**AEGM	-2	Amphibian
Amblyomma	YENPYG**C**PTDEGK**C**FDR **C**NDSEFEGGY**C**GGSYRA	-3	Tick
SpliDef	VS**C**DFEEANEDAV**C**QEH **C**LPKGYTYGI**C**VSHT**C**S **C**	-5	Insect

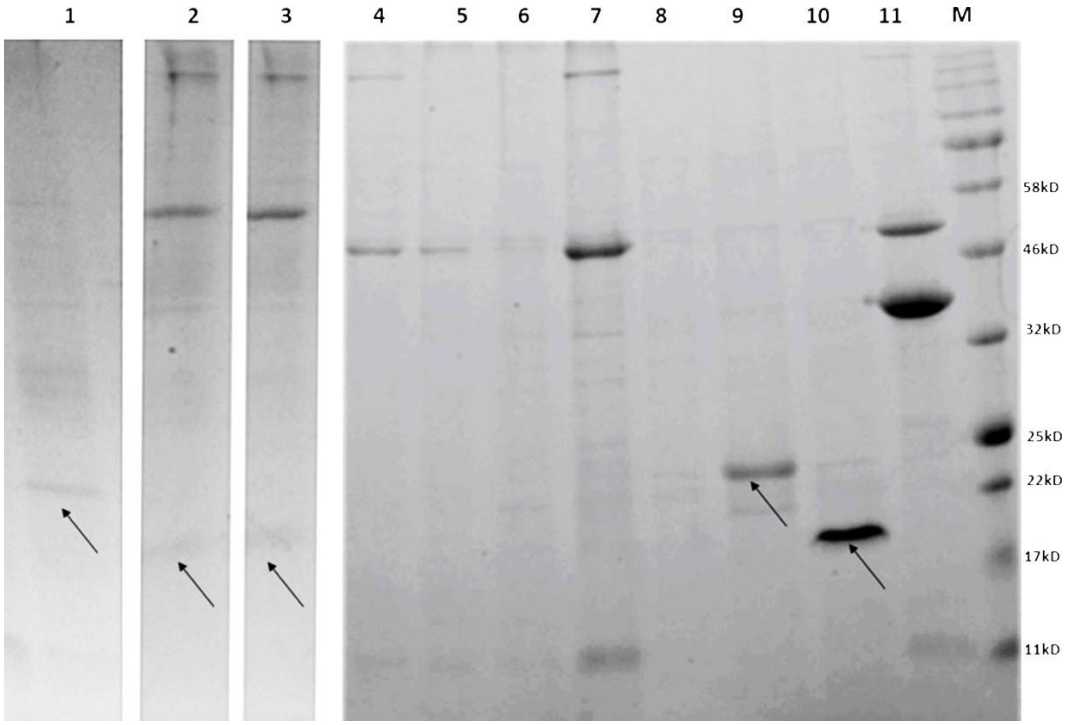

Fig. 4 SDS-PAGE/Coomassie blue analysis of AMP/ELP fusion peptides expressed in *Nicotiana benthamiana*. AMP fusion protein expression was detected only for the anionic AMPs (arrows). Expression was especially strong for laterosporulin and ADP2 (Lanes 9 and 10). Lane 1, Defensin-TK; Lane 2, PopuDef; Lane 3, SpliDef; Lane 4, Tachystatin B1; Lane 5, Charybdotoxin; Lane 6, Scapularsin-6; Lane 7, Plectasin; Lane 8, Eurocin; Lane 9, Laterosporulin; Lane 10 ADP-2; Lane 11, ELP-EGFP positive control (35 kDa).

Uncleaved AMP/ELP Fusions had Strong Antibacterial Activity

AMP/ELP fusion peptides of all of the tested anionic AMPs had unusually strong antibacterial activity as simple, unprocessed extracts. Against *Staphylococcus epidermidis*, the fusion peptides had MIC values that were consistently low (highly antibacterial), ranging from 0.22 - 0.29 μM for all AMP/ELP fusions (Figure 3). In contrast, the published MIC values against the related *Staphylococcus aureus* for the same AMPs purified from their source organisms are 7.5 μM for ADP-2 and 2 μM for laterosporulin [36,37], which shows greater than 10-fold less antibacterial activity. The eGFP/ELP vector control as well as the individual ELP fusion partner gave an average

MIC of 11.9 μM, demonstrating that the ELP carrier protein itself did not contribute significantly to antibacterial activity.

Attempts were made to find antibacterial activity in protease-treated extracts, but no activity was detected. To ensure that intact AMP was present after cleavage of the AMP/ELP fusion with TEV protease, protein analysis was performed by LC-EIS-MS for all fusions in the study that were successfully expressed in plants. For all of these, fully intact AMP was shown to be present in both the cleaved and uncleaved AMP/ELP fusion protein preparations (Supplementary Figures 5-40). Thus, strong antibacterial activity was demonstrated in the uncleaved AMP/ELP fusion proteins but no activity was found in the protease-treated extracts, despite the presence of intact AMP.

Discussion

As a protein expression system, plants bring the unique potential advantage of low-cost production and scalability. However, the yields of antimicrobial peptides reported from plant systems to date are far lower than those from *E. coli* and other competing expression systems, suggesting an intrinsic incompatibility between the plant hosts and the heterologous AMPs expressed. This presents a barrier to commercialization, with yields insufficient to take advantage of the scalability of plant systems (Table 2). As an example, the synthetic cationic AMP BP100 showed phytotoxicity in *Arabidopsis* seedlings and fitness reduction in rice plants [38], and had relatively low yield in *N. benthamiana* leaves [39]. As another example, seed expression systems often provide high yields and the expression of the AMP cecropin A in rice seed endosperm did not negatively impact seed physiology. Even so, the yield was low,

ranging from 0.5-6 µg per gram seed tissue weight [23]. Taking another approach, protegrin-1 (PG1) was expressed in the powerful magnICON tobacco mosaic virus (TMV) vector, with the AMP directed to the apoplast tabacum leaves, but no yield figures were reported [22]. Another powerful expression system involves chloroplast expression, which has the added advantage of being prokaryotic in nature. However, when protegrin was produced as a fusion with GFP in a chloroplast expression vector, the yield of the purified fusion protein was only 8 µg/g fresh weight of leaf tissue [21]. Finally, fusing AMPs to carrier proteins is normal practice in E. coli expression of AMPs and this was attempted with sarcotoxin IA, using GUS as the carrier protein for plant expression. However, the levels expressed were not sufficient for detection by SDS-PAGE [40].

This study appears to have broken the yield barrier for AMP expression in plants by observing a bias in peptide charge found naturally in plants and then experimentally demonstrating that, for our set of 10 AMPs, only the anionic AMPs could be expressed. The minimum yields for anionic AMPs were slightly above the highest reported AMP yields in plant expression systems to date; furthermore, the highest yielding AMPs delivered 10-fold as much. Though not a direct comparison, these yields from fresh plant tissue can be seen as comparable to those found in *E. coli* liquid cultures, with AMPs produced in the 10-100 mg range from a 1-liter culture [24]. In comparison, reported yields from the previously published plant expression systems cited above would correspond to 1 mg from a medium-sized harvest of 200 g of plant tissue. In contrast, this study reports an AMP yield in plants which is comparable to that achieved in *E. coli*. Here, a minimum yield of 20 µg and a maximum of 563 µ g per gram fresh weight was observed, which would correspond to 4 mg and 113 mg per 200 g of plant tissue,

respectively, equivalent to reported yields for *E. coli* expression systems. In perspective, the best yields of anionic AMP/ELP fusion peptides in our study also compare favorably to reports for the expression of the marker gene GFP in *N. benthamiana* plants (270-340 μg GFP/gfw) using a 35S promoter aided by the p19 silencing suppressor [41].

Net charge may be the sole deciding factor for whether the recombinantly produced AMP engages in an electrostatic interaction with the negatively charged components of the plant bio membrane, despite being sequestered in protein bodies of the ER by ELP-fused sequestering, similar to what was seen with BP100 expression [38]. This accumulation of recombinant peptide interacting with the plant tissue seems to trigger a phytotoxic response only when the AMP is not explicitly anionic. For its recombinant expression in plants, ELP protein accumulation at these levels have only ever been observed when the fusion protein is directed to the ER by the KDEL retention sequence, clustering around the ER and later released and free floating in the cytoplasm [34,35].

Furthermore, the AMP/ELP fusion peptides of our study possess an antimicrobial activity (0.22-0.29 μM) an order of magnitude stronger than these AMPs expressed from *E. coli* systems [42,43,44,45]. Thus, on a functional basis, the yield figures we report would be considerably higher in comparison to those of the *E. coli* systems.

The use of elastin-like polypeptide (ELP) as a fusion partner proved important for the yield and antibacterial activity of anionic AMPs expressed in plants in this study. ELP is an extracellular matrix protein found in vertebrate connective tissue. When targeted to the endoplasmic reticulum, ELP provides protein sequestering and stability to its fusion partner [26,46,47]. The ELP protein also provides a purification process using

inexpensive temperature shifts without the use of chemicals or chromatography [26,48]. In addition to benefiting yield and purification, we noted that the unusually high antibacterial activity was associated with the uncleaved ELP fusions. The loss of activity post-cleavage may have been due to loss of structural integrity maintained by ELP fusion partner in close proximity. Activity of uncleaved ELP/AMP fusions has not been previously reported in *E. coli* expression systems. In literature, fused ELP/AMP that has been expressed in *E.coli* has only once been reported to be biologically inactive before cleavage and separation , but with use of a larger ELP fusion partner than what this paper used [46]. Further studies are in progress in this lab using *E. coli* expression for faster turnover to elucidate the protein structural aspects of antimicrobial activity of AMP fused to the ELP carrier, focusing on variations of ELP fusion partner size, protease cleavage site employed for separation, net charge of AMP, and distance between the ELP and AMP using various lengths of linkers. The working theory for this phenomenon is that the steric hinderance of a larger ELP fusion partner inhibits fused AMP activity when still fused, and this effect is lessened or even lost when the ELP fusion partner is a smaller number of repeats.

Plant expression of AMPs seems well suited to large scale, low-margin applications and the effectiveness of AMPs has already demonstrated as poultry and livestock feed additives [49], food preservatives [50] and topical disinfectants [51]. The scalability of plant expression systems would allow for the production of large amounts of raw product, which could then be reduced to relatively pure protein by simple temperature shift cycles, potentially without the need for column chromatography, which increases post-production costs [25]. Alternatively, the AMPs might be expressed in

transgenic grain seed, which tends to have yields higher than seen in leaf tissue. Recombinant proteins remain stable using traditional seed storage technique [52,53,54]. Anionic AMPs used in the seed platform would address the increasing concern over the amount of antibiotics used with livestock [55,56,57]. Applications in animal feed have already shown limited success in protecting livestock against pathogens using antibodies and antimicrobial peptides [58,21]. Since the ELP/AMP fusion protein does not need to be cleaved, it would be expected that the resulting grain should be directly antimicrobial, without proteolytic processing. Proteins encapsulated in plant tissue may be expected to better survive the digestive system to arrive intact to eliminate gut pathogens [59]. Furthermore, AMPs would largely eliminate the environmental consequences of pesticide use. Being peptides, AMPs would be expected to have a very short half-life in soil or aquatic environments.

