Anti-Microbial Peptide Screen for Crop Protection

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Screening of Antimicrobial Peptides for Crop Protection

Sydney M Chen

Abstract

Vestaron currently uses an established and reliable peptide discovery and development research platform, particularly for the development of insecticidal peptides. The platform centers around a proprietary yeast expression system for peptide production (“Vestaron’s yeast expression system”). Vestaron aims to expand their portfolio and provide clean solutions in crop protection using peptide technologies to protect plants not only from insect pests but also microbial pests including phytopathogenic fungi and bacteria. Antimicrobial peptides (AMPs) have been studied extensively. However, it is questionable whether Vestaron’s yeast expression system can express AMPs heterologously. In this study, thirteen promising antimicrobial peptides were selected from literature reports to test the ability of Vestaron’s yeast expression system to heterologously express AMPs which maintained their antimicrobial activities. Five out of the thirteen AMPs were successfully expressed in the Vestaron yeast expression system. They all showed antibacterial activities against some agriculturally important phytopathogenic bacterial species. From this research, it has been confirmed that the Vestaron yeast expression system is usable for AMP heterologous expression. A few lead candidates for possible commercialization for plant health solutions have been identified from this program.

Introduction

Economically, safeguarding crops is essential to maintain yield, marketability, and quality products. Bacterial diseases cause devastating damage and significant economic loss. Collectively they cause over $1 billion worldwide food production chain losses every year (Mansfield et al., 2012; Kannan et al., 2015; Martins et al., 2018). California wine producers alone face losses of $104 million annually to *Xylella fastidiosa*, a bacterium that infects grapes and was targeted in this study. Crop protection solutions to bacterial pathogens are essential to maintain crop yields in the face of increasing demand while also helping to boost the economy.

While both gram-positive and gram-negative bacteria infect crop plans, gram-negative bacteria are harder to target and kill due to their cell wall composition. They are surrounded by a single layer of peptidoglycan which is protected by an outer lipid bilayer. The outer membrane protects the bacteria’s cell wall because the membrane is lipophilic. This makes it difficult for antibacterial agents to penetrate through the lipid bilayer and attack the peptidoglycan, which maintains the integrity of the cell. Furthermore, the current chemical antibiotic solutions that are available to treat some of these gram-negative plant pathogenic bacteria are losing efficacy.
Erwinia amylovora and Xanthomonas campestris are often treated with streptomycin, however, streptomycin resistant genes have been found in both strains (Sundin et al., 2018). Beyond antimicrobial peptides being an alternative for the reasons previously mentioned, they also offer a possible solution to the emerging antibiotic resistance. AMPs are part of the innate immune system of many organisms (Brogden et al., 2005). Due to their complex modes of action, resistance development to an AMP is more difficult than resistance development to chemical antimicrobials. Due to their economic relevance and difficulty to control, four gram-negative phytopathogenic bacteria were assessed in this study. These include *Escherichia coli*, *Erwinia amylovora*, *Xanthomonas campestris*, and *Xylella fastidiosa*.

Beyond the novel use of Vestaron’s yeast expression system to express AMPs, the platform posed another potential challenge to overcome. Plasmid DNA preparation proceeds using *Escherichia coli* competent cells to prepare the genetic material of the AMPs for yeast transformation. Due to the antibacterial nature of the selected AMPs, it was questionable if DNA preparation though *E. coli* was even possible as the AMPs can kill the bacterial vehicle result in a failed DNA preparation. Furthermore, due to the antimicrobial nature of the peptides, it was questionable if any yeast expression would be possible. With AMP production being reliant on yeast expression, this study tested the feasibility of using the Vestaron’ discovery platform for the production and development of AMPs.

