Vitis **42** (2), 95–97 (2003)

Effect of five antimicrobial peptides on the growth of *Agrobacterium tumefaciens*, *Escherichia coli* and *Xylella fastidiosa*

Z. T. Li and D. J. Gray

Mid-Florida Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Apopka, Florida, USA

Summary

Five lytic peptides, cecropin A (CA), cecropin B (CB), magainin I (MI), magainin II (MII) and Shiva-1 (a synthetic analog of CB), were evaluated for their antimicrobial activity against *Escherichia coli* and two pathogenic bacteria of grape, *Agrobacterium tumefaciens* and *Xylella fastidiosa*. Peptides were tested at 0.1, 0.5, 1.0, 5.0 and 10.0 µM concentrations in liquid nutrient medium-based assay to determine their relative efficacy in inhibition bacterial growth. A 24-h incubation of *E. coli* with the respective lytic peptides revealed that both CA and CB exhibited the highest antibacterial activity and completely inhibiting bacterial growth at concentrations as low as 0.5 µM. In contrast, MI, MII and Shiva-1 had no inhibiting effect on *E. coli* at the tested concentrations. Both CA and CB at concentrations greater than 0.5 µM were highly effective in inhibiting the growth of *Agrobacterium*, whereas complete inhibition was observed only at MI, MII and Shiva-1 concentrations greater than 5 µM. Growth inhibition profiles of *X. fastidiosa* were similar to those of *E. coli*, although Shiva-1 at 10 µM showed a modest growth inhibition.

K e y w o r d s : cecropins, growth inhibition, lytic peptides, magainins, Pierce's disease, Shiva-1.

Introduction

Many small lytic peptides possess potent antimicrobial activity and function in organisms as part of a self-defense mechanism in response to infection (Bechinger 1997). Among the most extensively studied lytic peptides are cecropins and magainins. Cecropins were originally isolated from the haemolymph of the giant silk moth *Hyalophora cecropia*. These peptides, in several forms (A, B and D), contain 35-37 amino acid residues. Their synthesis is induced in response to bacterial infection (Boman and Hultmark 1987). Magainins were originally isolated from the skin of the African clawed frog *Xenopus laevis*. Two forms of magainins, I and II, identified thus far contain 23 amino acid residues and are inhibitory to the growth of bacteria, fungi and protozoa (Zasloff *et al.* 1988). Both cecropin- and magainin-type peptides are attractive candidates for developing transgene-induced resistance characteristics in plants because they have a broad spectrum of antimicrobial activity and very little cytotoxicity to animal and plant cells, even at high concentrations (Bechinger 1997; Sharma *et al.* 2000). In addition, the small size of the proteins and corresponding DNA sequences makes manipulation and transfer of functional genes encoding lytic peptides relatively simple.

Although the relative efficacy of lytic peptides in inhibiting *in vitro* growth of various pathogenic bacteria has been determined (Moore *et al.* 1996; Alan and Earle 2002), there is a lack of information on their activity against xylem-limited bacteria such as *Xylella fastidiosa*. *X. fastidiosa* causes diseases in a broad range of vascular plants, most notably Pierce's disease (PD) of grapevine (Hopkins and Purcell 2002). PD causes devastating damage to the grape industries in many areas of the United States. To facilitate efforts to control PD disease in grapevine using a transgenic strategy, we evaluated 5 lytic peptides including 2 cecropins (A and B), 2 magainins (I and II) and Shiva-1, a synthetic analog of cecropin B (Jaynes *et al.* 1988) for their *in vitro* antibacterial activity against a PD strain of *X. fastidiosa*, *Agrobacterium tumefaciens* and *Escherichia coli*, using a liquid medium assay procedure.

Material and Methods

L y t i c p e p t i d e s a n d b a c t e r i a l s t r a i n s : Four purified lytic peptides including cecropin A (CA), cecropin B (CB), magainin I (MI) and magainin II (MII) were purchased from AnaSpec Inc. (San Jose CA, USA). Shiva-1 was chemically synthesized using the published amino acid sequence. *A. tumefaciens* strain EHA105 (Hood *et al.* 1986) and *E. coli* strain DH5α were used in this study. *X. fastidiosa* strain PD00-2 was isolated from a grapevine with Pierce's disease and kindly provided by Dr. D. L. Hopkins (MREC-IFAS, University of Florida, Apopka, FL, USA).