A final advantage to using AMPs is to avoid the development of resistance in target bacteria and the microbiota as a whole [60,61]. As already mentioned, amphipathic AMPs have a generalized mechanism for destroying bacteria by membrane disruption and other mechanisms, which inhibits resistance development [11,62]. Furthermore, it would be relatively easy to supply AMPs as "stacked drugs" by including several AMPs in the same treatment or in the same grain seed genome. In this way, resistance development is further forestalled since any resistance mutant that appears would be destroyed by another AMP with a divergent mode of action in the treatment mix. The sheer abundance of putative AMP sequences in genome databases is a vast resource for the rapid discovery and testing of large numbers of AMPs to support a stacked drug paradigm, as opposed to the slow development cycle of small molecule antibiotic drugs.

We have added to the capacity of this developmental pipeline by developing several algorithms for detecting AMPs from genomic databases. We can now predict sequential tri-disulfide peptides (STPs) from genomes using a support vector machine algorithm [30]. STPs are the predominant structural form of AMPs and are sturdy, robust structures [9]. We have developed a generalized algorithm based on natural language processing to classify protein sequence based on any input characteristic [63] and this has been used to predict protein function from genome sequences, such as picking AMP function while rejecting hemolytic activity to avoid human toxicity [64]. Anionic AMP candidate sequences from these genome searches can be expressed as ELP/AMP fusion peptides in plants with the expectation that a significantly proportion would express at high yield, as we observed in our present study. These fusion peptides could then be screened as extracts from a single thermal shift cycle, accelerating the screening workflow process.

Table 2. Low recombinant AMP previously expressed in plants.

Antimicrobial peptide	Yield (µg per gram plant tissue weight)
BP-100	N/A
Cecropin A	0.5-6
Protegrin-1	8
Sarcotoxin IA	N/A

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CHAPTER THREE

Modifying Antimicrobial Peptides to Selectively Kill Staphylococcus Bacteria using a Phage Display-derived Targeting Peptide

Abstract

Targeted therapies seek to selectively eliminate a pathogen without disrupting the resident microbial community. Bacteriophage peptide display technique provide a rich, well-documented source of target-specific peptides that can be fused to antimicrobial peptides (AMPs). Though resistance to a single targeted AMP may develop as with any antibiotic, the wealth of variants available in random peptide library allows for the use of multiple targeted AMPs in parallel, potentially circumventing resistance development.

Here, we target two cationic antimicrobial peptides (AMPs), plectasin and eurocin, by genetically fusing their coding sequence to that of the host-binding peptide A12C, which selectively binds to *Staphylococcus*. Targeting did not decrease the potency of the AMP when applied to the targeted staphylococci, *S. aureus* and *S. epidermidis*, but drastically decreased AMP potency against the nontargeted species, *Enterococcus faecalis* and *Bacillus subtilis*. This effect was equally evident across two different AMPs, two different species of *Staphylococcus*, two different negative control bacteria, and against biofilm and planktonic forms of the bacteria.

This is the first reported use of peptide sequences derived from phage display technique to modify established antimicrobial peptides and engineer its activity spectrum. Considering the near infinite sizes of random peptide library, this targeting approach should be generally applicable to a wide range of bacterial pathogens.

Introduction

A major drawback to traditional antibiotics is the massive disruption of the natural microbiota. Targeted antimicrobial peptides provide a solution by specifically reducing or eliminating the targeted pathogenic species allowing the remaining commensal population to occupy the deserted niche (Eckert *et al.,* 2012). A problem with the targeted antimicrobial peptide approach is finding targeting peptides suitably specific for the pathogen. Pheromone peptides used by bacteria to signal biofilm formation are species-specific sequences already identified for several bacterial pathogens and have been used to specifically target antimicrobial peptides (Eckert *et al.,* 2006; Mai *et al.,* 2011). In a large number of cases, the exact viral peptide sequence which specifically binds the host cell has been empirically determined (Nemesio *et al.,* 2011). If no viruses are currently known for a particular host species, a genomic analysis of host samples can produce viral or proviral sequences for use in mining targeting domains. Surprisingly, only one study has been published which utilizes this approach, and this involves specificity towards an insect pest rather than towards a microbial pathogen (Bonning et al, 2014). The spider toxin peptide, Hv1a, was fused to the peptide domain of pea enation mosaic virus that is responsible for virions moving from the gut lumen into the hemocoel. It was demonstrated that Hv1a itself was not orally toxic to aphids, but the targeted fusion protein was toxic.

A more universally available and diverse source of targeting moieties is the bacteriophage peptide display technique used by researchers to find and establish interaction between the peptide displayed on the surface of the phage and a target protein/cell (Drulis-Kawa et al., 2012; Lowman, 1997; Winter, 1994). This technique involves inserting the ORF of a desired peptide sequence in the genome of bacteriophages

in the gene encoding the minor or major coat peptide of the bacteriophage (Wu et al., 2016). Starting with a large library of random peptides, subsequent cycles of phage immobilization, washing of the unbound phages and the growth of the bound phages with a narrower range of display peptides lead to selection and enrichment of the ligands having specific affinity towards target proteins or cells (Wu et al., 2016). To improve the techniques, instead of using random libraries, we can curate a library to contain peptide sequences derived from natural sources that exhibit innate affinity towards that intended targets (Ryvkin *et al.*, 2018), which in our case are bacteria.

In our study, we utilized peptides derived from this approach to modify the specificity of antimicrobial peptides. A literature survey yielded a potent targeting domain (A12C) that was used to engineer a drug-carrying filamentous phage (Yacoby *et al.*, 2006). For targets, we chose the most common nosocomial biofilm-based pathogens that develop antimicrobial resistance, namely, *Staphylococcus epidermidis* and *Staphylococcus aureus* (Chalker *et al*., 2009). The binding affinity of A12C peptide covered filamentous phages towards staphylococci has been determined previously (Yacoby *et al.*, 2006) and we used this peptide to modify two well-studied antimicrobial peptides, plectasin (Mygind *et al.*, 2005) and eurocin (Oeemig *et al.*, 2012). We found a strong differential effect, with the targeted antimicrobial peptides retaining activity against the targeted staphylococci while being severally attenuated against the nontargeted bacteria *Enterococcus faecalis* and *Bacillus subtilis*. This demonstrates the general utility of this method of using binding domains derived from phage display studies to target antimicrobial peptides specifically to pathogens while preserving nontarget members of the microbiota.

Materials and Methods

Reagents

E. coli (BL21 and 10β) strains were purchased from New England Biolabs. The pE-SUMOstar vector used for *E. coli* expression was purchased from LifeSensors. The Ulp1 protease was expressed in *E. coli* using pFGET19_Ulp1 plasmid purchased from Addgene. The gBlock (gBlocks® Gene Fragments) containing *E. coli*-codon optimized sequences of plectasin, eurocin, and the A12C fusion peptide were purchased from IDT. Synthetic A12C peptide was purchased from Biosynthesis. We expressed native plectasin and eurocin and their A12C conjugated analogues for the primary microbicidal studies (See Table 1). As a control we also expressed plectasin and eurocin conjugated with the AgrD1 pheromone (Seq: YSTCYFIM) (Mao *et al.,* 2013) at the N- terminus. The strains of bacteria used for antimicrobial assay were obtained from S. J. Kim, Department of Chemistry and Biochemistry, Baylor University, and the Microbiology Laboratory, Department of Biology, Baylor University.

Construction and Cloning of Plasmid

The synthesized genes (Integrated DNA Technologies) were cloned into the pE-SUMOstar vector following the SUMO protease cleavage site (Figure 1). The recombinant plasmids were electroporated into *E. coli* 10β cells and positively transformed colonies were selected with kanamycin and screened via PCR. The prepared plasmids were extracted and transformed into chemically competent BL21 cells for expression (Pope *et al.,*1996).

Table 1: AMPs with and without viral targeting moiety from phage A12C.

Peptide	**Sequence**	**Molecular Weight (in Daltons)**
Plectasin	GFGCNGPWDEDDMQCHNHCK SIKGYKGGYCAKGGFVCKCY	4408
A12C- Plectasin	GVHMVAGPGREPTGGGHMGF GCNGPWDEDDMQCHNHCKSI KGYKGGYCAKGGFVCKCY	6137
Eurocin	GFGCPGDAYQCSEHCRALGG GRTGGYCAGPWYLGHPTCTCSF	4345
A12C-Eurocin	GVHMVAGPGREPTGGGHMGF GCPGDAYQCSEHCRALGGGR TGGYCAGPWYLGHPTCTCSF	6074
* The underlined sequence is the A12C targeting domain		

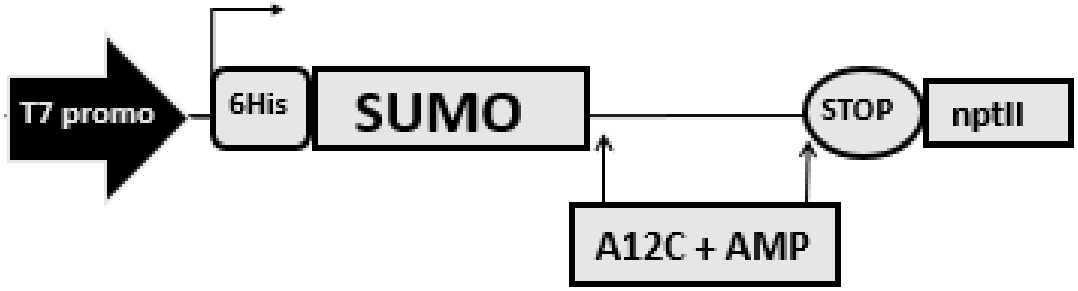


Figure 1: pE-SUMOstar/AMP *E. coli* vector. The SUMO protease cleavage site allowed the release of AMP (plectasin or eurocin) from the SUMO fusion partner. MCS, multiple cloning site (MCS).

Expression, Extraction and Purification of Proteins

Positive BL21 transformants were grown in 20 ml 2X YT broth (50 µg/mL kanamycin) at 37^{o}C overnight with shaking. The primary culture was used to inoculate a secondary culture of 500 ml 2X YT broth (50 µg/mL kanamycin). The secondary cultures

were grown at 37ºC with shaking (220 rpm) to an OD_{600} of 0.7. This was followed by four hours of induction with 0.1 mM IPTG at 180 rpm. The cells were harvested by centrifugation at 10,000 x g for 1 hour at 4ºC. The bacterial pellets were resuspended with PBS buffer containing 25 mM imidazole and 0.1 mg/ml lysozyme and then frozen overnight to facilitate lysis of bacterial cell. The frozen suspensions were thawed and sonicated at 40% amplitude with a probe sonicator. The lysed and sonicated slurry was then ultracentrifuged at 80,000 x g for 1 hour at 4ºC and the resultant supernatant was retained. The supernatant was then subjected to nickel column chromatography using PBS with 25 mM imidazole as the binding and wash buffer and PBS with 500 mM imidazole as the elution buffer. The eluents were screened for the presence of proteins by SDS-PAGE and the positive fractions were combined for storage at 4ºC. Before using the proteins, the SUMO fusion partner was removed using added Ulp1 protease (1U per 100 µg of substrate) at 4ºC overnight under mild nutation. The extent of cleavage was confirmed by SDS-PAGE. The gel bands corresponding to the AMPs were also excised and subjected to in-gel tryptic digestion (Thermo Fisher). After the digestion with trypsin, confirmation of the proteins' identity was performed by LC-ESI-MS (Synapt G2-S, Waters) at the Baylor University Mass Spectrometry Center using samples obtained by in-gel tryptic digestions of SDS-PAGE bands of the respective proteins. The analysis of the MS data was done by MassLynx (v4.1) The spectra of each protein, both non-targeted and targeted, were peak centered and MaxEnt3 processed and then matched against hypothetical peaks from peptides generated by simulated Trypsin digestion of the respective proteins (Supplementary Figure S1-S16).