This thesis project aimed to answer three main questions to achieve the goals of proof of concept, antibacterial activity, and leading candidate identification. Firstly, can the Vestaron yeast expression system be used to express AMPs? For the reasons mentioned above there was speculation that preparing, and expression of AMPs may not be possible. Secondly, after expression and purification do the peptides maintain their antibacterial activities? Post translational and expression modifications are common in various yeast expression systems. Sometimes, parts of the peptide will cleave and if these cleavages are in a pharmacophore region, then the peptide will not maintain activity. Other times, the peptide may fold in an unexpected way rendering the species inactive. Additionally, heterologous expression systems vary in the type and extent of glycosylation patterns on expressed proteins. There are many reasons why AMPs may express but may not maintain their desired activity. Finally, at the end of this project, will a leading candidate emerge for potential commercialization for crop protection? The process of the project needed to whittle down candidates. This process also needed to be robust enough to ensure that the lead candidate was the best expressor and had the best activity of the peptides that were screened. These three goals were successfully achieved throughout the project. Five of the original thirteen peptides were successfully expressed and when analyzed in in-vitro antibacterial bioassay, they were active. Of these five peptides, a lead candidate has been identified and further research is being performed in the hopes of future commercialization.
Methods

Figure 1: Schematic overview of the methods used to screen peptides from generation of expression vectors to in-vitro antibacterial bioassays. The methods used will be further described in the remainder of the methods section. Figure created with BioRender.com.

Generation of Expression Vectors

Thirteen AMPs with literature reported antibacterial activity were selected as candidates for heterologous expression in the Vestaron yeast expression system. The linear DNA sequences encoding the AMPs were synthesized after codon optimization for yeast expression, which were assembled into the expression vector through Gibson Assembly (GA). The GA reaction inserted the linear AMP DNAs into a backbone using Gibson Master Mix to facilitate the reaction. This mix included exonuclease which chewed back the 5’ end of the insert and backbone so they could overlap and anneal together. It also contained DNA polymerase which closed the gaps between the insert and backbone as well as DNA ligase which sealed any nicks where the insert and backbone were joined. After incubation in a Thermocycler at 50°C for one hour, the GA reaction was completed, and the AMP had successfully been inserted into the recipient plasmid. The assembled final plasmids containing the AMP expression cassette were transformed into *Escherichia coli* for DNA amplification and preparation.

DNA Preparation and Bacterial Transformation

Bacteria are used to amplify and prepare the plasmid that was assembled via GA. The bacterial workhorse used for this process was DH5α *E. coli* (DH5α, New England Biolabs). The assembled plasmid and competent DH5α cells were heat shocked to transform the plasmids into the cells. The cells were then chilled until they were recovered, and the entire reaction was plated on agar media that contained an antibiotic for selection and incubated at 37°C overnight. Colonies only formed from cells that had contained the desired plasmid during transformation because
the transformed expression vector includes an antibiotic resistance gene specific to the antibiotic used in the agar media. In addition to the bacterial transformations, a negative control of untransformed bacterial cells was plated. One or two colonies grew on the negative control plate, this growth was insignificant compared to the bacterial transformants which had 50-100 colonies indicating a successful Gibson Assembly of assembled plasmids. From the transformation plates, three colonies were picked and used to inoculate seed cultures of liquid media with antibiotics and incubated at 37°C overnight. The DNA from the seed cultures was then prepared using a Qiagen QIAprep Spin Miniprep Kit (Qiagen, Mississauga, Ontario, Canada). After performing the protocol provided by the kit, DNA was isolated in a pure and concentrated form. The DNA was quantified using an A280 program on a spectrophotometer to determine the concentration of double stranded DNA obtained. To confirm the appropriate DNA had been amplified by the bacteria, samples were sent to Eurofins for sequencing.

Yeast Expression Vector and Transformation

Once the appropriate sequence had been confirmed, the bacterial portion of the plasmid was removed using enzyme restriction digest. The plasmids contained two SacII sites which separated the bacterial DNA from the yeast integration fragment containing the DNA encoding for the heterologous expression of the AMPs. The SacII digested linear DNAs were transformed into competent yeast cells. This process was performed using electroporation to form pores in the yeast cell membranes so that the free-floating DNA could be taken up in the cells. After electroporating, recovering, and incubating the transformed yeast cells they were plated on yeast carbon base (YCB) agar with a selection marker. The plates were incubated at 30°C for transformant colony formation, growth did not exceed five days.

Deep-Well Plate Cultures

The transformant colonies were screened in a high throughput fashion using 48-well deep-well cultures. Eight colonies, or strains, were picked from each of the yeast transformant plates and used to inoculate individual wells containing defined medium that has been optimized for this yeast system. Each of the deep-well plates included control wells which were inoculated with untransformed yeast strains for comparison of expression. The deep-wells were incubated for six days in a shaking incubator to reach saturation. After the growth period, the deep-well cultures were broken down for further analysis.

