

**SYNTHESIS, CLONING AND EXPRESSION OF  
AN ANTIFUNGAL PEPTIDE, ESF1, IN  
*SACCHAROMYCES CEREVISIAE***

by

**VIVEKA VADYVALOO**

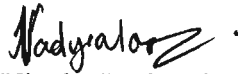
Submitted in fulfilment of the academic  
requirements of the degree of  
Master of Science  
in the School of Molecular and Cellular Biosciences,  
University of Natal

Pietermaritzburg  
2000

## PREFACE

The experimental work described in this thesis for M.Sc was carried out in the School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg, under the supervision of Professor John W. Hastings.

These studies represents the original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.



Viveka Vadyvaloo (Miss)

## TABLE OF CONTENTS

	<u>Page no.</u>
ABSTRACT	i
LIST OF FIGURES	ii
LIST OF TABLES	vii
ABBREVIATIONS	viii
ACKNOWLEDGMENTS	ix
CHAPTER ONE: LITERATURE REVIEW	1
1.1 An introduction to antimicrobial peptides	2
1.2 The nature of cationic antimicrobial peptides	2
1.3 Overview of antimicrobial peptides	3
1.3.1 Antimicrobial peptides from mammalian defense systems	3
1.3.2 Antimicrobial peptides from insect defense systems	5
1.3.3 Antimicrobial peptides from amphibians	9
1.3.4 Antimicrobial peptides from bees	11
1.3.5 Antimicrobial peptides from plant	13
1.3.6 Antimicrobial peptides from bacteria	15
1.3.7 Antimicrobial peptides from other species	17
1.4 Structure of cationic peptides	18
1.4.1 Structure-Function relationships	19
1.4.2 Mode of action	21
1.5 Applications of cationic antimicrobial peptides	23
1.5.1 Antimicrobial efficacy	23
1.5.2 Clinical applications	25
1.5.3 Other applications	25
1.5.4 Transgenic technology	25
1.6 Expression in yeast	26

1.6.1 Factors influencing foreign gene expression in yeast	27
1.6.2 Expression and secretion of heterologous proteins in yeast	29
1.7 The antifungal peptide, ESF1	32
<b>CHAPTER TWO: MATERIALS AND METHODS</b>	34
2.1 Strains, plasmids and culture conditions	35
2.2 DNA manipulations and transformations	35
2.2.1 DNA isolations	35
2.2.2 Agarose gel electrophoresis	36
2.2.3 Recovery of DNA from agarose gels	37
2.2.4 Enzymes	37
2.2.5 Transformation	37
2.3 Synthetic <i>ESF1</i> gene assembly	37
2.4 Cloning of the assembled <i>ESF1</i> gene into pUC18	38
2.5 Cloning of the <i>ESF1</i> gene into pVD4	38
2.6 Cloning of the ESF1/MF $\alpha$ 1 expression cassette into pTG3828	41
2.7 Verification of recombinant plasmids	41
2.7.1 Southern hybridization	41
2.7.2 Restriction analysis	42
2.7.3 Polymerase chain reaction	42
2.7.4 DNA sequencing	42
2.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	43
2.9 Reverse Transcriptase PCR (RT-PCR)	45
2.10 Purification of ESF1 peptide from the yeast pTGESF1 transformant culture supernatant	45
2.11 Bioassays	47
2.12 Tricine SDS-PAGE	47
2.13 Electroblothing of protein onto polyvinylidene fluoride (PVDF) membrane	48
2.14 Amino acid sequencing	48

2.15 Electro Spray Mass Spectrometry (ES-MS)	48
2.16 Purification of antibodies produced in chickens against the synthetic ESF1 peptide	49
2.16.1 Immunization of chickens	49
2.16.2 Isolation of IgY antibodies from chicken egg yolks	49
2.16.3 Purification of anti-ESF1 antibody	50
2.17 Western dot blot analysis of pTGESF1 yeast transformant culture supernatant	50
 CHAPTER THREE: RESULTS AND DISCUSSION	 52
3.1 Assembly of <i>ESF1</i> gene sequence and its cloning into pUC18	53
3.2 Construction of the pVDESF1 recombinant vector	53
3.3 pTG3828 containing the <i>Mfa1/ESF1</i> gene expression cassette isolated from <i>E. coli</i> transformants	57
3.4 pTG3828 containing the <i>Mfa1/ESF1</i> gene expression cassette isolated from <i>Saccharomyces cerevisiae</i> c13ABYS86 transformants	57
3.5 Transcription of the <i>MFα1 prepro</i> sequence and <i>ESF1</i> gene	58
3.6 Purification and detection of ESF1 peptide in PTGESF1 yeast transformant culture supernatant	59
 CHAPTER FOUR: CONCLUSION	 71
 CHAPTER FIVE: REFERENCES	 73

## ABSTRACT

ESF1 is a 2.052 kDa antimicrobial peptide, mimicking the charge distribution and amphipathic alpha-helical structure of magainin, pGLa, a naturally occurring antimicrobial peptide. ESF1 has been reported to display high activity against *Fusarium oxysporum* f. sp *lycopersici* and *F. oxysporum* f. sp *cubense* race 4, the tomato and banana crop plant, wilt-causing pathogens, respectively. To assess whether this synthetic peptide can be heterologously expressed in yeast in significant quantities, and still retain full bioactivity, within a eukaryotic system, the *ESF1* gene was designed and synthesized from five oligonucleotides, and cloned into pUC18. From the pUC18/ESF1 recombinant plasmid, the *ESF1* gene sequence was amplified and cloned into the pBluescript-based vector, pVD4, downstream of the yeast pheromone mating factor alpha (MF $\alpha$ 1) promoter, and in frame with the MF $\alpha$ 1 signal peptide sequence for expression and secretion in yeast. The expression cassette comprising the MF $\alpha$ 1 promoter and signal peptide sequence, and *ESF1* gene was subsequently cloned into the yeast/ *E. coli* shuttle vector, pTG3828 and transformed into *Saccharomyces cerevisiae*. Chicken IgY antibodies against ESF1 peptide were raised and immunoaffinity purified. Following this, western dot blot analysis and mass spectrometry confirmed the presence of ESF1 in partial HPLC purified fractions of the recombinant yeast culture supernatant.