Hemolytic Activity Assay

Targeted AMPs, non-targeted AMPs and synthetic A12C peptide were assessed for hemolytic activity via exposure to washed human erythrocytes. Red blood cells (RBCs) were collected a healthy volunteer was collected in 5 ml vacutainers. RBCs were isolated by gentle centrifugation (500 g for 5 min), washed with equal volume 150 mM NaCl twice and then with equal volume of 10 mM PBS (pH 7.4). The pellet was then diluted in equal volume of PBS and then diluted to a 1:50 dilution with the same PBS to have an approximate concentration of $5x10^8$ RBCs/ml. To initiate hemolysis, 190 µl of the cells was added to 20 µl of a 2-fold serially diluted peptide/ test reagent in PBS in a 96-well flat-bottom microtiter plate. Wells without peptide were used as negative controls, while wells containing 1% Triton X-100 were used as positive controls. The plate was incubated at 37°C for 1 h and centrifuged at 3,000 g for 10 min. An aliquot (120 µl) of supernatant from each well was transferred to a new plate to read the absorbance at 540 nm using a microtiter plate reader. The percentage of hemolysis was calculated by the following equation: (A541 of the peptide-treated sample - A540 of buffer-treated sample)/(A540 of Triton X-100-treated sample - A541 of buffer-treated sample) x 100% (Evans *et al.*, 2013).

In Vitro *Bactericidal Activity Assay*

The Ulp-1 protease-cleaved proteins were tested for antimicrobial assays against four strains of bacteria: *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* and *Bacillus subtilis*. These four species were selected because they are gram positive and the AMPs plectasin and eurocin are specifically active against gram positive bacteria (Mygind *et al.*, 2005; Oeemig *et al.*, 2012). The two controls used for the experiment were the free fusion partner SUMO protein dissolved in PBS as the vehicle and

synthetic A12C dissolved in PBS as well. Vancomycin was used as the positive control, which was experimentally determined to be active against these bacteria. The standard protocol for a microtiter plate assay with serial dilution was used (Sarker *et al.,* 2007). Briefly, the first well of the 12-well row in the 96 well microtiter plate contained 50 µl of the highest concentration of test protein/control solution with serial 2-fold dilutions leading to the last well having 2-11th of the concentration as the initial well. The serial dilution was done with PBS buffer and additional 30 µl of Tryptic-Soy Broth (TSB)/LB media was given to the wells before inoculating with 10 µl of the bacterial culture. For inoculation, the bacteria were grown in TSB/LB media overnight and then diluted in the same media to meet the McFarland 0.5 standard. After inoculation, the plates were grown at 37oC for 8 to 12 hours (depending on the strain). After the initial growth period, 10µl of resazurin solution (0.0015% w/v in DI water) was added. After adding resazurin, the plates were allowed to grow for 30 min to an hour before checking the progress. The results were reconfirmed by allowing the plates to grow further for a period of 12 hours and then checked for the change in coloration of the wells. Each test and control peptide were tested against each strain of bacteria for n>5 replicates.

In Vitro *Cell Kinetics Study*

The protease-cleaved peptides were assayed to determine their dynamic action against the bacteria in a growing culture. The bacteria assayed were *B. subtilis, S. epidermidis*, *S. aureus* and *E. faecalis* grown at 37°C with shaking and diluted in LB or TSB medium to ~$1x10^8$ CFU/ml. Antimicrobial peptides were then added to 2 ml of this culture and the culture was returned to 37°C with shaking for continued growth or decline

over 8 hours. For plectasin and eurocin, the concentration used was 3x the minimum

inhibitory concentration determined by the *in vitro* bactericidal activity assay described above. Targeted versions of these peptides were run at the same concentrations as the corresponding untargeted versions. The concentration of vancomycin was the mean of the molar concentrations of plectasin and eurocin (~7xMIC for both the bacterial species). To determine titers, samples of 10 µl were taken from each tube at specific time intervals from 2 hour to 10 hour post. The samples were diluted in LB or TSB media (1500x, 22500x, 45000x or 90000x) and spread on Mueller-Hinton agar plates. After an overnight growth period, the number of colonies formed were recorded and titers calculated.

In Vitro *Biofilm Inhibition Assay*

In addition to testing the efficacy of the AMPs against the planktonic bacterial cultures, they were also evaluated on how effectively they can inhibit the growth of biofilms of the 4 bacterial species in a microtiter plate. The assay was performed following the protocol established in previous articles (Merritt *et al.*, 2005; O'Toole, 2011). Briefly, the bacterial culture grown overnight in TSB/LB media were diluted 1:100 and 100 µl of the dilution were added to 100 µl of serially diluted AMP/ antibiotic control solution in PBS and allowed to grow for 24-36 hours to form a visible biofilm. The supernatant cultures from the wells were carefully aspirated and the underlying films were washed gently with PBS, dried over air and fixed with methanol. On the evaporation of methanol, the plates were washed again with PBS, air-dried and 125 µl of 0.1% crystal violet was added to the wells. Crystal Violet stains the cell wall of the bacteria in the biofilm. After 10-15 minutes, the plates were washed again, dried and treated with 100 µl of 30% acetic acid to dissolve the attached crystal violet stain. The absorbance of the wells were quantified at 540 nm with 30% acetic acid solution as blank. The absorbance data was

tabulated against the concentration of the AMPs/control reagent in each well with at least 3 or more replicates for each test. The absorbance reading of crystal violet indicates the quantity of the biofilm that had formed in that well.

Results

Protein Expression and Purification

AMPs with or without the targeting domain and the SUMO fusion partner, at 4-6 kDa and ~17 kDa respectively, were highly expressed, successfully cleaved and clearly visualized with SDS-PAGE (Figure 2). For further peptide identification, peptides were extracted from the SDS-PAGE gel bands, digested by trypsin and detected by mass spectrometry. Peptide identities were confirmed using the MassLynx (v4.1) application (Waters), which created hypothetical MS peaks by virtual trypsin digestion of the four protein sequences and matched them with the spectrum generated experimentally. The hypothetical peaks simulated from the four peptides overlapped satisfactorily with the MS peaks generated in the spectrometer and hence confirmed the presence of the peptides in our samples. Supplementary Figure S1, S3, S5, S7, S9, S11, S13 and S15 show the peptide list generated by the simulated trypsin digestion and their hypothetical m/z values (in red) with the matched peaks appearing in black. Supplementary Figures S2, S6, S10 and S14 show the MaxEnt3 processed deconvoluted mass spectrum of each peptide while Supplementary Figure S4, S8, S12 and S16 show the mass corrected (green) and peak centered (red) mass spectra of each peptides. The average yields (n>=3) of the proteins plectasin, A12C-plectasin, eurocin and A12C-eurocin were between 15 to 26 mg/L of bacterial culture or between 3 to 4 µmoles per L of culture. These were

calculated from the SDS-PAGE data, using NIH ImageJ to measure band density and the marker lane bands for mass reference.

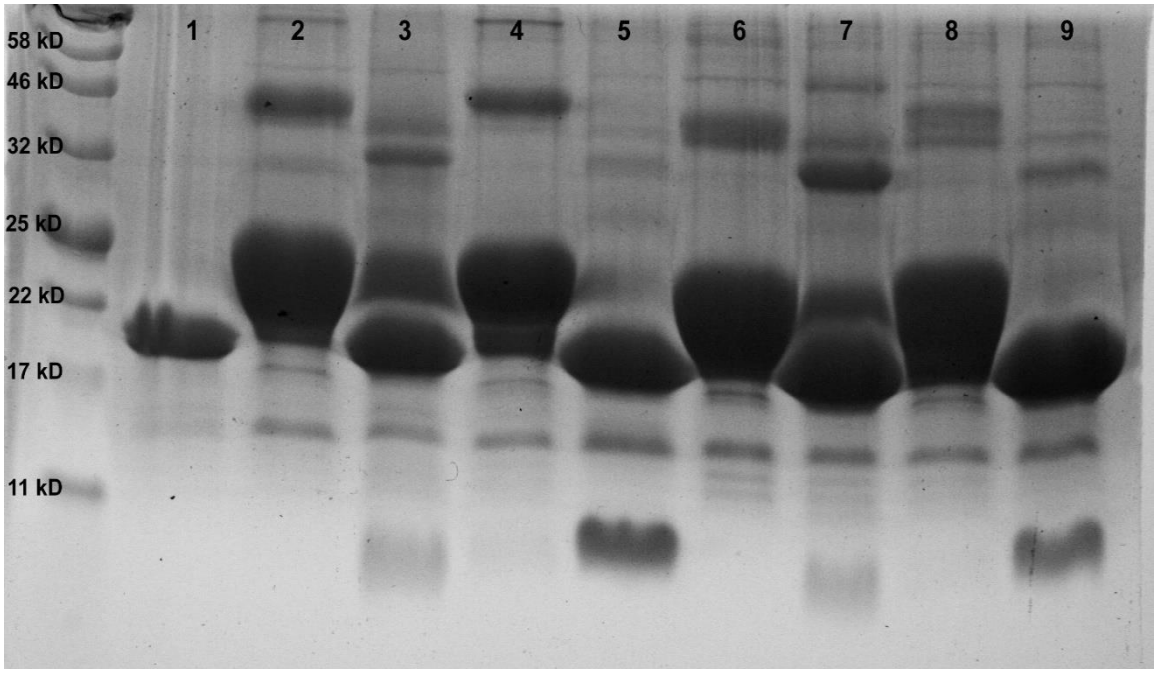


Figure 2: Expression of SUMO/AMP in *E. coli* and cleavage of AMP free of SUMO fusion partner. Plectasin (lane 2), A12C-plectasin (lane 4), eurocin (lane 6), A12C-eurocin (lane 8) expressed with the SUMO fusion partner. On cleaving with SUMO protease (Ulp1), the cleaved SUMO protein can be seen at 17 kDa on lanes 3, 5, 7 and 9; free SUMO protein control is in lane 1. The released AMPs, with and without targeting moieties, are in the same lanes as with the cleaved SUMO below 11 kDa.

Hemolytic Activity Assay

In concordance with previously published individual studies on both AMPs plectasin and eurocin (Mygind *et al.*,2005; Oeemig *et al.*, 2012; Yacoby *et al.*, 2006), both targeted and untargeted fusion peptides along with the free A12C peptide displayed no hemolytic effect on human erythrocytes (data not shown) in comparison to a 20% Triton-X positive control.

In Vitro *Bactericidal Activity Assay*

Differential toxicity between targeted and non-targeted peptides was observed, with the addition of the viral A12 targeting domain driving a loss of activity against the non-target species rather than a gain of activity against the target species. A12C-AMPs retained their toxicity against both staphylococci bacterial species but showed a dramatic decrease in toxicity (presented logarithmically in Figure 3) against non-target species relative to natural AMPs (Figure 3). This data is presented in tabular format in Supplementary Table S1. Purified SUMO dissolved in PBS and free A12C in PBS were used as negative controls for all experiments and showed no antimicrobial activity. For the non-target bacterium *E. faecalis* and *B. subtilis*, the attachment of the A12C targeting domain lowered the antimicrobial efficacy by increasing the mean MIC values for both plectasin and eurocin to over 70 μM compared to <10 μM seen without the targeting moiety ($p<0.001$; ANOVA 2-tailed test). For *S. aureus* and *S. epidermidis*, however, no significant rise in MIC values was observed upon attachment of the fusion partner for either eurocin or plectasin. Peptide analogues with AgrD1 for both plectasin and eurocin had either lower or non-significantly different MIC values compared to non-targeted plectasin and eurocin against all 4 bacteria (data not shown).