Reverse-Phase High Performance Liquid Chromatography (rpHPLC)

The desired peptide expression was designed to be secreted out of the cell into the surrounding medium during cell culture. The spent medium was separated from the cell debris using a centrifuge. The centrifugation left a cell pellet separate from the peptide rich spent media supernatant. The spent medium was removed from the pellets and the pellets were resuspended in 20% glycerol so they could be frozen and stored for later use. The spent medium was prepared for rpHPLC by filtration into a plate compatible with a ThermoFisher UltiMate 3000 BioRS System. This HPLC was equipped with 0.1% TFA in HPLC grade water and 100% HPLC grade acetonitrile as
the mobile phases. 20 microliters of filtered spent medium was injected on a C18 column. A 5-60% acetonitrile gradient was applied to the column to resolve the components of the injected sample. Absorbance was read at 220 nanometers to detect the peptide bonds. The resulting chromatogram was used to verify and quantify peptide expressions. Each of the strain’s chromatograms were compared to an untransformed yeast control strain. Any non-endogenous peaks were then compared to the rest of the strains from the same peptide to ensure this expression was specific to the peptide. Uniform peaks were noted and the strain with the largest unique peak area was selected for collection. To collect the peaks, the same sample was injected multiple times while watching the real time chromatogram. The waste line was bypassed so that the material could be collected. This was repeated multiple times for each sample and for each of the peptides.

**New Active Ingredient Confirmation via Liquid Chromatography/Mass Spectroscopy (LC/MS)**

Once enough material had been collected off HPLC, the samples were freeze-dried using a Labconco Lyophilizer. The samples were dried until no liquid was left in the sample and only powder remained. The powder was resuspended in water and prepared for liquid chromatography/mass spectroscopy (LC/MS). Freeze-drying the peaks and then resuspending them in water offered a concentrated sample to be injected onto LC/MS. A Shimadzu LCMS-2020 was used to characterize and identify the peaks to confirm or deny the presence of a new active ingredient (AI). 15-20 microliters of the freeze-dried and resuspended peaks were injected onto the LC/MS. The machine generated mass spectrum with mass/charge (m/z) ratios could be matched with calculated m/z ratios. Identical or very similar m/z ratios indicated successful AMP expression.

**Ion Exchange Chromatography (IEX) Peptide Purification**

The ion exchange chromatography (IEX) protocol used to purify the successfully expressed peptides was optimized for the AMPs purification process. Each of the successfully expressed AMPs were cultured in large batch shake flask cultures for seven days, the spent media without cells was collected and sterile filtered. Roughly 1.0 liters of peptide rich sterile spent media was obtained from these cultures. Nuvia HR-S resin (Bio-Rad), a strong cation exchange chromatography resin that employs SO$_3^-$ was used to perform the IEX procedure. Sterile deionized (DI) water was used to equilibrate the resin. 30 millimolar (mM) sodium citrate (NaCit) at pHs of 3.3 and 4.0 were used as wash buffers before loading the spent media. The AMP rich spent media was loaded on the resin and the flowthrough was collected and stored. NaCit at both pHs was used to wash the resin. Then 1.0 and 2.0 molar (M) sodium chloride (NaCl) solutions were used to elute the peptide from the resin. Elutes were collected in 1-2 mL fractions which were analyzed using rpHPLC to determine which fractions the peptide eluted into. The AMP rich fractions were consolidated and loaded into dialysis cassettes. These cassettes were placed in UltraPure water baths and dialyzed for at least two days with four water changes. After dialysis, the excess salts had been removed from the AMPs. The AMPs were removed from the dialysis cassettes and were freeze-dried using a Labconco Lyophilizer to obtain a concentrated powder. The powder was resuspended in sterile DI water. The 1.0 L
shake flask cultures spent media yielded 500 µL of purified and concentrated AMPs. A small sample was injected onto LC/MS to confirm the AI hadn’t been lost during the purification process. The remaining material was then determined to be successfully IEX purified and ready for bioassay.

![Diagram of purification process]

**Figure 2:** Schematic overview of the purification process followed to obtain IEX purified AMPs for the successfully expressed and LC/MS confirmed AIs. This process was repeated each time purified peptide material needed to be replenished. Figure created with BioRender.com.