## LIST OF FIGURES

**Figure 1** Examples of structures of four classes of cationic peptides. (a)  $\beta$ -strand human defensin-1, (b)  $\alpha$ -helical cecropin mellitin hybrid, (c) extended coil indolicidin, (d) loop structure bactenecin. Positive charges denoted by (+), amino termini denoted by (N), and disulfide bridges are present in (a) and (d). In (a) the three  $\beta$ -strands are denoted by pairs of one, two or three lines on the backbone ( Hancock, 1997). page 22

**Figure 2** Proposed mechanism of action of cationic antimicrobial peptide with Gram negative outer membrane and the cytoplasmic membrane of bacterial cells. The hypothesized 'self-promoted uptake' pathway of cationic peptide into the cell, occurs when there is an association of the cationic peptide with the negatively charged outer membrane, creating cracks through which the peptide moves across the outer membrane (A); or binding to the divalent cation binding sites on LPS and disrupting the membrane (B). The peptide consequently folds into amphipathic structure (C), once bound to the negatively charged surface of the cytoplasmic membrane. Several peptide molecules insert and span the membrane after forming aggregation complexes (D), or move across the membrane under the influence of the large transmembrane potential (E). (Hancock and Chapple, 1999). page 24

**Figure 3** Construction of the *ESF1* gene. The five oligonucleotides VV1, VV2, VV3, VV4 and VV5 were designed to compose the *ESF1* gene sequence. Annealing allows the joining of the complementary oligonucleotides VV1 and VV2 of the 5' to 3' strand to VV3, VV4 and VV5 of the 3' to 5' strand, while ligation facilitates joining of the VV1 to VV2, VV3 to VV4, and VV4 to VV5 oligonucleotides. page 53

**Figure 4** 12.5% polyacrylamide gel of putative pUC/*ESF1* clones restricted with *Bam*HI/*Pst*II to give a 78 bp fragment containing the *ESF1* gene. Lanes: (1) *Eco*RI/*Hind*III cleaved  $\lambda$ -DNA; (2) pUC/*ESF1* transformant 1; (3) pUC/*ESF1* transformant 2. page 54

**Figure 5** pBluescript-based vector pVD4. The vector has been assembled from a basic

pBluescript vector by the introduction of the MF $\alpha$ 1 promoter and prepro leader sequences to form an expression cassette, with the insect defensin A sequence cloned in frame with the expression cassette. This will allow expression and secretion of the cloned peptide in yeast. The underlined *Kpn*I site contained within the insect defensin A sequence is not present in the *ESF1* sequence. This allows determination of the probable replacement of insect defensin A by the *ESF1* gene during restriction analysis of putative pVDESf1 clones. The arrows indicate the site of insertion for the *ESF1* gene (W.F Broekaert, personal communication).

page 54

**Figure 6** Agarose gel (3%) of PCR amplification of *ESF1* gene from pUCESf1 clone. Lanes: (1) 1018 bp fragment and its multimers plus pBR322 fragments; (2) pUCESf1, 1.5mM MgCl<sub>2</sub>; (3) pUCESf1, 2mM MgCl<sub>2</sub>; (4) pUCESf1, 2mM MgCl<sub>2</sub>; (5)pUCESf1, 3mM MgCl<sub>2</sub>; (6) pUCESf1, 3.5mM MgCl<sub>2</sub>; (7) no template; (8) pMAL-p2, 1.5mM MgCl<sub>2</sub>.

page 55

**Figure 7** Agarose gel (left) and corresponding Southern blot (right) of pVD4/ESf1 (*Kpn*I) putative hybrids. Lanes: (1) 1018 bp fragment and its multimers plus pBR322 fragments; (2) pVD4; (3) pVD4 (*Kpn*I); (4) transformant 6b; (5) transformant 7b; and (6) transformant 8b. Digoxigenin-labeled probe VV3, which hybridizes to *ESF1* was used at a hybridization temperature of 55°C.

page 56

**Figure 8** Yeast/*E. coli* shuttle vector pTG3828. The multiple cloning site contains the *Sal*I and *Bgl*III sites indicated by arrows at which the MF $\alpha$ 1/*ESF1* gene expression cassette can be cloned. The expression cassette will then be flanked at the 3' end by the PGK transcriptional terminator thus providing stability to the MF $\alpha$ 1 prepro leader and *ESF1* mRNA strand. The underlined *Cla*I site will lie 3' to the inserted expression cassette, and following cleavage of pTGESf1 DNA with *Sal*I and *Cla*I, an approximate 1,3 kb fragment should be produced (Achstetter *et al.*, 1992).

page 56

**Figure 9** Agarose gel (left) and corresponding Southern blot of putative pTGESf1 recombinants. Lanes: (1) digoxigenin-labeled *Eco*RI/*Hind*III cleaved  $\lambda$ -DNA; (2)



pVDESf1; (3) pTG3828; (4, 5, 6, 7, 8) putative pTGESF1 clones. Digoxigenin-labeled probe, proMF, hybridizing to the *MF $\alpha$ 1 pro* sequence, was used at a hybridization temperature of 42°C for Southern hybridization. page 57