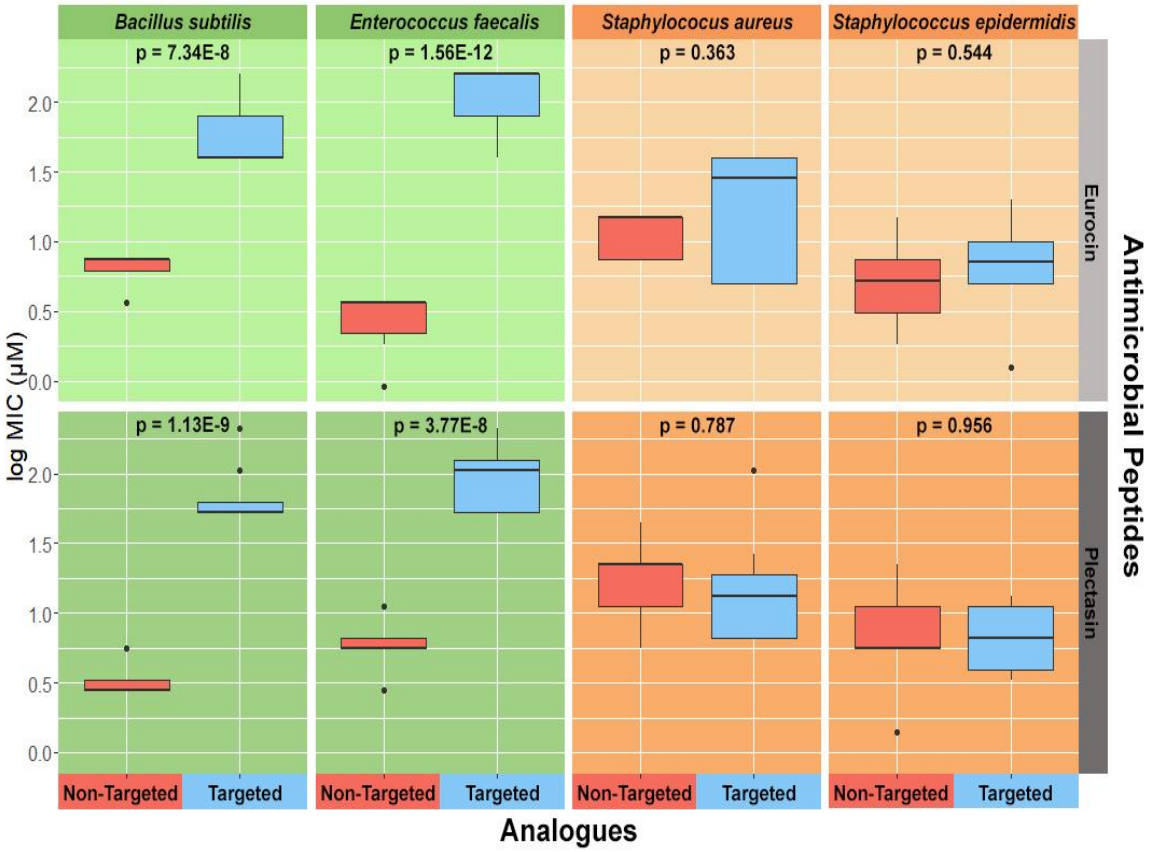
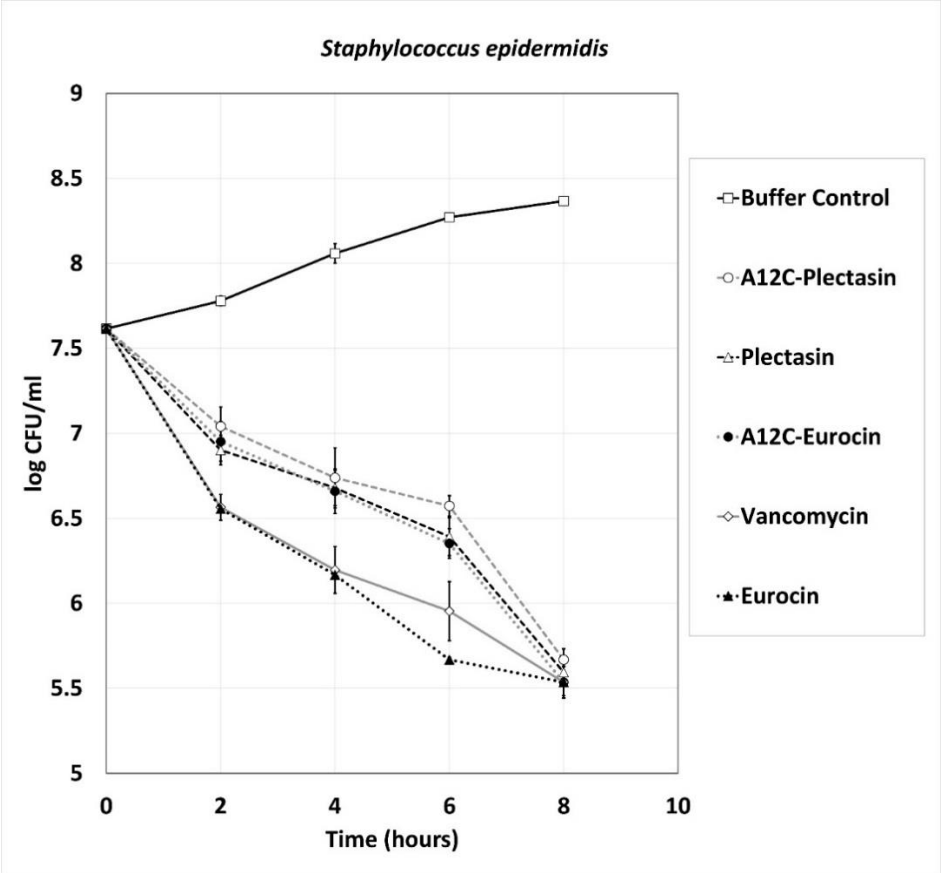


Figure 3: Log values for minimum inhibitory concentrations (MIC) in μM for non-targeted (red) and targeted (blue) analogues of eurocin and plectasin against *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. The boxed regions represent 50% of the values while the bars represent 95%.

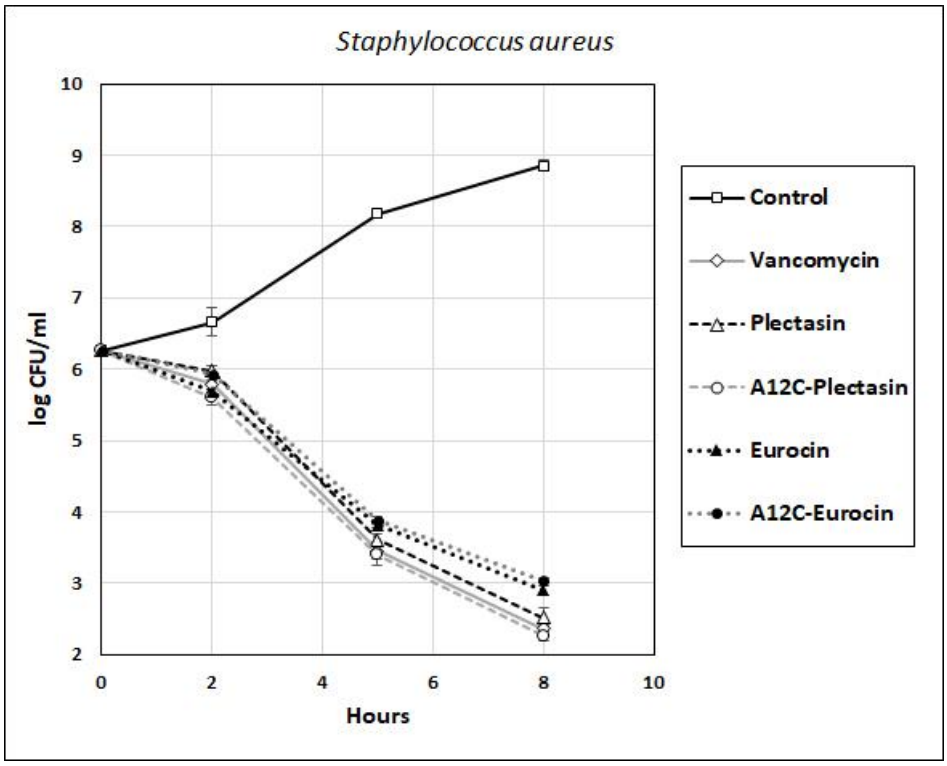
In Vitro *Cell Kinetics Study*

Growth kinetics over an 8 to 10 hour period further demonstrated the loss of antimicrobial competence of the AMP against non-staph post targeting. All peptides - both targeted and non-targeted - demonstrated a strong bactericidal effect, as did the vancomycin positive control, against the target bacteria *S. epidermidis* and *S. aureus* over an 8-hour period (Figure 4a and 4b). In contrast, for the nontarget bacteria *B. subtilis* and *E. faecalis*, the bactericidal effect was seen only with nontargeted plectasin and eurocin peptides, with a toxicity similar to vancomycin. The A12C-targeted analogues did not

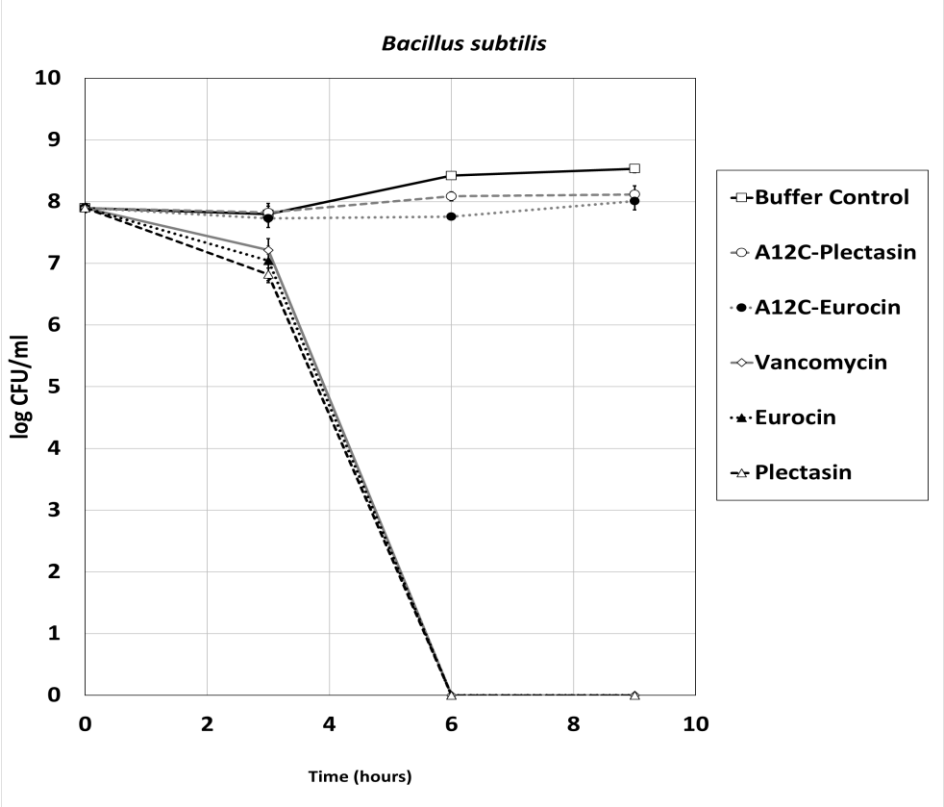
induce any decline in *B. subtilis* and *E. faecalis* cultures, which lagged only slightly behind the buffer-control treated cultures (Figure 4c and 4d). The relatively flatter growth curve for the *B. subtilis* control cultures reflects its growth kinetics, which is far slower than that of other bacteria.