**In-Vitro Antibacterial Bioassay Setup**

The successfully expressed and purified AMPs were used to treat four gram-negative pathogenic bacterial strains in in-vitro antibacterial bioassays. These four bacterial strains were recovered and stored in glycerol stocks which were used to inoculate bacterial seed cultures 24-48 hours before bioassay setup. The bioassays were performed in sterile 96-well cell culture plates. When possible, each AMPs activity was tested in two different media types. Lysogeny broth (LB) medium is a full medium and Low Salt Medium (LSM) is a minimal medium. The full medium is crude and contains excess salt while the minimal medium, like its name suggests, is low in salt. This was intentionally decided to compare the activities of the AMPs in an environment with salt and with low salt. AMPs are known to have a cation inhibition effect which decreases their potency and effects against bacterial strains.

For the bioassay setups, the generic setup remained the same, the medium and type of bacteria used to inoculate the plates were the only components that changed. A multichannel pipette was used to pipette 100 µL of LB medium or LSM into each of the wells from columns #2-#12. Column #1 was left empty. A single pipette was used to pipette the appropriate amount of medium and AMP to total 200 µL for the initial dose of AI for serial dilution. The final row(s) of the bioassay plate did not receive any AI treatment. A multichannel pipette was used to serially dilute the AIs ten times using a 2x dilution by pipetting 100 µL from column #1-#11. After the final serial dilution, 100 µL was discarded from column #11. Column #12 did not receive any AI treatment. Then each well was inoculated with 5 µL of diluted bacterial seed culture. The final row(s) of the bioassay plate was not inoculated with bacteria. Therefore, each bioassay plate included at least one blank row that consisted of just LB medium or LSM, no inoculum or AMP treatment. Each plate also included an untreated control column that consisted of just LB medium or LSM inoculated with bacterium but not treated with an AMP. Finally, each bioassay plate included a positive control row that consisted of LB medium or LSM, bacterial inoculum, and treatment by AMP713, the synthesized control peptide known for its antibacterial effects. The AMPs were serially diluted ten times at a 2x dilution and cultured with four different bacterial strains. The bioassay plates were incubated at 27°C in a plate shaker. Optical density 600 (OD600)
readings were taken at 24 and 48 hours to determine bacterial cell growth and generate dose-
response curves. A SpectraMax M2 Plate Reader with multi-detection capabilities was used to
read the ODs. Igor Pro software was used to generate the dose-response curves and estimate the
half maximal inhibitory concentration (IC50) to determine the potency of each of the AMPs.

Figure 3: General template used for all the antibacterial bioassays performed. Rows F-G represents the blank rows with only
growth medium. Row A represents the positive control row using AMP713, a synthesized peptide. Column 12 represents
untreated controls of inoculum in growth medium without antibacterial peptide treatment.

Figure 4: Generic dose-response curve. The x-axis depicts the concentration in parts
per million (ppm) of AMP treatment from left, lowest concentration, to right, highest
concentration. The y-axis depicts the cell density using OD600 readings. The IC50
value is determined using the dose-response curve as depicted by the red lines on
the figure. Figure created with BioRender.com.

Gram-Negative Bacterial Strain Overview

Each of the IEX purified peptides were tested in in-vitro bioassays against four gram-
negative bacterial strains. Three of the strains are phytopathogenic bacteria obtained from ATCC. They were selected for their commercial relevance as in-field pathogens. *Erwinia amylovora*
causes Fire Blight in pome fruits like apples, pears, plums, etc. (Zwet et al., 1979). *Xanthomonas campestris* causes Black Rot in brassicas like cabbage, cauliflower, broccoli, etc. (Vicente et al., 2012). And *Xylella fastidiosa* which causes Pierce’s Disease of grapes and Leaf Scorch in almonds and olives (Davis et al., 1980). The fourth bacterium, *Escherichia coli* (DH5α, NEB) is both an in-field and post-harvest pathogen that often causes gastrointestinal infections in humans (Makvana et al., 2015).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Catalog number</th>
<th>Disease(s) caused</th>
<th>Species Effected</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Erwinia amylovora</em></td>
<td>ATCC 49946</td>
<td>Fire Blight</td>
<td>Pome fruits (Zwet et al., 1979)</td>
</tr>
<tr>
<td><em>Xanthomonas campestris</em></td>
<td>ATCC 33913</td>
<td>Black Rot</td>
<td>Brassica species (Vicente et al., 2012)</td>
</tr>
<tr>
<td><em>Xylella fastidiosa</em></td>
<td>ATCC 35879</td>
<td>Pierce’s Disease and Leaf Scorch</td>
<td>Grapes, almonds, and olives (Davis et al., 1980)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>NEB C2987</td>
<td>Gastrointestinal infections</td>
<td>Humans (Makvana et al., 2015)</td>
</tr>
</tbody>
</table>