**Figure 10** Agarose gel (left) and corresponding Southern blot (right) of yeast pTGESF1 transformants. Lanes: (2) digoxigenin-labeled *EcoRI/HindIII* cleaved  $\lambda$ -DNA; (3) pTG3828 (*E. coli*); (4) pTGESF1 (*E. coli*); (5, 6, 7) pTGESF1 transformants (yeast); and (8) pTG3828 (yeast). Digoxigenin-labeled probe proMF, hybridizing to the *MF $\alpha$ 1 pro* sequence was used as a probe at a hybridization temperature of 42°C in Southern hybridization page 58

**Figure 11** Agarose gel (3%) of DNA fragments from a PCR amplification of DNA isolated from pTGESF1 transformants. Lanes: (1) 1018 bp fragment and its multimers plus pBR322 fragments; (2) transformant A5 DNA, 1.5mM MgCl<sub>2</sub>; (3) A5 DNA, 2.5mM MgCl<sub>2</sub>; (4) A5 DNA, 3.5mM MgCl<sub>2</sub>; (5) A5 DNA, 4.5mM MgCl<sub>2</sub>; (6) pTGESF1 positive control DNA, 1.5mM MgCl<sub>2</sub>; (7) no template. page 59

**Figure 12** RT-PCR of yeast total mRNA (A) and Southern blot (B) of the gel indicating mRNA transcription of the *ESF1* gene. Lanes: (1) Digoxigenin-labeled *EcoRI/HindIII* cleaved  $\lambda$ -DNA; (2) 1018 bp fragment and its multimers plus pBR322 fragments; (3) pBR322 (*HaeIII* digested); (4) RT-PCR of pTGESF1 transformed yeast mRNA; (5) RT-PCR of pTG3828 transformed yeast mRNA; and (6) PCR of pVDESf1. Amplification products are noted for the reverse transcribed pTGESF1 transformed yeast mRNA and the positive control pVDESf1 DNA (contains recombinant MF $\alpha$ 1/ESF1 expression cassette). page 60

**Figure 13** Elution profile of synthetic ESF1 peptide using a C<sub>18</sub> Brownlee column. The retention time of the largest peak which corresponds to ESF1 activity is 31.883 minutes, and elution concentration is 41.883% acetonitrile in acidified water. page 61

**Figure 14** Elution profile of pTGESF1 (A) and pTG3828 (B) transformed culture supernatant using Bondapak-C<sub>18</sub> preparative HPLC column, monitored by absorbance at 220nm, using acetonitrile in acidified water as the solvent. Eluent at 30-40 minutes retention time was collected since the visible protein from the recombinant yeast culture supernatant (A) has a retention time of 33.090 minutes, which correlates with the retention time 31.883 for synthetic ESF1 peptide from a C<sub>18</sub> analytical HPLC column. **page 62**

**Figure 15** Elution profile of pTGESF1 (solid line) and pTG3828 (dotted line) transformed yeast culture supernatant using the C<sub>18</sub> analytical HPLC column monitored at A<sub>220</sub>, using acetonitrile/ acidified water as solvent. A fraction with 31-34 minutes retention time was collected, lyophilized and activity against *Carnobacterium mobile* was displayed. The peak at the retention time of 31.667 minutes is thought to represent ESF1 peptide. **page 63**

**Figure 16** Spot-on-lawn assay of HPLC analytical column, lyophilized fractions of pTGESF1 and pTG3828 transformed yeast culture supernatants. 5μl of pTGESF1 (1) and pTG3828 (2) culture preparations were spotted on brain-heart-infusion agar seeded with the indicator organism, *Carnobacterium mobile*. Only the pTGESF1 culture supernatant shows activity against the indicator bacterium. **page 63**

**Figure 17** Tricine (10% C; 3% T) SDS-PAGE of HPLC analytical column, lyophilized fractions of both pTGESF1 and pTG3828 transformed yeast cultures. The arrow indicates the position of the 2.052 kDa ESF1 peptide, and this band was blotted to PVDF membrane and subjected to amino acid sequencing. Lanes : (1) Promega low range protein marker, (2) Promega low range protein marker, (3) empty, (4) pTG3828 yeast culture supernatant (10μl), (5) and (6) pTGESF1 transformed yeast culture supernatant (20μl), (7) pTGESF1 transformed yeast culture supernatant (10μl). **page 64**

**Figure 18** Progress of immunization with ESF1 peptide as determined by ELISA. ESF1 peptide was coated at 1μg/ml to microtitre plates and incubated with antibody dilutions of 500 μg/ml, 100 μg/ml, 50 μg/ml and 10 μg/ml, collected after 4 (●), 6 (■), 8 (▲), and 9 (◆), and non-immune IgY (+). Binding was visualized by incubation with rabbit anti-

chicken HRPO-linked secondary antibody as described by Coetzer *et al.* (1991). Each point is a mean at  $A_{405}$  of duplicate samples. page 65

**Figure 19** Representation of different immunoaffinity purified ESF1 IgY concentrations with ESF1 peptide as determined by ELISA. ESF1 peptide was coated at  $1\mu\text{g/ml}$  to microtitre plates and incubated with antibody dilutions of  $100\mu\text{g/ml}$ ,  $50\mu\text{g/ml}$ ,  $10\mu\text{g/ml}$ , and  $1\mu\text{g/ml}$  collected during immunoaffinity purification. Binding was visualized by incubation with rabbit anti-chicken HRPO-linked secondary antibody as described by Coetzer *et al.* (1991). page 66

**Figure 20** Western dot blot of synthetic ESF1 peptide ( $1\mu\text{g}$ ), leucocin A peptide ( $1\mu\text{g}$ ), and preparative and analytical HPLC column lyophilized fractions of pTG3828 and pTGESF1 yeast culture supernatants. (1) ESF1 peptide. (2) pTGESF1 culture supernatant, preparative column fraction. (3) pTG3828 culture supernatant, preparative column fraction. (4) leucocin A peptide. (5) pTGESF1 culture supernatant, analytical column fraction. (6) pTG3828 culture supernatant, analytical column fraction. (7) empty. page 67