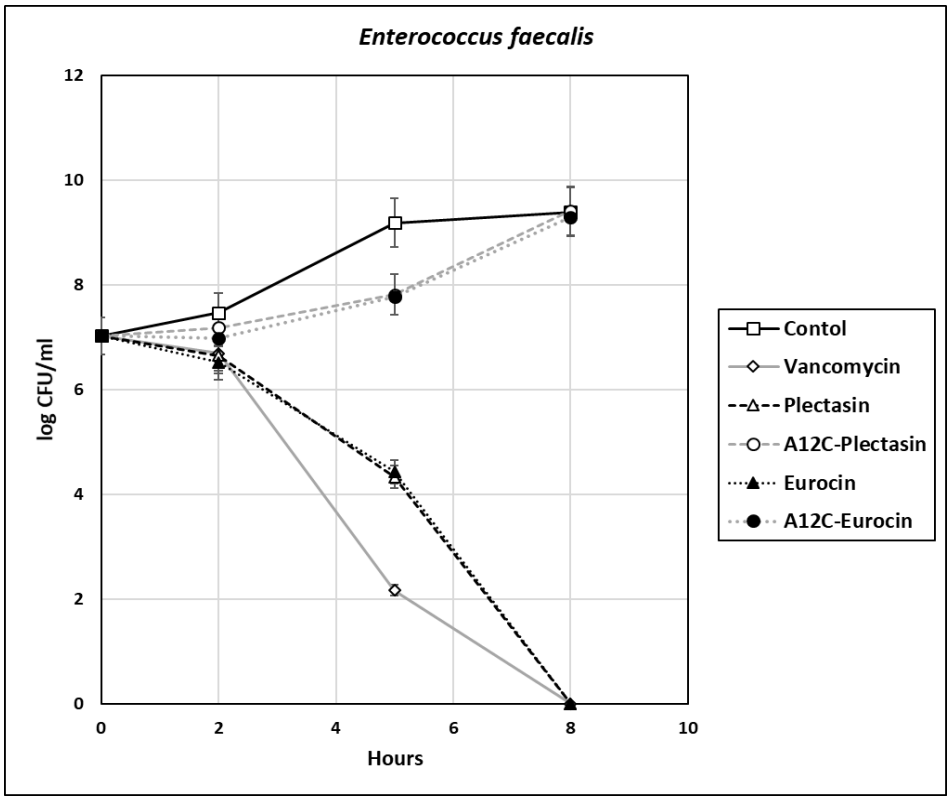
(a)


Staphylococcus aureus
log CFU/ml
Hours
Control
Vancomycin
Plectasin
A12C-Plectasin
Eurocin
A12C-Eurocin

(b)


Bacillus subtilis
log CFU/ml
Time (hours)
Buffer Control
A12C-Plectasin
A12C-Eurocin
Vancomycin
Eurocin
Plectasin

(c)

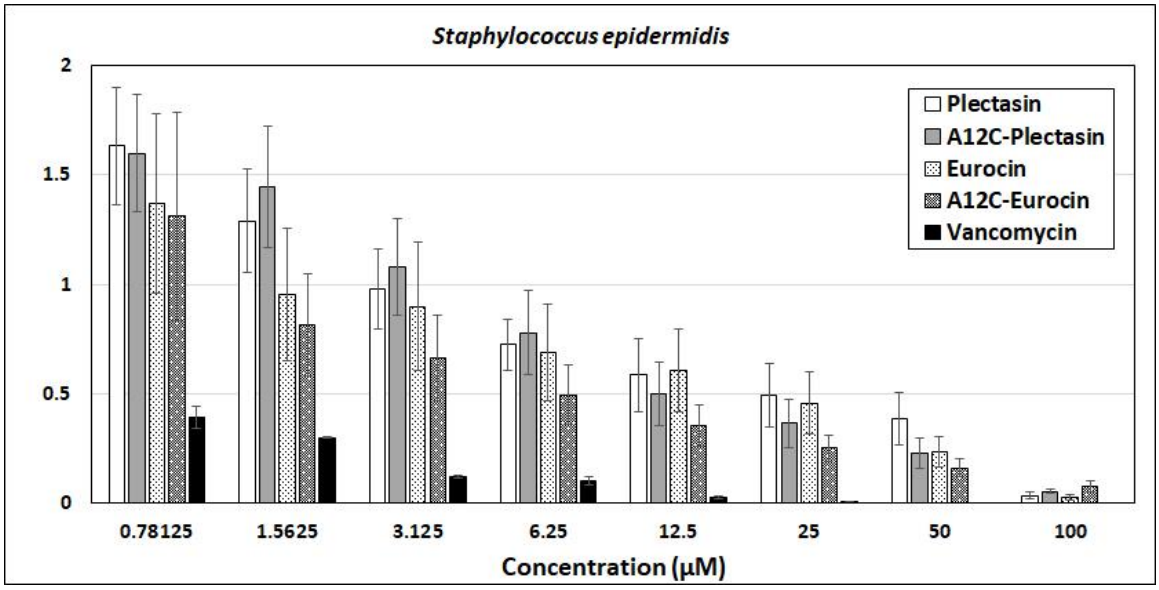


(d)

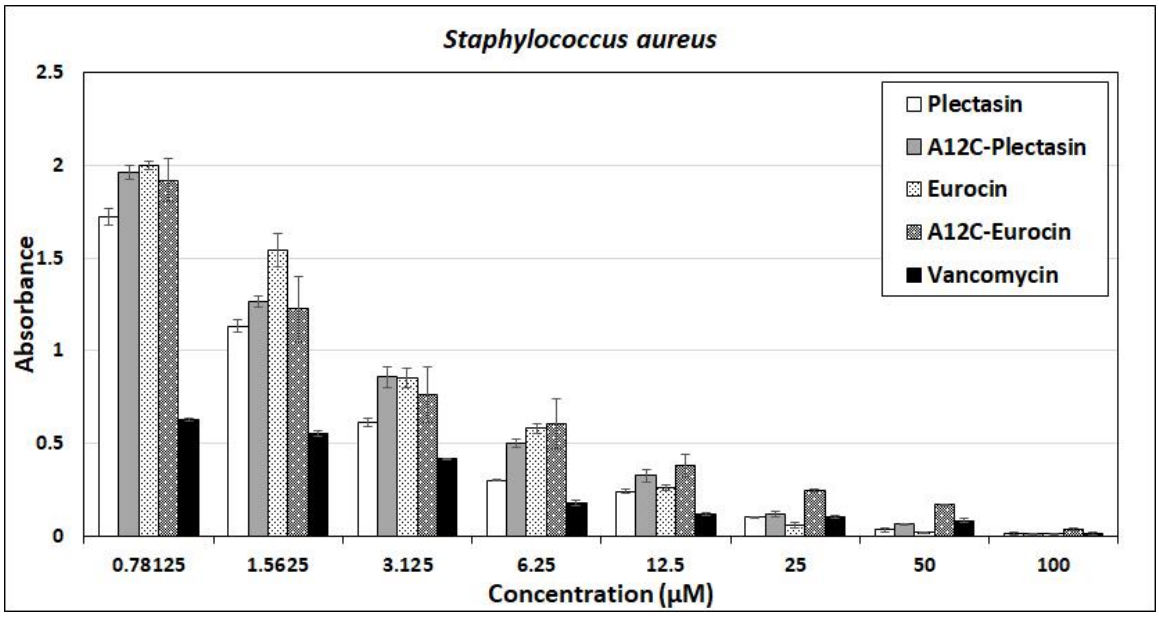
Figure 4: The cell-kinetic profile. *S. epidermidis* (a), *S. aureus* (b), *B. subtilis* (c) and *E. faecalis* (d) created by plotting log CFU/ml of the bacteria grown in the presence of each peptide for 8-10 hours collected in 2-3 hour intervals.

In Vitro *Biofilm Inhibition Assay*

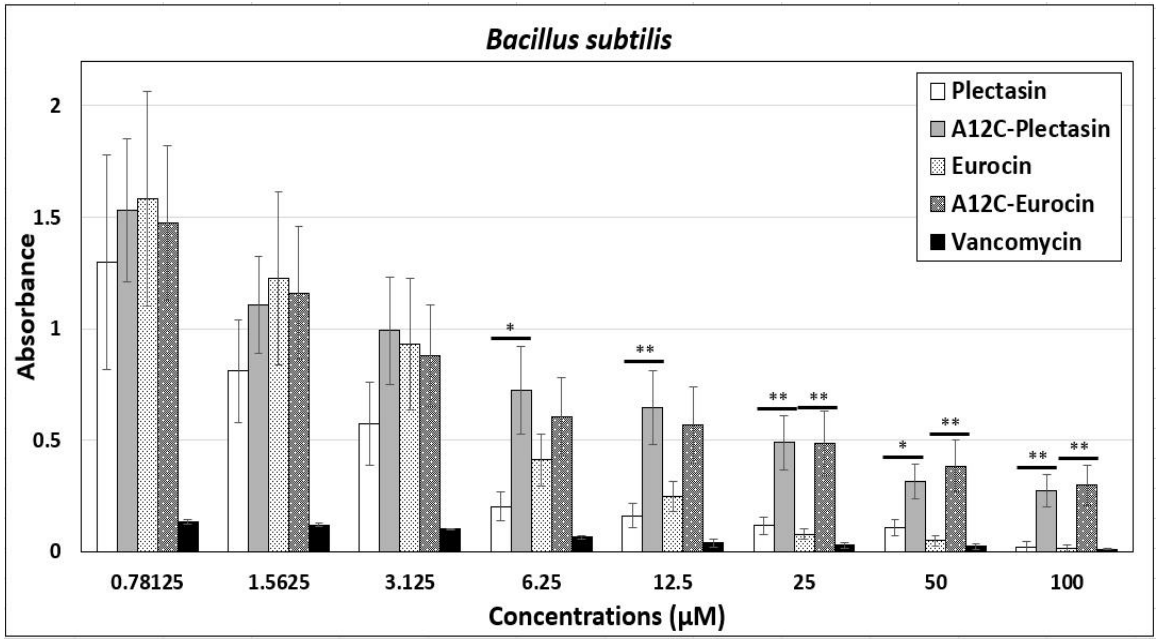
Growing bacterial cultures with the peptides demonstrated the preferential inhibition of bacterial biofilm of the *Staphylococcus* strains (Figure 5a and 5b) by the targeted AMPs over the non-*Staphylococcus* bacteria. The absorption reading (hence, the quantity of biofilm formed) decreased with the increase in peptide concentration for all the 4 bacteria when treated with non-targeted peptides but the targeted peptides did not have similar effects on *B. subtilis* (Figure 5c) and *E. faecalis* (Figure 5d) with significant ($p < 0.10$ or $p < 0.05$) difference in the absorbance values between targeted and non-targeted AMPs at concentrations beyond 6.25 µM.