A n t i b a c t e r i a l a c t i v i t y : Stock cultures were initiated by inoculating 25 ml liquid PD2 medium (Davis *et al.* 1980) with respective bacterial strains in a 125 ml flask and culturing them in a rotary shaker (260 rpm) at 28 °C for 1-2 d. The PD2 medium was chosen because it provides a favorable growth environment for *X. fastidiosa* as well as other types of bacteria. 10 µl of the stock culture were mixed with 10 ml of fresh PD2 medium and incubated for an additional 4 h under the same conditions in order to facilitate harvest of cells at

Correspondence to: Dr. D. J. Gray, Mid-Florida Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 2725 Binion Road, Apopka, FL 32703-8504, USA. Fax: +1-407-814-6186. E-mail: djg@mail.ifas.ufl.edu

the mid-logarithmic phase of cell growth. Cultures of *A. tumefaciens* and *E. coli* were diluted with fresh PD2 medium to a final cell density of 1 x 10^7 cfu ml^{-1}, whereas cultures of *Xylella* were adjusted to a final density of 1 x 10^8 cfu ml^{-1}. Higher initial inoculum density of *X. fastidiosa* was employed to account for its relative slower growth rate. Diluted bacterial cultures were mixed with an equal volume (100 µl) of 2-fold serial concentration dilution of the test peptide in fresh PD2 medium. The final culture mixture was placed in Eppendorf tubes and incubated under the previously described culture conditions for growth inhibition analysis.

At the end of culture period, bacterial cultures in Eppendorf tubes were thoroughly mixed by a brief vortex and then transferred to a 96-well microplate (100 µl per well) for absorbance measurement. Bacterial growth was determined by monitoring the increase in optical density (OD) at 600 nm as measured with a Sunrise absorbance microplate reader (Tecan, Phenix Research Products, Hayward, CA, USA) after 0, 24 and 48 h incubation. Readings from the 0 h incubation time were used to determine the absorbance due to media and inoculum, which was subtracted from 24 and 48 h data to determine a no-growth baseline. Experiments were repeated three times with three replicates for each treatment. With the exception of *X. fastidiosa*, only data taken at 24 h are presented because growth inhibition profiles of *A. tumefaciens* and *E. coli* remained relatively unchanged at 48 h.

Results and Discussion

To verify the effectiveness of the liquid PD2-based assay system in studying phytopathic bacteria, non-phytopathic *E. coli* cells were first utilized. 24 h after inoculation, differences in cell growth were observed in cultures containing each of the 5 tested lytic peptides. As illustrated in Figure, A, concentrations of CA or CB at 0.5 µM or higher effectively inhibited *E. coli* cell proliferation. These results were similar to findings using other assay systems (Moore *et al.* 1996; Alan and Earle 2002). For example, Oh *et al.* (1998) used an LB medium-based assay to show that 0.3-0.4 µM CA or CB was lethal to *E. coli* within 1 h. In the present study, similar concentration of CA and CB continued to exhibit antibacterial activity for up to 100 h (data not shown). In contrast to results obtained with CA and CB, *E. coli* growth was not inhibited by MI, MII or Shiva-1 concentrations of up to 10 µM (Figure). This lack of antibacterial activity of MI, MII and Shiva-1 against *E. coli* collaborates previous reports that concentrations from 10-100 µM of magainins or Shiva-1 were required to completely inhibit the proliferation of various pathogenic microbes (Moore *et al.* 1996; Alan and Earle 2002).

A. tumefaciens was included in this study because it is a major pathogen of grapevine, causing crown gall disease. Reactions of this microbe to both CA and CB were nearly identical to that of *E. coli* (Figure). Similarly, growth of *A. tumefaciens* was not affected by MI, MII and Shiva-1 at concentrations of up to 1 µM. However, *A. tumefaciens* differed from *E. coli* in that 5 or 10 µM of magainins and Shiva-1 resulted in complete growth inhibition (Figure, B). The 5 µM concentration of MII is lower than the 12 µM reported by Alan and Earle (2002) to be necessary for complete growth inhibition. Differences in antibacterial activity might be attributed to the differences in the bacterial strains used, source of lytic peptides, or assay systems. Nonetheless, results of the present study are in agreement with Alan and Earle (2002) in that CB was more inhibitory to the growth of *Agrobacterium* than MII.