**Figure 21** Electro spray mass spectrometry of synthetic ESF1 peptide and a Bondapak- $\text{C}_{18}$  preparative column HPLC preparation of pTGESF1 yeast culture supernatant. The  $684.27$  ( $m/z$ ) ration in (B, HPLC ESF1 preparation) corresponds to the  $684.65$  ( $m/z$ ) ratio in (A, synthetic ESF1 peptide).  $684$  ( $m/z$ ) is calculated to be  $2052\text{ Da}$ , which reflects the ESF1 peptides mass, and this shows that ESF1 peptide is contained within preparative column fraction of the pTGESF1 culture supernatant. page 68

## LIST OF TABLES

<i>Table 1</i> Bacterial peptide classes as characterized by Klaenhammer (1993).	<b>page 16</b>
<i>Table 2</i> Transgenic cationic antimicrobial peptides providing disease resistance in plants.	<b>page 28</b>
<i>Table 3</i> List of strains and plasmids used in this study.	<b>page 36</b>
<i>Table 4</i> Components of the PCR reaction mixture for amplification of the <i>ESF1</i> gene from the recombinant pUC/ESF1 vector.	<b>page 39</b>
<i>Table 5</i> Components of the PCR reaction mixture for amplification of the <i>ESF1</i> gene from the recombinant vector pTGESF1.	<b>page 44</b>
<i>Table 6</i> Components of the PCR reaction mixture for amplification of the <i>ESF1</i> gene from cDNA of the <i>ESF1</i> gene sequence from the vector pTGESF1 isolated from yeast transformant A5.	<b>page 46</b>

## ABBREVIATIONS

A <sub>220</sub>	Absorbance at 220nm
ABTS	2,2'-azino-di-(3-ethyl)-benzothiazoline sulfonic acid
AMV-RT	Avian myeloid virus reverse transcriptase
BCIP	5- bromo- 4-chloro- 3- indoyl- phosphate
bp	Base pair
BSA	Bovine serum albumin
BSA-TBS	Bovine serum albumin tris buffered saline
Da	Dalton
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ESMS	Electro spray mass spectrometry
f. sp	formae species
HPLC	High pressure liquid chromatography
HRPO	Horse radish peroxidase
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
IPTG	Isopropyl- $\beta$ - D- thio- galactopyranoside
kDa	Kilodalton
mRNA	Messenger ribonucleic acid
NBT	Nitroblue tetrazolium chloride
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
UV	Ultraviolet
X- gal	5- bromo- 4- chloro- 3- indoyl- $\beta$ - D- thio- galactopyranoside
cDNA	copy DNA

## ACKNOWLEDGMENTS

I would like to express my sincere thanks and appreciation to the following people and institutions for their contribution to this thesis :

Professor John Hastings for his guidance, advice, supervision and input as supervisor during my research study, and compilation of this thesis.

Mervyn Beukes for his unending support, encouragement, advice, and brainstorming during the most trying times of my research study, and also for the invaluable friendship, willingness to help, and space in his office.

Professor Karl Kunert for his assistance and supervision at the initial stage of this project.

Drs, Theresa Coetzer, and Dean Goldring for sharing their antibody expertise with me, and also for assisting me and providing invaluable advice on production and purification of antibodies.

Ramola Chauhan, for her technical assistance with DNA and amino acid sequencing, encouragement and friendship.

Dr Thinus van der Merwe of the University of Stellenbosch, for the ES-MS analysis.

Manil Ramnath, for his support, friendship, confidence, willingness to help, for enduring my foul moods, and for his 'tin fish' sandwiches.

Thuli Kunene and Gina Geornaras who helped preserve my sanity, and gave me days of sunshine and happy faces, encouragement and friendship.

Fellow colleagues in the 'lab 212', that have passed by during my research study, for the fun and laughter.

Megan Kufal for her kindness, efficiency, willingness to help, smiles and thoughtfulness.

The African Explosives and Chemical Industries (AECI) for their financial assistance.

Andrew Graham, for being the calm after the storm.

My family, for their unending love, support, understanding, inspiring belief in me and encouragement to persevere.

# **CHAPTER ONE**

## **LITERATURE REVIEW**



## **1.1 An introduction to antimicrobial peptides**

Plants and animals have to survive in a world laden with pathogenic microorganisms. In order to prevent and overcome infection, plants and animals have developed many defense mechanisms. In animals, including humans, mediation of defense occurs by events such as phagocytosis, complement activation, the immune response, and the release of small, low molecular weight antimicrobial peptides. However, in lower organisms, like amphibians and insects, it is clear that small antimicrobial peptides play a major role in warding infection (Bevins and Zasloff, 1990). Thus, antimicrobial peptides have become recognized as important contributors to nonspecific host defense for both vertebrates and invertebrates. These antimicrobial peptides are easily and rapidly synthesized and have a broad specificity against prokaryotic cells and general lack of toxicity against eukaryotic hosts (Boman, 1995).

The growing incidence of antibiotic resistance amongst pathogenic microorganisms, has stimulated the search for alternative sources of antimicrobial agents that occur naturally in the environment. Antimicrobial peptides have properties that make them a potentially ideal source of new effective therapeutic agents, and this has evoked efforts to elucidate new structures, of which, more than 70 have already been identified (Boman, 1995).