Staphylococcus epidermidis
2
1.5
1
0.5
0
Plectasin
A12C-Plectasin
Eurocin
A12C-Eurocin
Vancomycin
0.78125
1.5625
3.125
6.25
12.5
25
50
100
Concentration (μM)

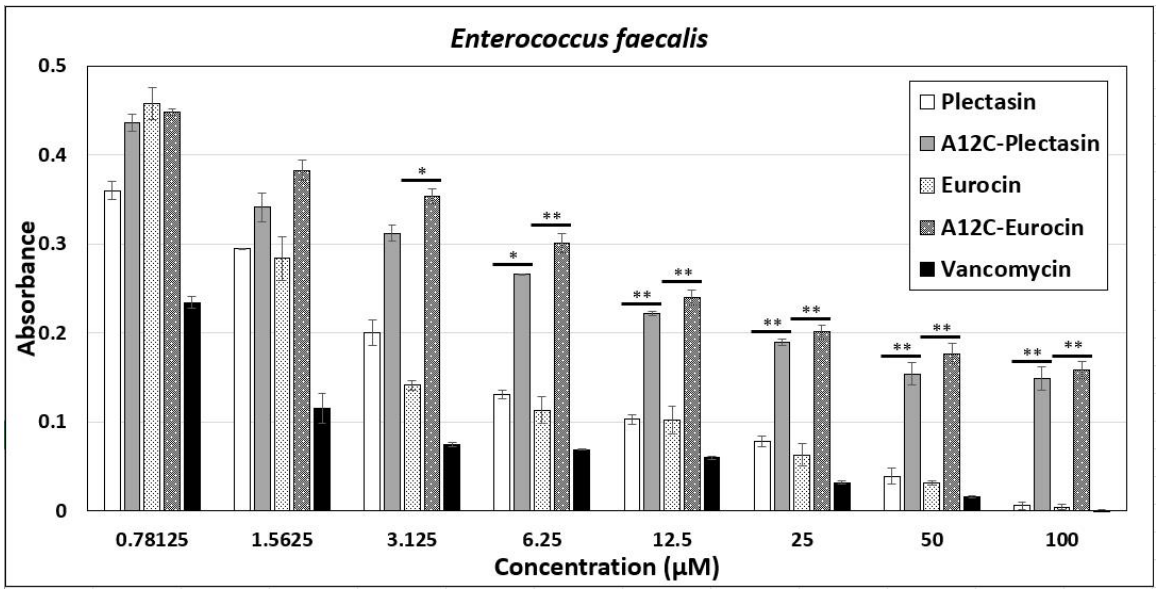
(a)


Staphylococcus aureus
2.5
2
1.5
1
0.5
0
Absorbance
Plectasin
A12C-Plectasin
Eurocin
A12C-Eurocin
Vancomycin
0.78125
1.5625
3.125
6.25
12.5
25
50
100
Concentration (μM)

(b)



(c)



(d)

Figure 5: Biofilm inhibition activity. Evaluated by plotting the absorbance of crystal violet (540 nm) against the concentration of 4 AMPs on the 4 bacteria - *S. epidermidis* **(a)**, *S. aureus* **(b)**, *B. subtilis* **(c)** and *E. faecalis* **(d)**. (* = $p<0.1$, ** = $p<0.05$, $n>=3$).

Discussion

With the rise of antibiotic-resistant bacterial infections, the discovery of new antimicrobial agents has become essential. The AMP discovery and development cycle is greatly aided by the vast resource of natural AMPs produced by a wide range of taxa. This resource is now accessible at the genome level due to the advancement of sequencing technology and predictive algorithms (Islam *et al.*, 2015; Islam *et al.*, 2017; Xiao *et al.*, 2013). This allows for data mining and the collection of large libraries of presumably well-adapted and functional native AMPs. Our lab has been a leader in developing these algorithms for the discovery of AMPs and other peptides.

In the present study, we address the targeting of AMPs, which can be easily done at the genetic level by the addition of targeting sequences. Tremendous health advantages would come with any targeted antimicrobial drug since only the specific targeted pathogen would be eliminated, leaving the rest of the microbiota undisturbed. Disturbing the microbiota can lead to the rise of opportunistic pathogens and decreased health outcomes generally.

Despite these obvious benefits, only a select number of studies have reported the development of targeted antimicrobial peptides. Specifically, these AMPs were targeted against *Streptococcus mutans* (Eckert *et al.*, 2006), *Enterococcus faecalis* (Qui *et al.*, 2005), *Staphylococcus aureus* (Mao *et al.*, 2013) and plant-pathogenic fungi of the genus *Fusarium* (Peschen *et al.*,2004). The targeting moieties were either derived from scFvs or quorum sensing peptides. However, an AMP fused to a targeting domain derived from phage display technique has, to our knowledge, not been reported. The phage peptide display approach opens a wide array of random sequences that can be explored to find out

novel targeting domains starting with millions of possible peptides to a few candidates within four to five cycles of "biopanning" (McGuire *et al.*, 2009). This technique has been employed in many drug lead discoveries, cancer immunotherapies, imaging molecules for diverse target molecules and even to screen for novel antimicrobial peptides (Rami *et al.*, 2017). For the last purpose though, there is already a pipeline of numerous experimentally proven candidate peptides occurring naturally as stated previously. Also, the commercially available phage display libraries contain peptides usually ranging between 4 to 15 amino acids in length. This is length is shorter for conventional AMPs but is ideal for targeting domains which can be fused to said AMPs, thus targeting their activity towards the bacterial species the phages were screened against. And since the screening technique mimics the innate ability of bacteriophages to exclusively find and bind their target cells through the phage coat peptide interactions, the targeting domains obtained through phage display should be comparable if not superior to existing targeting moieties (Huang et al., 2012).

Choice and curation of peptide library will lead to less randomness in the final products and also finetune the screening process. Libraries populated with BLIP, C7C cyclic peptides, scFvs, Fabs have been previously explored for developing anti-infective agents for a variety of pathogens (Huang et al., 2012). For finding suitable targeting domains of selected pathogens we can look towards the bacteriophages itself. Most pathogenic bacteria are vulnerable to a specific phage with many variants, as the phage and host bacterium evolve around each other (Curtis *et al.*, 2002). Several virus species infect a single bacteria, often more than 10 for many species (Grose and Casjens, 2014; Blasche *et al.*, 2013). Thus, we have a vast reserve of phage-based targeting domain for any desired

pathogen if we explore the genomes of those phages for coat-peptide sequences (Gao *et al*., 2017; Amgarten *et al.,* 2018) to create the library for screening. Even for lesser-studied bacterial pathogens that as yet have not been surveyed for viruses, it is now possible for a genomic search to quickly discover dormant prophage sequences (Krupovic *et al.,* 2011.) with annotated conserved domains that the phages use to differentiate and bind with their hosts. Thus, phages constitute an abundant and widely applicable source of targeting peptide libraries (Elbreki *et al.,* 2014; Matsuzaki *et al.,* 2005; Viertel *et al.,* 2014) that can be screened by phage display to direct AMPs against specific bacterial pathogens, and, as well, a bank of variants that can be used to as a source of alternate targeting peptides that can be used in the "stacked gene" format to prevent the development of resistance against the targeted antimicrobial peptides (Rivero *et al.,* 2012).

To demonstrate the straightforward nature of using targeting sequences derived from phage display to target AMPs to specific bacterial pathogens, we selected an important pathogen with know antibiotic resistance, *S. aureus*, and quickly noted a previously published host-binding 12 mer sequence with affinity towards *Staphylococcus*-A12C (Yacoby *et al.,* 2006). There is a large literature base describing established bacteriophage host-binding sequences, including several databases, so building a phage library and discovering targeting sequences against pathogenic bacteria is less daunting then one might think. For less well-studied bacteria, the process of finding viruses as been greatly accelerated by next generation sequencing, with many examples of the determination of viral or proviral sequences from clinical samples of bacteria.

Once we had chosen the A12C sequence, we fused it to the well-established broad spectrum AMPs plectasin (Mygind *et al.,* 2005) and eurocin (Oeemig *et al.,* 2012) and

tested for differential antimicrobial activity. As controls, we used bacteria from the same order (*Bacillus subtilis, Bacillales*) or phylum (*Enterococcus faecalis, Firmicutes*) as the targeted bacteria, using two species of *Staphylococcus* to test the breadth of the antimicrobial activity. Though both plectasin and eurocin in native form were highly potent against the nontargeted *Enterococcus faecalis* and *Bacillus subtilis*, the A12C version of these AMPs showed little to no activity against these nontarget bacteria. In contrast, both non-targeting and targeting AMPs exhibited strong potency against the targeted staphylococci (see Figure 3 and Supplementary Table S1). Thus, the phage-derived targeting protected the nontarget bacteria but did not affect the potency of the AMP against the target bacteria. This differential action was echoed in the cell kinetics assay and the biofilm inhibition assay. Hence, we can assume that the actions conferred to the AMPs by the fusion peptide A12C acts similarly with both planktonic form of the bacteria and static biofilms formed by them.

It is challenging to express high quantities of soluble, correctly folded and biologically active AMPs in *E. coli* (Ingham *et al.*, 2007). Nevertheless, we were able to harvest AMPs at relatively high concentrations (see supplementary TableS1) using the SUMO fusion partner. We used the SUMO expression system and obtained a high concentration of the target proteins which also displayed the expected activity following the protease cleavage and separation from their SUMO fusion partner. An equal concentration of SUMO alone lacked toxicity, demonstrating that the toxicity was the property of the AMP and not the fusion partner. As well, free A12C peptide used as a control showed no inhibitory action towards any bacteria we tested.

Continued investigation of targeting moieties for targeted AMPs is necessary to keep pace with the constantly increasing number of antibiotic-resistant bacterial infections. The vast natural resources of evolutionarily-tested natural AMP sequences now easily accessible from any reference genome, including the human genome, via advanced predictive algorithms makes AMPs a favorable choice for antibiotic substitutes. We have demonstrated how such sequences can be genetically modified to create targeted AMPs using targeting sequences procured from bacteriophage display technique, which are accessible and customizable for any pathogenic bacterium.

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CHAPTER FOUR

Separation Anxiety: Retained Fusion of Small Elastin-like Polypeptide to Cysteine-stable Antimicrobial Peptides Reveals Novel Antibacterial Application

Abstract

Failing antibiotics and a consistent decrease in novel drug discovery has lead to the investment into structurally stable peptide therapeutics as a viable alternative. Small naturally occurring antimicrobial peptides (AMPs) offer an alternative that may address the rise of resistant strains in hospital settings, though they only occur in nature at low concentrations and purification from source organisms are not a commercial option. Theses peptides can be produced recombinantly at commercially viable concentrations with cost-effective aggregation and purification tags like the non-antimicrobial elastin-like polypeptide (ELP) consisting of the VPGVP pentamer subunit repeats. ELP acts as a clean purification tag utilizing temperature sensitive inverse phase transitioning which forgoes costs in chromatography during extraction. Previous studies have shown AMPs produced with ELP provides relatively high yield, but never has a study thoroughly tested antimicrobial efficacy pre-cleavage. In this study we observe that the smaller size (<30 subunit repeats) ELP fusion partner determines that the antimicrobial activity of an ELP/AMP fusion is functional before cleavage. This strategy of AMP expression and purification provides a clean alternative to peptides more effective against antibiotic resistant pathogens and innovates on how recombinant therapeutic protein expression can be handled post-production.

Introduction

Antimicrobial peptides (AMPs) are host defense peptides which possess broad-spectrum activity due to their generalized targeting of microbial membranes (Mansour *et al.*, 2014;), resulting in an exceptionally low rate of development of microbial resistance to AMPs, in contrast to traditional small-molecule antibiotics. Since the discovery of the first defensin in 1981 (Patterson-Delafield *et al.*, 1981), the AMPs discovered have typically been small cationic amphipathic peptides and have been found to be part of the defense repertoire of vertebrates, insects, plants and bacteria. Anionic AMPs have also been discovered and are especially effective in their roles as immune effectors (Harris *et al.*, 2009). Structurally, AMPs include alpha helical peptides and also cystine-stabilized peptides, with beta sheets given a resilient structure due to disulfide bonding (Yount and Yeaman, 2004). The most common form of the cystine-stabilized AMPs is the sequential tri-disulfide bond peptides (STPs) (Islam *et al.*, 2015), which includes the knottins and cyclotides. This class is richly populated by AMPs, but also venoms from taxa ranging from corals to snakes and spiders. STPs are strongly resistant to high temperatures and pH change, ideal properties for venoms and AMPs that must be produced and stored ahead of time and then must be stable external to the source organism. These properties also make STPs ideal for medical applications involving harsh environments like the gut.