Table 1: Summary of the bacterial strains, where they were obtained from, the disease(s) that they cause, and the literature to support the correlation between bacteria and species susceptible.
Results

Antimicrobial Peptides Selected from Literature

To determine which AMPs would be screened in this project an extensive literature search was performed. From this search thirteen peptides were selected for their reported antibacterial activities and to represent five different categories of AMPs (Table 1). Six of the peptides selected were linear cationic helix peptides, three were defensins, one fragment peptide (also known as plant defense reduction peptide), one anionic peptide, and two cationic peptides rich in specific amino acids. These peptides were isolated from a variety of different species including but not limited to mammals, plants, insects, and amphibians. AMP713 was not included in the initial screen as it was originally synthesized to be used as the positive control in this study. However, it was eventually screened and will have its own results section separate from the twelve other peptides.

<table>
<thead>
<tr>
<th>Table 2: Antimicrobial Peptides Selected for Screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP Category</td>
</tr>
<tr>
<td>Linear cationic helix peptide</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>AMP713</td>
</tr>
<tr>
<td>Defensin</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Fragment (plant defense reduction) peptide</td>
</tr>
<tr>
<td>Anionic peptide</td>
</tr>
<tr>
<td>Cationic peptide rich in specific AA</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Table 2: AMPs selected from literature search: AMP701 – AMP712 were the twelve peptides selected for screening from the literature search. AMP713 was selected for its well reported antimicrobial activities. This peptide was synthesized by GenScript and used as a positive control in antibacterial bioassays. The names/numbers assigned to the peptides are arbitrary to maintain anonymity.

Deep-Well Plate Screen for Expression

One of the AMPs did not successfully transform into competent yeast cells and was eliminated from the study. Three AMPs showed no unique peaks when compared to the negative control strain (Figure 5). Unique peaks were identified in the eight remaining AMPs and their chromatograms are displayed in Figure 6. These eight peaks were collected for LC/MS confirmation.
Figure 5: AMPs that showed no peptide expression: The x-axis represents time, and the y-axis represents absorbance in milli absorbance units (mAU). (A) depicts the HPLC chromatogram analysis of the beer of AMP702. The blue trace is the AMP, and the black trace is the untransformed yeast control strain. (B) depicts AMP703 and (C) depicts AMP705. All three of these AMPs have chromatograms that have no unique peaks when compared to the control. These strains did not express the peptide of interest and were eliminated from the study.
Figure 6: AMPs that showed peptide expression: The x-axis represents time, and the y-axis represents absorbance in milli absorbance units (mAU). (A) Depicts the HPLC chromatogram analysis of the spent media of AMP704. The blue trace is the AMP’s chromatogram, and the black trace is the untransformed yeast control strain. The circled area depicts the unique peaks that were collected and analyzed via LC/MS. (B) Depicts AMP706 and the collected unique peak. (C) Depicts AMP707 and the collected unique peaks. (D) Depicts AMP708 and the collected unique peaks. (E) Depicts AMP709 and the collected unique peaks. (F) Depicts AMP710 and the collected unique peaks. These peaks were collected and analyzed on LC/MS separately. (G) Depicts AMP711 and the collected unique peaks. (H) Depicts AMP712 and the collected unique peaks. These peaks were collected and analyzed on LC/MS separately.