## **1.2 The nature of cationic antimicrobial peptides**

Numerous antimicrobial peptides from a variety of species have been isolated and studied. These antimicrobial peptides have been found to be typically cationic. They have a net positive charge of at least +2 at neutral pH, a minimum of four lysine and/or arginine residues, are amphipathic and usually range between 12 and 45 amino acids in length (Hancock and Lehrer, 1998; Hancock, 1997). These peptides are produced either in response to infection, or constitutively. There are two basic structural classes of cationic peptides : (1) the alpha ( $\alpha$ )-helical peptides, especially in the presence of structure enhancing solvents, like trifluoroacetic acid, or membrane-mimicking environments;

(2) the beta ( $\beta$ )-sheet peptides which usually contain structure stabilizing cysteine-disulphide bonds. Some peptides contain both  $\beta$ -sheet elements, as well as, an associated  $\alpha$ -helical domain, while others may contain numerous proline, tryptophan or histidine residues. However, within the great structural diversity of peptides, common structural patterns have emerged that assist in classification of these cationic antimicrobial peptides.

### 1.3 Overview of antimicrobial peptides

#### 1.3.1 Antimicrobial peptides from mammalian defense systems

Mammals have a microbicidal host defense mechanism that has, as contributing factors to defense, numerous protein molecules, and these include the mammalian defense peptides that are cationic in nature. Mammalian defensins generally consist of about 29-35 amino acids, including approximately six cysteine residues that contribute to three intramolecular disulfide bonds. Mammalian defensins fall into two categories:  $\alpha$ - or 'classic' defensins, and  $\beta$ -defensins (<http://bsd.uchicago.edu/~immunobio/papers/defensin/lab.html>). The  $\alpha$  and  $\beta$ -defensins differ with respect to the positions and bonding pairs of their cysteine residues. They also differ with respect to their sites of expression and type of precursors they may form (Hancock and Lehrer, 1998). The cDNAs of a member from each class of defensin have been cloned. Defensins have been found to be synthesized initially as preprodefensins, the prepiece being a signal peptide for directing of the cationic peptide to the endoplasmic reticulum, and an anionic propiece, implicated in neutralizing the effect of the cationic defensin, before cleavage from the preproregion. Defensins have been isolated from granules of neutrophils of humans, rabbits, rats, guinea pigs, and hamsters (Mak *et al.*, 1996).

As a first line of defense against invading pathogens, phagocytic neutrophils are deployed in the body. Defensin peptides are found within the neutrophil cytoplasmic granules, composing about 5-15% of the total cellular protein (Hancock *et al.*, 1995). To date there

has been six human defense peptides isolated, designated HP 1-6. HP 1-4 are  $\alpha$ -defensins, and they exhibit varying degrees of antimicrobial and/ or virucidal properties *in vitro* (Zenno *et al.*, 1994; Inouye, 1994). HP peptides have their first and last cysteines linked and are therefore considered to be cyclic (Selsted and Harwig, 1989). In addition, they are considered to be amphipathic, since they form dimers with charged and hydrophobic molecules, clustered on two different faces of the molecule. HP-1 is able to permeabilize the outer and inner membranes of *Escherichia coli*, forming voltage gated ion channels (Kagan *et al.*, 1990).

Human beta-defensin 1 (HBD-1) has been identified in several mucosal epithelia. The constitutive expression of HBD-1 in the gingival epithelial tissue was shown by reverse transcriptase-polymerase chain reaction of HBD-1 mRNA from both inflamed and non-inflamed tissues (Krishnaprakornkit *et al.*, 1998). It was concluded from this that HBD-1 may play a role in the maintenance of normal gingival health. Human airway epithelia also express HBD-1 which may contribute to the antimicrobial activity of airway surface fluid and mucosal defenses of the lung (McCray and Bentley, 1997). Inflamed skin has been shown to make HBD-2, whose properties have not been clearly determined as yet (Harder *et al.*, 1997). The murine homolog of HBD-1, called mouse beta-defensin (MBD-1), has been isolated and characterized (Bals *et al.*, 1998). MBD-1 is present in the respiratory system and the mucosal surfaces of mice.

$\beta$ -defensin peptides that are structurally related were isolated from bovine neutrophils (Selsted *et al.*, 1993). Tracheal antimicrobial peptide (TAP) was the first  $\beta$ -defensin 27 residue, bovine peptide to be isolated and characterized from bovine tracheal mucosa (Diamond *et al.*, 1991).  $\beta$ -defensins are clearly distinguishable by consensus sequences at the gene and protein levels, and their disulfide array, although they are similar in size, *in vitro* antibiotic activity, and structural features to other  $\beta$ -defensins (Gallagher *et al.*, 1995). It was recently found that TAP and lingual antimicrobial peptide (LAP), also a  $\beta$ -defensin, isolated from bovine tongue, can be inducibly expressed by inflammatory mediators like bacterial lipopolysaccharide (Russell *et al.*, 1996).

Structurally distinct from the  $\beta$ -defensins of bovine neutrophils, are 2 potent novel antimicrobial peptides also isolated and characterized from the larger cytoplasmic granules of bovine neutrophils. The peptide, indolicidin, is a 13-residue tridecapeptide amide, having potent antibacterial activity (Subbalakshmi *et al.*, 1996). The primary structure of indolicidin is unique to other peptides in that it contains 5 tryptophan residues interspersed with proline residues. The other novel antimicrobial peptides from bovine neutrophils are the batenecins, which are arginine-rich peptides (Romeo *et al.*, 1988, Gennaro *et al.*, 1989). Batenecins display antibacterial activity against Gram negative and Gram positive bacteria. Two of the batenecins can be grouped together because of their high proline content. The other batenecin is a dodecapeptide forming a cyclic loop structure because of a disulfide bridge, formed by its 2 cysteines (Romeo *et al.*, 1988).