Although some AMPs have reached clinical trials (Mahlapuu *et al.*, 2016), the main commercial issues center on delivery, yield and production expense (Kumar *et al.*, 2018). In the present work, we focus on the latter two issues. Expression of some AMPs is commercially viable by extraction from the source organism, such as nisin, a common antimicrobial peptide added in the production of cheese and other food products (Hansen

1994). Another production route is the chemical synthesis of AMPs, but this is commercially practical only for smaller, helically-structured AMPs (Eckert *et al.,* 2006). The larger, resilient STPs are more economically produced in a heterologous expression system such as E. coli (Li *et al.,* 2010) or yeasts (Cipakova and Hostinova, 2005). In such systems, a carrier protein is always fused to the AMP. Yields have varied, with the best yields being achieved recently using the SUMO carrier protein (Li *et al.,* 2009). However, there remains the issue of production expense, primarily due to the cost of protein concentration and purification, as is the case with most heterologous expression systems (Parachin *et al.,* 2012).

To address the barrier of production expense of STP-AMPs, we have utilized elastin-like polypeptide (ELP) as a carrier peptide for AMP production. The ELP subunit is derived from mammalian elastin peptide (VPVPG), and, in a pentamer ranging from 100s to 10s of subunit repeats, has been shown to serve as an expression chaperone for AMPs (Yang *et al*., 2012; Hu *et al.,* 2010) via its solubility-enhancing and aggregation-promoting properties (Li 2011). ELP carrier protein utilizes an inverse transition cycling protocol which greatly reduces the cost of protein concentration and initial purification (Floss *et al.,* 2009). In the presence of salt, ELP becomes insoluble above a Tm (usually above 30°C) in solution and can be isolated from host proteins by centrifugation. This solubility change is reversible, as the ELP can once again become soluble when brought below 4ºC. Thus, repeated cycles of temperature shifts and centrifugation can cumulatively increase purity. At this point, the protein would be concentrated and relatively pure, reducing the scale and expense of the final affinity column step or would be available as is for an application requiring only partial purification. One drawback

with this approach is that AMPs have never been shown to retain their antimicrobial potency while fused to a carrier protein; in fact, one study has made it a point to report in its results that the fused AMP/ELP protein has no antimicrobial efficacy pre-cleavage (Sousa *et al.*, 2016). An additional step of proteolysis for antimicrobial is needed, requiring an expensive protease and the purification of the AMP free from the carrier protein and the protease protein (Shamij *et al.*, 2007; Hassouneh *et al.*, 2012).

In this study, we demonstrate the production of an AMP fused to the C-terminus of a shortened 28x ELP carrier pentamer repeat at high yield. We also demonstrate that this AMP is strongly antimicrobial while fused to the ELP carrier protein, and we investigate whether this property is conserved using a larger ELP fusion partner (60 subunit repeats), as well as increasing the molecular distance between the AMP and ELP to observe any steric hinderance using rigid linkers. The AMP selected for expression was *Popu*Def (Wei *et al.*, 2015), an anionic AMP never before expressed in a heterologous system. The AMP Human Beta Defensin 2 was also expressed in the same vectors as a control peptide (Xu *et al.*, 2006). This system illustrates the possibility of producing AMPs at high yield and at low production cost using the *E. coli* heterologous expression system.

Materials and Methods

Reagents

Human erythrocytes obtained from Baylor University, donated by Ankan Choudhury. TEV protease was recombinantly produced in house using BL21 DE3 bacterial expression. Factor Xa protease and Enterokinase protease were purchased from

New England Biolabs. All restriction enzymes for cloning and ligation purchased form New England Biolabs. ELP x28 sequence cloned from the plant binary vector pCaMterX supplied from the Menassa lab (University of Western Ontario). ELP x60 sequence cloned from purchased Addgene vector pMAL-c5X-ELP[V5A2G3-60].

Vector Construction

The *Popu*Def coding sequence was incorporated into two expression vectors under the T7 promoter. Both the TEV protease and enterokinase protease cleavage sites were placed in tandem next to the ELP x28 pentamer sequence (Figure 1). The *Popu*Def gene was cloned onto either the N-terminus or C-terminus of the ELP ORF while maintaining the TEV and IEGR protease cleavage sites between them (Figure 1). Empty ELPx28 and ELPx60 plasmids were used as a control. Positive colony selected by the NptII kanamycin resistance gene present in both vectors.

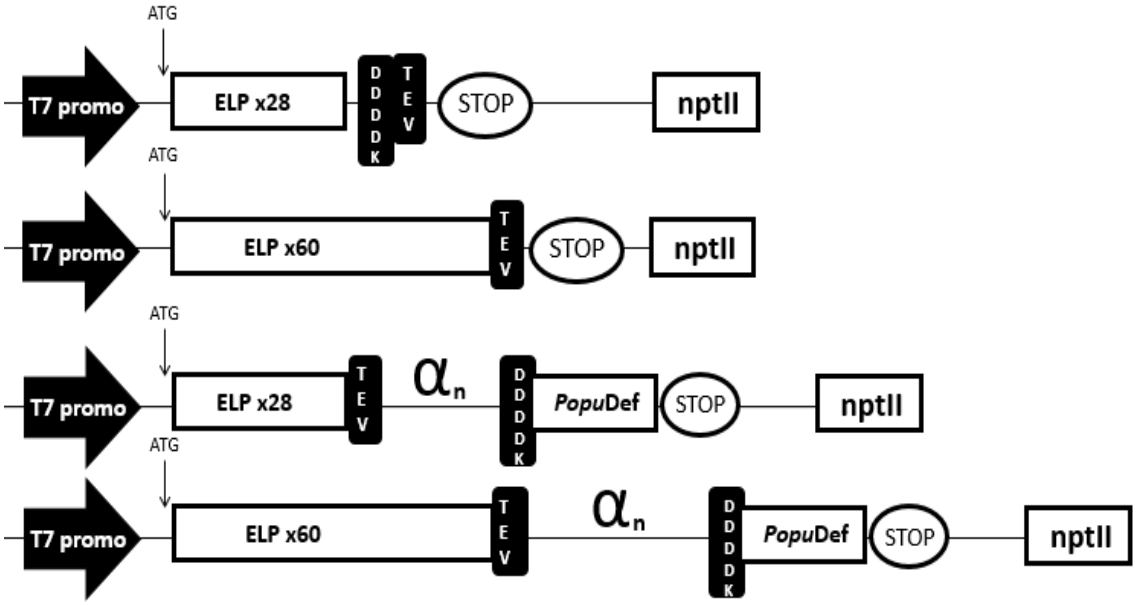

Figure 1 The STP AMP coding sequence expressed in two separate ELP vectors varying ELP fusion partner size. T7, promoter; ATG, start codon; DDDDK, enterokinase cut site; TEV, tobacco etch virus protease recognition site (ENLYFQ); nptII, kanamycin resistance gene; *α*n, *n* (1-3) amount of rigid alpha linker between the AMP coding sequence and its ELP fusion partner.

Expression of ELP/AMP Fusions

Once newly constructed plasmids were successfully transformed into 10β *E.* coli and plasmid was collected, constructs were transformed into BL21 *E. coli* for protein expression. Colonies were shaken overnight in 20 mL of 2X YT media with kanamycin (50 µg/mL). Then, 20 mL of culture was added to 500mL of 2X YT media with kanamycin (50 µg/mL) and shaken at 220 rpm at 37^{o}C untill the OD600 reached 0.7-1.0 (approximately 4 hours). Expression of protein was then induced by 0.1 mM isopropyl-thio-galactoside (IPTG) and shaken overnight at 14^{o}C. Cells were then centrifuged at 8000 rcf for 1 hour at 4^{o}C and resuspended in 20 mL ice cold 1xPBS, lysed with 0.1 mg/ml lysozyme, sonicated on ice at 60% amplitude for 30 seconds then rested for 30 seconds, and finally centrifuged for 45 minutes at 50,000 rcf at 4^{o}C. Expressed protein was present in collected supernatant.

ELP ITC Purification

Collected supernatant was warmed to 37°C and NaCl was added at a concentration of 3 M in solution and incubated for at least 15 minutes. Solution was then centrifuged at 20,000 rcf at 37°C for 10 minutes in 3 mL aliquots, then pellet resuspended in 1 mL ice cold 1x PBS, split into 1 mL aliquots and again centrifuged at 8000 rcf for 10 minutes at 4°C, concluding one cycle of phase transition. Process repeated 2-3 times for pure protein extraction (MacEwan *et al.*, 2014). Proteins were assessed and identified by SDS PAGE assay.

Antimicrobial Assay

Cleaved and uncleaved protein was screened for antibacterial efficacy against the gram positive *Staphylococcus epidermidis*. Minimum inhibitory concentration (MIC) was determined by serial dilution across a microtiter plate against bacteria grown in LB media. 50µl of AMP at 4mg/mL was aliquoted into the first well then diluted across the row, along with 50µl test bacterium at OD600 0.125. Inhibition of bacterial growth was determined by resazurin dye application. MIC was determined by the furthest well exhibiting dye change and dot formation of surviving bacteria.

Hemolytic Assay

All peptides including AMP/ELP, AMP/ELP separated and cleaved, and individual ELP were exposed to human erythrocytes for measuring hemolytic activity as previously described (Brovedani *et al.*, 2016). Blood cells were received pre-washed and centrifuged at a low speed of 500 g for 5 minutes, washed with 150 mM NaCl then washed with 10 mM PBS at pH 7.4. Once centrifuged one last time, pellet was

resusended in PBS followed by a 1:50 dilution yielding a $5x10^8$ RBCs/ml concentration. Application of test proteins were added in serial dilution of 1:2 to a 96 well plate microtiter plate, with 1% (v/v) triton-x serving as a positive hemolytic control and 1xPBS as a negative control. After 2 hour incubation in 37degreee, absorbance was taken of the supernatant to measure percent hemolysis at 540 nm absorbance using the following equation: A541 of the peptide-treated sample - A540 of buffer-treated sample)/(A540 of Triton X-100-treated sample - A541 of buffer-treated sample) x 100%.

Results

Creating Expression Vectors

The gene for *Popu*Def along with the TEV and enterokinase protease cleavage sites were subcloned into modified pMAL-c5X vectors (Figure 1). Initial testing discovered ELP sequence must be attached to the N-terminus of the AMP coding sequence for proper protein expression. Rigid α linkers (EAAAK) attachment to separate AMP from fusion partner were cloned in using specific primers. All genes were verified by PCR and cloned into BL21 *E. coli*.

Expression Levels of ELP/AMP Fusion Not Affected By ELP Size

Yield for ELP28-*Popu*Def and ELP60-*Popu*Def proteins (at all three α linker lengths) were moderate (around 250 µg/mL: 0.25 $mgL^{-}1$), with the fusion protein weighing in at approximately 21 kDa when quantified (Figure 2). Once cleaved, *Popu*Def can be detected around 4-5 kDa, and ELP at 11 kDa. SDS PAGE confirms yield for ELP/*Popu*Def fusion was highest when the AMP was bound to the C-terminus of ELP. This study marks the first instance of recombinant production for the *Popu*Def AMP,

with yield markedly higher than what is typically collected from skin secretion (Wei *et al.*, 2015).