The reaction of *X. fastidiosa* to the various lytic peptides was evaluated using 24 and 48 h incubation periods due to the relatively slower growth rate of this bacterium in the *Xylella*-specific PD2 medium (Davis *et al.* 1980). After 24 h of incubation, significant inhibition of cell proliferation (up to 95 % reduction in OD_{600} increase) was observed in the presence of 0.5 and 1 µM of CA or CB; growth inhibition was even greater in the presence of 5 and 10 µM of cecropins (Figure, C). No cell growth was observed after 48 h in the presence of 0.5 µM or higher concentrations of CA and CB (Figure, D). In contrast, no growth inhibition was evident in medium containing MI, MII and Shiva-1 at all concentrations tested in both 24-h and 48-h assays, except for 10 µM of Shiva-1 (Figure, C and D). At 24 h, 10 µM of Shiva-1 completely inhibited growth of *X. fastidiosa*, whereas, at 48 h, about 30 % of the cell growth (70 % inhibition) had occurred (Figure, D).

This study demonstrated that CA and CB at low concentrations provided longterm and stable growth inhibition to three tested bacteria. Lytic peptides are cationic and have a high degree of electrostatic affinity to anionic lipopolysaccharide rich bacterial cell membranes (Bechinger 1997). Once in contact with such lipid membranes, these peptides undergo spontaneous conformational changes that lead to the formation of pore structures across the lipid bilayers. These pores subsequently permeate the membranes, disrupt the cellular environment and eventually cause cell death of affected organisms (Bechinger 1997). Many lytic peptides have different cationic properties and affinities to anionic lipid membranes. As a consequence, they often require different *in vitro* concentrations to achieve their most effective antimicrobial activity.

Data from this study indicate that, while MI, MII and Shiva-1 were the least effective lytic peptides for impeding the growth of all the tested bacteria, Shiva-1 had limited antibacterial activity against *X. fastidiosa* at the highest concentration tested (10 µM). In contrast, both CA and CB totally inhibited cell growth of all the tested bacteria at concentrations as low as 0.5 µM. The relatively high levels of antibacterial activity of cecropins may be due to their unusual physiological influence on bacterial cell growth, in addition to their pore-forming capability. Recent studies suggest that cecropins at sublethal peptide concentrations are capable of inducing high levels of transcriptional activation of stress promoters in *E. coli*, leading to the continuous excessive expression of stress proteins, and ultimately stasis and cell death (Oh *et al.* 1998). Our data suggest that the long-term significant inhibition of *X. fastidiosa* growth by low concentrations of cecropins also may have been de-