Potent antibacterial cationic peptides, called protegrins, have been identified from porcine leukocytes (Kokrayakov *et al.*, 1993). Protegrins share some similarities in sequence with mammalian defensins. Protegrin-1 is 18 amino acids in length and contains an amidated carboxyl terminus, and 2 disulfide bridges. Protegrins do however maintain a  $\beta$ -sheeted tertiary structure (Harwig *et al.*, 1995). These peptides have activity against important sexually transmitted disease-causing pathogens, significantly *Neisseria gonorrhoeae* (Qu *et al.*, 1996), *Chlamydia trachomatis* (Yasin *et al.*, 1996), and human-immunodeficiency virus type I (Yasin *et al.*, 1996). Protegrins are noted for their ability to maintain antimicrobial efficacy at physiological concentrations of sodium chloride (Harwig *et al.*, 1996).

### **1.3.2 Antimicrobial peptides from insect defense systems**

Insects tend to show effective resistance against infection by pathogenic microorganisms. Antimicrobial activity of the hemolymph of insects seems to be induced after the insect is challenged by microbial penetration or by injury within the humoral immune system (Hoffmann and Hoffmann, 1990). Insect defense peptides can be grouped into 4 convenient categories: (1) the cecropins which are typical amphipathic  $\alpha$ -helical peptides approximately 4 kDa in size, and having activity against both Gram negative and Gram

positive bacteria (Hoffmann and Hetru, 1992); (2) polypeptides ranging in size from 8 kDa to 27 kDa, often rich in glycines: these include coleopteracin (Bulet *et al.*, 1991), dipteracin (Reichhart *et al.*, 1989), sarcotoxins (Okado and Natori, 1985; Ando and Natori, 1988), 20kDa attacins (Hancock *et al.*, 1995). The molecules are either bactericidal or bacteriostatic against Gram negative bacteria; (3) proline-rich peptides that have been isolated from Hymenoptera (Casteels *et al.*, 1989) and Diptera (Bulet *et al.*, 1993), and a Hemiptera bug *Pyrrhocoris apterus* (Cociancich *et al.*, 1994); and (4) the insect defensins, which contain six cysteines that form 3 intramolecular disulfide bonds (Hoffmann and Hetru, 1992). Insect defensins occur throughout the insect kingdom, in insect orders including Diptera, Coleoptera, Hymenoptera, Hemiptera and Odonata. These inducible antibacterial peptides seem to be primarily active against Gram positive bacteria.

Cecropins were first isolated from the hemolymph of the giant silkworm moth, *Hyalophora cecropia* (Hultmark *et al.*, 1980). The 3 principal insect cecropins are cecropin A, B and D, and they are between 35-37 amino acids long, with a structure made up of 2 amphipathic segments joined by a glycine-proline hinge (Holak *et al.*, 1988). Gram negative bacteria are more sensitive to cecropins, than are gram positive bacteria (Boman and Hultmark, 1987). Cecropin B has been shown to disrupt the bacterial outer membrane, whereas protoplasts of human pathogenic *Staphylococcus* strains were resistant to cecropin B, suggesting that the cytoplasmic membrane of Gram positive bacteria are less susceptible to the peptide (Moore *et al.*, 1996). Cecropins are thought to exert their activity by forming pores in the bacterial outer membrane, thus causing lysis of bacterial cells (Christensen *et al.*, 1988). A 31-residue cecropin-like peptide, cecropin P1, isolated from the pig intestine, may suggest the existence of mammalian cecropins (Lee *et al.*, 1989). Cecropin P1 offers also a variation from the  $\alpha$ -helical basic structural class of cationic peptides, in that it has an uninterrupted 24 residue amphipathic helix which is bounded by 2-4 residues at the – and C-termini (Sipos *et al.*, 1992).

Proline-rich peptides range from 15-34 residues in length, 25% of which are proline residues (Cherynsh *et al.*, 1996). Proline-rich peptides usually contain arginine-proline or lysine-proline doublets, but may also contain an arginine-proline-arginine triplet which

appears in mammalian proline-rich peptides e.g. batenecins. The first inducible antibacterial peptide, drosocin, from the fruit fly *Drosophila*, is a small, 19 residue peptide that requires an O-glycosylation in order to retain its full biological activity (Bulet *et al.*, 1993). Besides the proline-rich peptides from the honeybee, drosocin and pyrrhocoricin both carry O-glycosylated substitution, N-acetylgalactosamine-galactose disaccharide. Metalnikowins, recently isolated and characterized from the hemipteran insect, *Palomina prasina*, are also proline-rich peptides that lack the O-glycosylation (Cherynsh *et al.*, 1996). Metalnikowins seem to be more bacteriostatic in nature, rather than bactericidal like drosocin and pyrrhocoricin (Bulet *et al.*, 1993; Cociancich *et al.*, 1994).

The smaller of the glycine-rich insect defense peptides that have been isolated, are the 8 kDa dipterocins, from *Phormia* and *Drosophila* species, which have 18% and 22% of glycine residues, respectively. The fat body of *Phormia* has been shown to be the site of biosynthesis of dipterocins (Reichhart *et al.*, 1989). Coleopterocin, is a 74 residue 18% glycine-rich molecule that has been isolated from the large tenebrionid beetle, *Zophobas atratus* (Bulet *et al.*, 1991). The sarcotoxin IIA glycine-rich peptide isolated from the flesh-fly *Sarcophaga peregrina* was reported to share 30% homology at the carboxyl terminus with basic attacins, attacins being 20 kDa peptides isolated from *Cecropia* (Okado and Natori, 1988). The mode of action of sarcotoxin IIA was investigated, and it was shown that the 24 kDa peptide, affects the morphology of cells, by inhibiting cell wall synthesis, including septum formation (Ando *et al.*, 1987). Similarly, attacins are suggested to act on the outer membrane of cells, consequently affecting the bacterial division cycle.