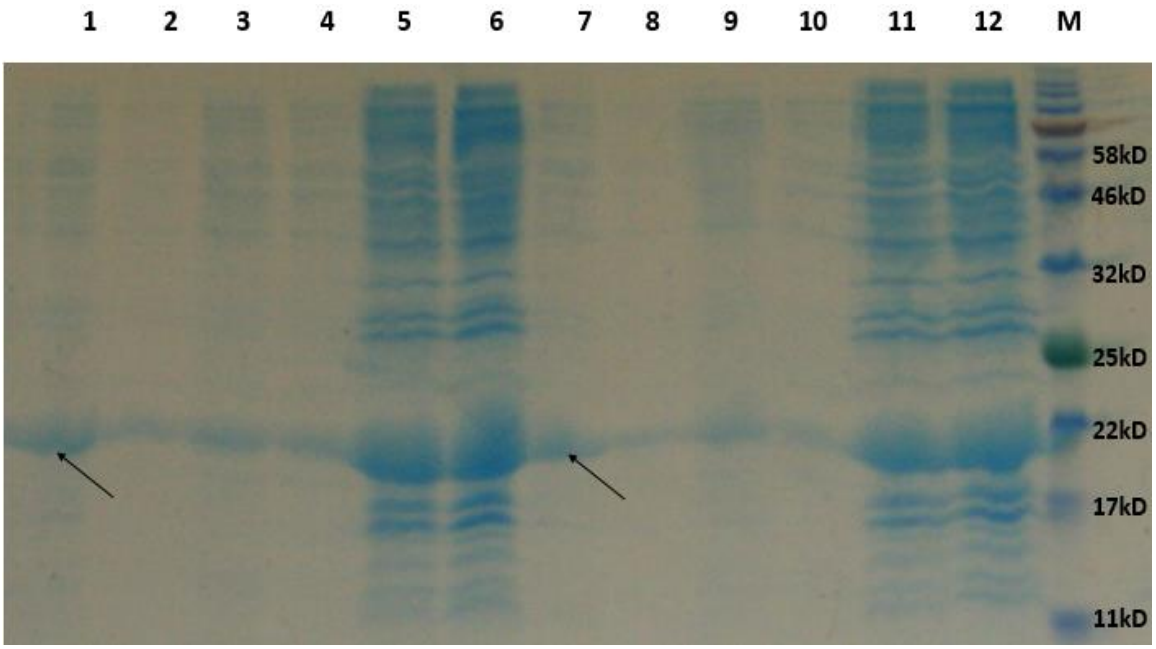

Figure 2 SDS-PAGE/Coomassie blue analysis of ELP-αLinker-AMP fusions optimized for *E. coli* expression and purification. Protocol was optimized to with initial volume reduced ½ in 3 mL aliquots (Lanes 1 and 7). Lane 1, *Popu*Def α2 ½ volume; Lane 2, *Popu*Def α2 full volume; Lane 3, *Popu*Def α2 ½ volume no salt added; Lane 4 *Popu*Def α2 full volume no salt added; Lane 5, *Popu*Def α2 unpurified no salt added; Lane 6 *Popu*Def α2 unpurified; Lane 7, *Popu*Def α3 ½ volume; Lane 8, *Popu*Def α3 full volume; Lane 9, *Popu*Def α3 ½ volume no salt added; Lane 10, *Popu*Def α3 full volume no salt added; Lane 11, *Popu*Def α3 unpurified no salt added; Lane 12 *Popu*Def α3 unpurified; M, molecular ladder.

ELP/AMP Fusions Remain Highly Cytotoxic While Exhibiting No Hemolytic Effect

When conducting MIC testing, both cleaved and still fused ELP/*Popu*Def and ELP/HbD2 fusion peptides were assessed to determine whether separation was necessary or even detrimental to antimicrobial competence. In the case of the ELP28-*Popu*Def peptide, maintained fusion exhibited low MIC concentration (0.75μM) in spite of linker length, while loss of the ELP fusion partner directly led to a loss of antibacterial activity. The ELP60-*Popu*Def however showed no antimicrobial activity. ELPx28/AMP exhibited

more antimicrobial potency against *S. epidermidis* than previously reported from pure *Popu*Def (Wei *et al*., 2015), with all active ELP/AMP fusions showing MICs less than 1 μM, while the ELP fusion partners by themselves showing almost none.

Discussion

With a cost effective and clean method of producing stable antimicrobial peptides in mass quantity, recombinant AMPs are seen as feasible alternatives to rapidly failing antibiotics. AMP purification from host organisms are impractical for commercialization, and chemical synthesis for more complex AMPs that require post-translational modification are impractical (Münzker *et al.*, 2017). AMP recombinant expression in *E.*coli faces hurdles in the AMP payload affecting the host microbe and instability of the AMP itself. We've addressed these issues with the use of a cysteine-stabilized antimicrobial peptide fusion to ELP. For efficient yield and ease of protein purification, as well as aggregating and protecting the host cell from antibacterial, recombinant production of a cysteine-stable AMP in conjunction with the ELP fusion partner is optimal. Testing with the two most common ELP configurations, 28 and 60 subuint repeats, helped to elucidate why our retained activity pre-cleavage of the smaller ELP tag was unique amongst other AMP-ELP studies. The loss of activity with the more commonly used 60 repeat ELP using the same AMPs confirmed what previous work had reported.

Orientation of the protein of interest in relation to the ELP has been contested in previous literature (Christensen *et al.* 2009), it would appear that in our investigation there was a skew towards the AMP C-terminus fusion for a cystine-stabilized peptide. This is in direct opposition of some previous studies (Sousa *et al.*,2016) that stated this

manner of fusion would increase misfolding amidst translation in *E.coli* (Christensen *et al.,* 2009).

Historically, few studies have demonstrated successful co-expression of an AMP with the cationic ELP carrier protein, and those AMPs are typically cationic themselves and not structurally stable in volatile pH and temperature (Yang *et al.,* 2012; Hu *et al.,*2010). Investigation into cystine-stabilized AMPs as sturdy antimicrobial agents is a valid avenue that's gained traction recently (Mishu *et al.* 2015). The anionic STP-AMP *Popu*Def was chosen as the fusion partner to observe if the recently characterized peptide can be recombinantly produced, and if antimicrobial efficacy can be enhanced with a stabilizing fusion partner. Traditionally, recombinant AMP expression in *E. coli* concludes with the separation of the AMP from its fusion partner whether it be SUMO, Thr or even ELP (Li *et al.,* 2010). Previous work have explicitly reported activity was appeared to be inhibited while still in a fused state (Sousa *et al.,* 2016).

This manuscript marks the first reported instance of antibacterial efficacy maintained before carrier partner separation. Furthermore, our work shows that ELP removal from the AMP package may not be necessary for medical application, further decreasing the cost of downstream purification. This may prove to be of interest as use in topical treatments, such as foot ulcers (Lipsky *et al.,* 2014).

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CHAPTER FIVE

Conclusion

The feasibility of discovering new antibiotics for augmenting or perhaps even replacing current therapies may hinge not on the chemical modification of current antibiotics, but in optimizing natural antimicrobial agents for industrial grade processing production and molecular targeting. Both plants and *E. coli* can serve as bioreactors for commercial application, but low protein yield and non-specific antimicrobial activity have been identified as limiting factors. The ELP fusion tag serves not just as a low cost purification tag, but also as a non-reactive accumulation tag that fosters proper folding, post-translational modification and increased recombinant protein yield. Our studies not only displayed successful ELP/AMP expression for the first time in plants, but also elucidated that the net charge of a cysteine-stable AMP dictates whether the recombinant protein is detectable post plant tissue harvest and processing. This phenomenon was observed in spite of the ELP fusion partner forming sequestered protein bodies around the ER (Conley *et al.*, 2009). A likely explanation for this may be that a strong electrostatic interaction between the negatively charged plant membrane and the positively charge antimicrobial peptide is triggered by the accumulation of these recombinant proteins, and the plant itself responds by necrotizing tissue where this accumulation takes place, similar to what was seen in the expression of BP100 in plants (Nadal *et. al.*, 2012).

The discovery high overexpression of biologically active anionic STP AMPs is optimal for commercialization in seed expression for use as a livestock feed additive (Bundó *et al.,* 2014). Seed expression has been seen as an attractive platform due to long term storage properties and stability (Sabalza *et al.,* 2013). Previous work with the saliva derived AMP lactoferrin has already been applied in rice bran for piglet feed, showing a positive effect transgenic rice expressing AMP have on piglet growth and immunity (Lee *et al.,* 2010). This proof of concept in animal feed, along with our successful expression of PB-forming anionic antimicrobial peptides in plant tissue could be applied to poultry in targeting the avian influenza, and previous recombinant AMP expression studies with lactoferrin has already been made progress in this direction with the use of broad spectrum activity (Tanhaiean *et. al.,* 2018). Chicken feed could be augmented in a similar fashion as the piglet rice bran to target specific strains of avian flu based on peptide targeting hemagglutinin of influenza A H5N1.

We addressed the specificity issue of broad spectrum AMPs with the fusion of a 12 amino acid peptide derived from phage display previously confirmed as specific towards *S. aureus*. Placed at the N-terminus of our STP AMP, it was found there was no enhancement of activity against target *Staphyloccoci*, contrary to previous pheromone targeted studies (Eckert *et al.,* 2006), but a complete loss of antimicrobial activity against non-target genus *Enteroccus* and *Escheria*. These results provide proof of concept for exciting topical applications of a highly selective antimicrobial, specifically in catheters. Bacteria may infect the bladder by forming biofilms along the inside and outside of the catheters, with *Pseudomonas aeruginosa* being the most common opportunistic pathogen (Feneley *et al.,* 2015). Catheter-associated urinary tract infections (CAUTIs) is generally

facilitated by well characterized extracellular polysaccharides to form biofilms *in vivo* (Cole *et al.,* 2014). Interestingly, our AMPs mode of actions have been shown to specifically reduce biofilm formation in target *Staphylococcus* (Islam *et al.,* IN PRESS). Targeting our AMPs to *P. aeruginosa* by either utilizing a phage display library to bind these extracellular polysaccharides or generating quorum quenching antibodies specific to the bacteria (Kaufmann *et al.,* 2011) to discover specific targeting domains offers a modular and effective therapeutic model to answer CAUTIs.

An unexpected result of these recombinant ELP/AMP proteins produced in plants was the retention of antibacterial activity pre-protease cleavage followed by a complete loss of activity post-cleavage when separating the fusion partner from the AMP. This observation not found in the SUMO/AMP expression paradigm. This phenomenon has since been explored in *E. coli* with both anionic and cationic STP AMPs. Preliminary results confirmed that the same ELP/STP AMP motif is biologically active only when still fused. Literature has expressly mentioned no activity in the fused state of an AMP when bound to the ELPx60 fusion partner containing 60 repeats of the VGVPG pentamer motif (Sousa *et al.,* 2016). Our studies had until recently used the ELPx28 repeat fusion partner typically used in plant expression (Saberianfar *et al.,* 2015), and when compared to the 60 ELP repeat used in *E. coli* we found a difference biological activity. Once established, a topically applicable ELP/AMP peptide therapeutic can be developed for mass production while circumventing almost all post-production purification costs.

This dissertation has described a workflow of experimental design informed by computational assessment and confirmed in wet-lab confirmation and discovery in the pursuit of targeted therapeutic alternatives to failing antibiotics currently in use. Anionic

AMPs may now be seen as a viable option in treating the most resilient of infectious diseases, and applying a targeting moiety may keep these treatments flexible enough to adapt to the ever-evolving diseases they treat.

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