Liquid Chromatography/Mass Spectroscopy

Unique peaks were characterized and identified to confirm if the unique expressions were the AI. Four of the eight AMPs with unique rpHPLC expressions resulted in confirmed AIs (Figure 7).
Figure 7: The unique peaks were analyzed using liquid chromatography/mass spectrometry to determine if the unique peaks were the AI of interest. Four of the collected peaks resulted in matching mass/charge (m/z) ratio calculations. The m/z values are redacted to maintain trade secret. The x-axis represents m/z ratio, the y-axis represents relative intensity. (A) The LC/MS data for AMP706. (B) The LC/MS data for AMP707. (C) The LC/MS data for AMP708. (D) The LC/MS data for AMP711.
Summary of Expressible AMPs

Four of the twelve originally screened AMPs were confirmed to be successfully expressed in the Vestaron yeast expression system. AMP706 and AMP707, both defensins, isolated from a leafy green; AMP707, also a defensin, isolated from an insect; and AMP711, a cationic peptide rich in specific amino acids. These four peptides were then funneled through a purification process.

Table 3: Successfully Expressed AMPs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Origin</th>
<th>AMP category</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP706</td>
<td>Leafy green</td>
<td></td>
</tr>
<tr>
<td>AMP707</td>
<td>Insect</td>
<td>Defensin</td>
</tr>
<tr>
<td>AMP708</td>
<td>Insect</td>
<td>Cationic peptide rich in specific AA</td>
</tr>
<tr>
<td>AMP711</td>
<td>Insect</td>
<td>Cationic peptide rich in specific AA</td>
</tr>
</tbody>
</table>

Table 3: summary of screened AMPs confirmed by LC/MA to be expressible in the Vestaron yeast expression system.

Antimicrobial Peptide Activities in Anti-*Escherichia coli* Bioassays

*E. coli* was grown in both LB medium and LSM under AMP treatment. OD600 readings were taken at 24 and 48 hours after incubation at 27°C in a plate shaker. The control peptide, AMP713, was active against *E. coli*, with IC50 values around 10-20 ppm. Its antibiotic potency was slightly higher in LSM than LB, but the difference was not significant. None of the purified AMPs showed any anti-*E. coli* activities (Figure 8: A and B).

Figure 8: The dose-response curves for the anti-*E. coli* bioassays. For both graphs, AMP706 is represented by the teal trace, AMP707 is the red trace, AMP708 is the blue trace, AMP711 is the green trace, and AMP713 is the black trace. (A) Depicts the dose-responses for the AMPs and control peptide in LSM. The four purified AMPs had no antibacterial activities. However, the control peptide, AMP713, showed potent antibacterial activity with an estimated IC50 of 13.8 ppm. (B) Depicts the dose-responses for the AMPs and control peptide in LB medium. Once again, the four purified AMPs had no antibacterial activities. The control peptide, AMP713 had potent antibacterial activity with an IC50 of 17.4 ppm.

Antimicrobial Peptide Activities in Anti-*Erwinia amylovora* Bioassays

*E. amylovora* was grown in both LB medium and LSM under AMP treatment. OD600 readings were taken at 24 and 48 hours after incubation at 27°C in a plate shaker. The control peptide, AMP713, had potent antibacterial activity against *E. amylovora*. Its antibiotic potency was higher in LSM than LB medium, but the difference was not nearly as significant as the other peptides (Table 4). AMP706, AMP707, and AMP711 had weak anti-*E. amylovora* activity in LSM. These purified AMPs had little to no activity in LB medium. AMP708 had no anti-*E. amylovora* activity in LSM.
activity up to 10 ppt. Overall, the AMPs showed more potent activities in LSM than LB medium (Figure 9).

Table 4: IC50 Values for Anti-\textit{Erwinia amylovora} Bioassays

<table>
<thead>
<tr>
<th>Medium</th>
<th>AMP713 (control)</th>
<th>AMP706</th>
<th>AMP707</th>
<th>AMP708</th>
<th>AMP711</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSM</td>
<td>6.6 ppm</td>
<td>826 ppm</td>
<td>747.5 ppm</td>
<td>Not active</td>
<td>445 ppm</td>
</tr>
<tr>
<td>LB Medium</td>
<td>38.2 ppm</td>
<td>7385.6 ppm</td>
<td>4016.2 ppm</td>
<td>Not active</td>
<td>Not active</td>
</tr>
</tbody>
</table>

Table 4: Summary of the IC50 values for the control and purified peptides in both media types.