Insect defensins are the only group of the insect defense peptides that have disulfide bridges. These peptides have some sequence homology to rabbit lung macrophage bactericidal peptides, therefore the name 'insect defensins' (Lambert *et al.*, 1989). Insect defensins differ from mammalian defensins in that they are secreted into the blood and are considered to be acute phase reactants. Insect defensins are moderately cationic (pI 8-8.5), non-glycosylated peptides, comprising 38-43 amino acids (Lepage *et al.*, 1991). The general structure of insect defensins shows an antiparallel  $\beta$ -sheet linked to an amino-terminal loop by a disulfide bond, and the  $\alpha$ -helix is stabilized to the second strand of the

$\beta$ -sheet by the other 2 disulfide bonds (Hoffmann and Hetru, 1992). The variability in structure of defensins from the dipteran, coleopteran and paleopteran insect orders is the differing number of residues in the amino terminal loop (Hoffmann and Hetru, 1992). Insect defensins are also synthesized, at first, as preprodefensins. The prepiece is responsible for insertion of the peptide into the cytoplasmic reticulum, and the propiece for stabilization of the peptide (Ganz and Lehrer, 1995). Insect defensins display predominant antibacterial activity against Gram positive bacteria (Hoffmann and Hetru, 1992). The larvae of the beetle, *Allomyrina dichotoma* produces a 43 amino acid defensin that exhibits antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from a patient (Miyashonita *et al.*, 1996). This may become significant as a potential drug for staphylococcal infection, especially with the growing number of resistant strains of medically important pathogens.

Sapecin B isolated from *Sarcophaga* is the only sapecin detected in larval brain, although it is induced similar to the sapecins in response to larval body injury. Sapecin B expression has been implicated in regulation of potassium channels in the brain, while on injury of the larval body, it is expressed in the fat body and secreted into the hemolymph to prevent bacterial infection (Lee *et al.*, 1995).

A novel, highly basic, 42 amino acid, antibacterial peptide has been isolated from the hemolymph of the silkworm, *Bombyx mori* (Hara and Yamakawa, 1995). The peptide was named, moricin, and shows no similarity with antibacterial peptides. Moricin showed higher activity than cecropin B (also a peptide from the silkworm) to Gram positive organisms, especially *S. aureus*. The N-terminal part of the moricin molecule assumes a predicted  $\alpha$ -helix which increases bacterial membrane permeability (Hara and Yamakawa, 1995).

Thanatin, a 21-residue peptide isolated from the insect, *Podiscus maculiventris*, exhibits the largest antimicrobial spectrum observed so far in an insect defense peptide (Fehlbaum *et al.*, 1996). Thanatin is bactericidal and fungicidal, and has sequence homology to brevinins from frog skin (Fehlbaum *et al.*, 1996).

### 1.3.3 Antimicrobial peptides from amphibians

Through approximately 30 years of research, Vittorio Erspamer and colleagues revealed that the skin of frogs has amazing significance as a source of biologically active peptides (Bevins and Zasloff, 1990). The dermatus glands of the frog synthesize and release an extensive variety of pharmacologically active peptides and antimicrobial peptides. The pharmacologically active peptides include mammalian-like hormones and neuropeptides, including angiotensin and bradykinin (Bevins and Zasloff, 1990), while the neuropeptide variety includes opioids and deltorphins (Charpentier *et al.*, 1998). Production of cytolytic and antimicrobial cationic peptides in large amounts, is suggested to be significant in the naked skin of the frog against microbial invasion and assistance in wound recovery.

Antimicrobial peptides of the frog skin are grouped into 3 broad categories, on the basis of their sequence and structure characteristics (Nicolas and Mor, 1995): (1) Linear amphipathic helix-forming peptides, e.g magainins and related peptides from the African claw frog *Xenopus laevis*, and the dermaseptins from the South American arboreal frogs, *Phyllomedusa sauvagei*. The linear amphipathic peptides show broad spectrum antimicrobial activity against yeast, protozoa, bacteria and fungi, but are not hemolytic/cytolytic. (2) Four different groups of related peptides isolated from a variety of Ranidae family species, namely brevinins-1 and -2, esculentins -1 and -2, and gaegurins. These peptides differ from linear amphipathic peptides in that they contain 2 cysteines at the carboxyl terminus that are linked in a disulfide bridge. (3) Small antimicrobial peptides of about 10-13 residues in length isolated from the *Rana temporari*, called temporins, make up the third category.

It is noted that for a particular species of amphibia, a host of unique antimicrobial peptides will be produced, having overlapping structural features, but each targeting a specific microorganism. The presence of these structurally related peptides at the same time, is thought to suggest a synergism of antimicrobial activity which lends to a more enhanced shield against a wide variety of organisms. This type of functional synergism was found to be the case when the magainins pGLa and magainin-2 were added together, in an attempt



to dissipate membrane potential against *E. coli*, permeabilize protein free liposomes for glucose, and to test for the toxic effects of tumor cells (Westerhoff *et al.*, 1995).

Magainins are grouped in the first category known as, linear amphipathic helix forming peptides. The first two magainins were named magainin-1 and -2, and were isolated from the ventral skin of the frog *Xenopus laevis* (Zasloff, 1987). Magainins are also peptides that are synthesized as precursor molecules with a conserved 20 amino acid signal sequence (Zasloff, 1987; Bevins and Zasloff, 1990). The family also shares a common amino terminal processing motif, ARG-XXX-VAL-ARG, indicating a similar enzymatic cleavage activity in the processing of the mature peptide. The peptides also have an acidic amino-terminal pro region (Bevins and Zasloff, 1990; Hancock *et al.*, 1995). The dermaseptin family comprise, lysine rich, amphipathic  $\alpha$ -helical, 28-34 residue peptides, isolated from the skin secretions of *Phyllomedusa bicolor* (Charpentier *et al.*, 1998), and *Phyllomedusa sauvagei* (Mor and Nicolas, 1994). While screening to assess relatedness of dermaseptin genes, a gene sequence coding for a novel dermaseptin was identified by Fleury and colleagues (1998). This novel dermaseptin was called Drg3, and exhibited 23-42 % amino acid identity with the other members of the family (Fleury *et al.*, 1998). The first coding exon of the *Drg3* gene contains conserved nucleotides that encompass the 25 residue preproregion of precursors of several opioids and various amphibian originating peptides (Fleury *et al.*, 1998).