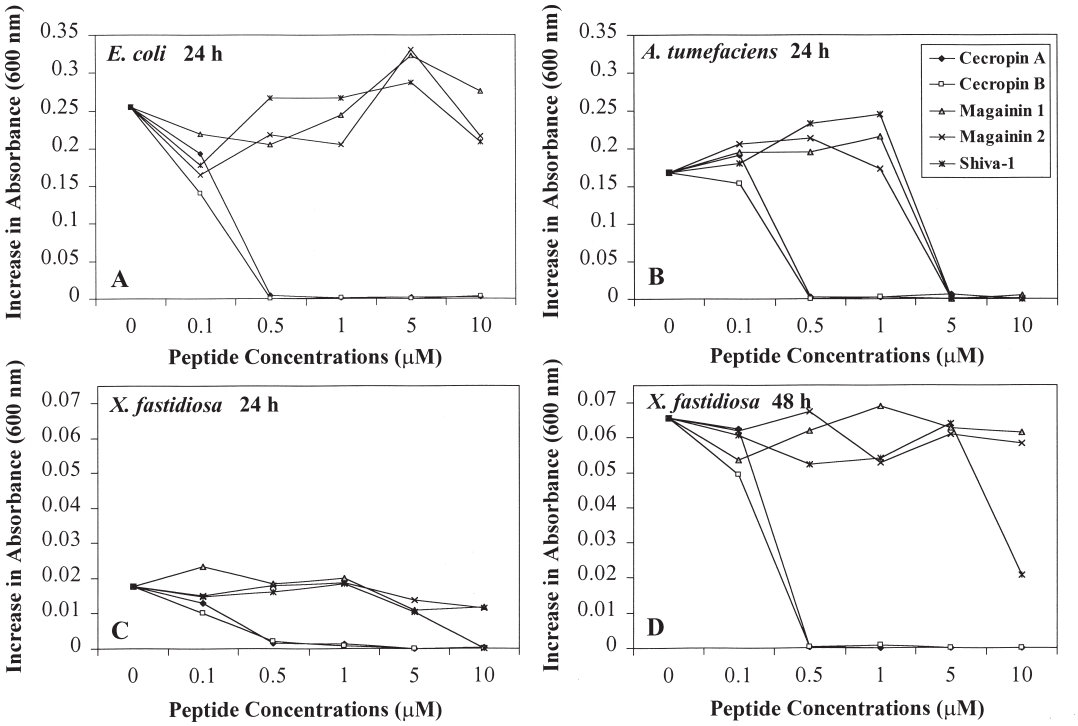

Figure: Influence of different concentrations of lytic peptides on the growth of *E. coli*, *A. tumefaciens* and *X. fastidiosa*. Bacteria were incubated at 28 °C for 24-48 h in a rotary shaker (260 rpm) in liquid PD2 medium containing various concentrations of tested lytic peptides. Optical absorbance of the cultures at 600 nm was determined. Experiments were repeated three times with three replicates per treatment. Data points represent mean values of OD changes at the end of culture period, which were adjusted to remove background absorbance due to inoculated medium. **A**: 24-h inoculation of *E. coli*; **B**: 24-h inoculation of *A. tumefaciens*; **C**: 24-h inoculation of *X. fastidiosa*; **D**: 48-h inoculation of *X. fastidiosa*.

rived, in part, from similar excessive expression of induced stress proteins. Thus, cecropins may be useful in controlling PD in transgenic grape due to their effective antimicrobial activity and very low toxicity to animals.

Acknowledgements

This research was supported by the Florida Agricultural Experiment Station and a grant from The Florida Department of Agriculture and Consumer Services' Viticulture Trust Fund, Tallahassee, Florida and approved for publication as Journal Series No. R-09363.

References

Alan, A. R.; Earle, E. D.; 2002: Sensitivity of bacterial and fungal plant pathogens to the lytic peptides, MSI-99, magainin II, and cecropin B. Mol. Plant-Microbe Interactions **15**, 701-708.

Bechinger, B.; 1997: Structure and functions of channel-forming peptides: Magainin, cecropins, melittin and alamethicin. J. Membr. Biol. **156**, 197-211.

Boman, H. G.; Hultmark, D.; 1987: Cell-free immunity in insects. Annu. Rev. Microbiol. **41**, 103-126.

Davis, M. J.; Purcell, A. H.; Thomson, S. V.; 1980: Isolation medium for Pierce's disease bacterium. Phytopathology **70**, 425-429.

Hopkins, D. L.; Purcell, A. H.; 2002: *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. Plant Dis. **86**, 1056-1066.

Hood, E. E.; Helmer, G. L.; Fraley, R. T.; Chilton, M. D.; 1986: The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J. Bacteriol. **168**, 1291-1301.

Jaynes, J. M.; Burton, C. A.; Barr, S. B.; Jeffers, G. W.; Julian, G. R.; White, K. L.; Enright, F. M.; Klei, T. R.; Laine, R. A.; 1988: *In vitro* cytocidal effect of novel lytic peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*. FASEB J. **2**, 2878-2883.

Moore, A. J.; Beazley, W. D.; Bibby, M. C.; Devine, D. A.; 1996: Antimicrobial activity of cecropins. Antimicrob. Chemother. **37**, 1077-1089.

Oh, J. T.; Cajal, Y.; Dhurjati, P. S.; Van Dyk, T. K.; Jain, M. K.; 1998: Cecropins induce the hyperosmotic stress response in *Escherichia coli*. Biochim. Biophys. Acta **1415**, 235-245.

Sharma, A.; Sharma, R.; Imamura, M.; Yamakawa, M.; Machii, H.; 2000: Transgenic expression of cecropin B, an antibacterial peptide from *Bombyx mori*, confers enhanced resistance to bacterial leaf blight in rice. FEBS Lett. **484**, 7-11.

Zasloff, M.; Martin, B.; Chen, H. C.; 1988: Antimicrobial activity of synthetic magainin peptides and several analogues. Proc. Natl. Acad. Sci. USA. **85**, 910-913.

Received February 17, 2003