Figure 9: The dose-response curves for the anti-\textit{E. amylovora} bioassays. For both graphs, AMP706 is represented by the green trace, AMP707 is the blue trace, AMP708 is the red trace, AMP 711 is the brown trace, and AMP713 is the black trace. (A) Depicts the dose-responses for the AMPs and control peptide in LSM. AMP706, AMP707, and AMP711 had antibacterial activities. AMP708 had no antibacterial activity. The control peptide, AMP713, showed the most potent antibacterial activity. (B) Depicts the dose-responses for the AMPs and control peptide in LB medium. AMP706 and AMP707 had weak antibacterial activities. AMP708 and AMP711 had no antibacterial activities. The control peptide, AMP713, had potent antibacterial activity.

Antimicrobial Peptide Activities in Anti-\textit{Xanthomonas campestris} Bioassays

\textit{X. campestris} was grown in both LB medium and LSM under AMP treatment. OD600 readings were taken at 24 and 48 hours after incubation at 27°C in a plate shaker. In LSM, \textit{X. campestris} grew poorly with a three-fold cell density decrease compared to LB medium growth. The purified AMPs all showed anti-\textit{X. campestris} activities in LSM and weak anti-\textit{X. campestris} activities in LB medium (Table 5). The control peptide, AMP713, had potent anti-\textit{X. campestris} activities in both media types. Overall, the AMPs showed more potent activities in LSM than LB medium (Figure 10).

Table 5: IC50 Values for Anti-\textit{Xanthomonas campestris} Bioassays

<table>
<thead>
<tr>
<th>Medium</th>
<th>AMP713 (control)</th>
<th>AMP706</th>
<th>AMP707</th>
<th>AMP708</th>
<th>AMP711</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSM</td>
<td>1.1 ppm</td>
<td>13 ppm</td>
<td>33.4 ppm</td>
<td>90.8 ppm</td>
<td>8.86 ppm</td>
</tr>
<tr>
<td>LB Medium</td>
<td>2.93 ppm</td>
<td>788 ppm</td>
<td>1878 ppm</td>
<td>7448 ppm</td>
<td>1445 ppm</td>
</tr>
</tbody>
</table>

Table 5: Summary of the IC50 values for the control and purified peptides in both media types.
Antimicrobial Peptide Activities in Anti- *Xylella fastidiosa* Bioassays

*Xylella fastidiosa* was incapable of growth in LSM. Therefore, it was grown in only LB medium under AMP treatment. OD600 readings were taken at 24 and 48 hours after incubation at 27°C in a plate shaker. AMP706 and AMP707 showed very weak anti-*X. fastidiosa* activities in LB medium. AMP708 had no anti-*X. fastidiosa* activity up to 10 ppt (Table 6). The control peptide, AMP713, had potent anti-*X. fastidiosa* activity in LB medium (Figure 11).

<table>
<thead>
<tr>
<th>Medium</th>
<th>AMP713 (control)</th>
<th>AMP706</th>
<th>AMP707</th>
<th>AMP708</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Medium</td>
<td>21.3 ppm</td>
<td>9434 ppm</td>
<td>4491 ppm</td>
<td>Not active</td>
</tr>
</tbody>
</table>

Table 6: Summary of the IC50 values for the control and purified peptides in LB medium.

Figure 11: The dose response curves for the anti-*X. fastidiosa* bioassay in LB medium. AMP706 is represented by the blue trace, AMP707 the green trace, AMP708 the red trace, and AMP713 is the black trace. AMP706 and AMP707 had weak antibacterial activities. AMP708 had no antibacterial activity. The control peptide, AMP713, showed the most potent antibacterial activity.
Expression of AMP713

Due to the effectiveness and spectrum of the synthesized control peptide, AMP713, the same peptide expression pipeline was used in attempt to express AMP713. This AMP was successfully cloned, vectored, transformed, and deep-well screened. Unique expressions were confirmed using rpHPLC (Figure 12) and AI was confirmed using LC/MS (Figure 13). Currently, confirmation of successful expression has been achieved. Antibacterial bioassays with treatment using expressed AMP713 are on the horizon.

Figure 12: The x-axis represents time, and the y-axis represents absorbance in milli absorbance units (mAU). The figure depicts the HPLC chromatogram analysis of the spent media of expressed AMP713. The blue trace is synthetic AMP713, the black trace is expressed AMP713, and the pink trace is an untransformed yeast control. The circled area depicts the unique peak that was collected and analyzed via LC/MS.

Figure 13: The unique peak was analyzed using liquid chromatography/mass spectroscopy to determine if the unique peak was the AI of interest. The collected peak resulted in matching mass/charge (m/z) ratio calculations depicted by the red stars. The m/z values are redacted to maintain trade secret. The x-axis represents m/z ratio, the y-axis represents relative intensity.
Discussion

From this study, five AMPs were successfully expressed using the Vestaron yeast expression system. Four of the AMPs were successfully purified and tested in in-vitro antibacterial bioassays. AMP706, AMP707, AMP708, and AMP711 all showed some antibacterial activities against *E. amylovora*, *X. campestris*, and *X. fastidiosa* when assayed in LSM. These four AMPs also consistently showed decreased antibacterial activities when assayed in LB medium suggesting that the antibacterial effects of all candidate AMP AIs were inhibited by the cation inhibition effect. Regardless, these peptides still had some weak activity even in an unfavorable environment. The fifth peptide that was successfully expressed was AMP713 and further purification and bioassay research will be conducted. This AMP was originally synthesized to be used as the positive control in the in-vitro antibacterial bioassays. After seeing how potent and how broad the spectrum of its activity was, it too became a candidate of interest. Not only was synthetic AMP713 effective against the three gram-negative phytopathogenic bacterial strains, it also was effective in controlling the human pathogen, *E. coli*. Furthermore, synthetic AMP713 did not have dramatic antibacterial activity loss when exposed to cations like the other four peptides had. AMP713, was selected as the leading candidate because it was effective against all the bacterial strains tested in this study. It had the broadest spectrum, was consistently the most potent, and was not as significantly impacted by the cation inhibition effect.

These results suggest that while bacterial and fungal vehicles are used in the Vestaron yeast expression system pipeline, AMPs can be successfully expressed. Even extremely potent AMPs like AMP713 can be expressed in this way. More generally, some of the bacterial strains that were targeted in this study do not have reliable prevention methods besides cutting back infected crops to prevent spread to healthy crops. Some of the strains do not have any treatment at all or the treatment is heavily reliant on antibiotics which are failing due to emerging antibiotic resistance. AMPs offer an alternative to prevent and possibly treat in-field bacterial infections. They offer a more complex mode of action than the chemical antibiotics that are currently available, which reduces the chances of resistance. In some cases, they also offer a completely new path for growers because there is no commercial product available.

While the results of the study are exciting, they are limited. Efficacy of the AMPs were only tested in in-vitro bioassays, these results do not necessarily translate to in-situ, in-vivo, or in-field assays. In the in-vitro bioassays, the AI was cultured with the bacteria and there was confidence they would interact directly. In-situ, in-vivo, or in-field infections can be on or in the plant which can make targeting the bacteria more difficult. Furthermore, the efficacy of AMP713 is based solely on a synthetically produced peptide. It is possible that the expressed and purified version of AMP713 will not be as effective as synthetically produced AMP713.

AMP713 will continue to be researched and engineered. As depicted in Figure 12, there are multiple expressions that occurred when the AI was transformed and expressed in the Vestaron yeast expression system. Separating, characterizing, and identifying the peaks should be prioritized. While the lead candidate has been determined to be effective against at least four
gram-negative phytopathogenic bacteria, further spectrum studies should be conducted. Mammalian toxicity should be analyzed to determine the safety of AMP713. Stability and melting point studies should be performed to conclude if this peptide is able to withstand uncontrolled conditions after production. To promote a peptide product from lab to field is not simple even if the initial screening results are promising.

**Conclusion**

The completion of this study provided evidence to support the proof of concept that AMPs can be expressed in the Vestaron yeast expression system. Not only can they be expressed successfully, but they can then be used to control bacterial growth in in-vitro bioassays. Furthermore, from this study an AMP has been selected as a leading candidate for possible commercialization. In the future, a peptide-based bactericide may be available to combat the economic losses caused by phytopathogenic bacteria. The specific bacteria that were studied were selected because they are of commercial interest. Like human pathogens, treatment for phytopathogenic bacteria is an antibiotic, whether that be peptide-based or chemically produced. Bactericides would prevent and possibly reverse the negative effects of plant pathogenic diseases. There are plans to continue developing this AMP program and penetrate a new market in the coming years.

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