Besides the 3 cytolytic peptides brevinin-1, brevinin-2 and esulentin, 10 new peptides, ranging in size from 24-46 residues, all containing the carboxyl terminus intramolecular disulfide bond, were isolated from the European frog, *Rana esculenta* (Simmaco *et al.*, 1994). The antimicrobial peptides of *Rana esculenta* are divided into 4 subfamilies: (1) Brevinin-1E peptides are highly hemolytic, with 6 positions occupied by the same residue in all these peptides. They are 24 residue peptides containing a proline at position 3 and a pair of basic residues at the carboxyl terminus; (2) the type 2 brevinins generally consist of 33-34 residues, except for brevinin-2Ed and -2Ee which contain a nonapeptide at the carboxyl terminus; (3) esculentins 1a, -1b and -1, are all 46 residues in length, with only 2 or 3 amino acid substitutions, respectively; (4) this group consists of the esculentins-2a and

-2b, which are 37 amino acid peptides (Simmaco *et al.*, 1994).

### 1.3.4 Antimicrobial peptides from bees

Field bees are largely exposed to plant-associated microorganisms when gathering pollen and nectar. It has been found that bees produce numerous antimicrobial peptides in their hemolymph, upon challenge with foreign material. Bees belong to the insect order Hymenoptera. Similar to the other insect species discussed earlier bees also contain inducible immune systems. Bee peptides have been identified as a unique family of peptides therefore they are classed separately from other insect antimicrobial peptides. The honeybee *Apis mellifera* produces some novel antimicrobial peptides, namely, magainins (Zasloff, 1987), abaecin (Casteels *et al.*, 1990), hymenoptaecin (Casteels *et al.*, 1993), royalisin (Fujiwara *et al.*, 1990), bee defensin (Casteels *et al.*, 1993) and the apidaecins (Hancock *et al.*, 1995).

The apidaecins isolated from the lymph fluid of the honeybee was discovered to contain 3 different isoforms, apidaecin IA, IB and II. Apidaecin consists of 18 amino acids which includes 6 proline residues and 3 arginine residues (Taguchi *et al.*, 1994). Apidaecins are extremely stable at high temperature and low pH, and exhibit activity primarily against Gram negative and plant associated bacteria (Hancock *et al.*, 1995). Mutational analyses of the apidaecin gene in a secretory expression system was carried out in order to determine structure-function relationship of the peptide. This study showed that the proline residues play an important role in expression of activity of apidaecin (Taguchi *et al.*, 1994).

Besides the 3 apidaecins, a fourth bee immune response antibacterial peptide has been identified. The peptide was named abaecin, and is a 34-residue peptide with an almost 30 % proline residue composition (Casteels *et al.*, 1990). The abaecin amino terminal is related to apidaecin and the proline motifs are similar (Casteels *et al.*, 1990), but the abaecin differs from apidaecins in that it displays lower specific activities against Gram negative plant pathogens, and lacks the ability to inhibit bacteria at medium ionic strength.

Furthermore its highest specific activity is against an apidaecin-resistant *Xanthomonas* strain, and unlike the immediate activity that apidaecins displays, abaecin shows a delayed activity (Casteels *et al.*, 1990). This could suggest different mechanism of action for abaecin, although structurally similar to apidaecins.

Hymenoptaecin, is a 93 amino acid polypeptide isolated also from the honeybee (Casteels *et al.*, 1993). Hymenoptaecin shows no structural similarities to any of the other bee peptides isolated. It is 19 % glycine rich and contains no cysteines, although showing similarities to the insect defensin, dipteracin. This large peptide inhibits viability of both Gram negative and Gram positive bacteria under physiological concentrations (Casteels *et al.*, 1993).

Royal jelly secreted from the pharyngeal glands of honeybees has extensive antimicrobial activity. Activity against both Gram negative and Gram positive bacteria, molds and fungi has been noted (Fujiwara *et al.*, 1990). Royalisin is the 51 residue peptide isolated from royal jelly. It is an amphipathic molecule and contains 3 intramolecular disulfide bridges, and sequence homology to the insect defensins, sapecin and phormicin. Royalisin is suggested to be representative of the insect defensin peptide family in bees (Hancock *et al.*, 1995).

*Apis mellifera*, also produces as the major protein component of its venom, the highly hemolytic broad spectrum antibacterial peptide, mellitin (Hancock *et al.*, 1995). Mellitin is composed of 26 amino acids and has an amide at the C-terminus (Juvvadi *et al.*, 1996). Mellitin is an amphipathic,  $\alpha$ -helical molecule. The C-terminal segment has a basic hydrophilic, highly charged nature and is composed of 4 adjacent basic amino acids, spanning positions 21 to 24 (Juvvadi *et al.*, 1996). Mellitin is one of the best studied peptides. Two  $\alpha$ -helical segments comprise the mellitin chain at residues 1 - 10 and 13-26, with the shape of the molecule being that of a bent rod at residues 11 and 12. Mellitin displays the property of killing eukaryotic cells. Studies on mellitin have assisted in relating the structure of peptides to their activity.

The venom of the bumblebee, *Megabombus pennsylvanicus*, has also been shown to contain 5 structurally related, hydrophobic heptadecapeptides. These 5 peptides are called bombolitins, and although not structurally related to other bee-derived peptides, they do display functional similarities to them (Argiolas and Pisano, 1985).

### 1.3.5 Antimicrobial peptides from plants

Similar to the animal kingdom, the plant kingdom has also evolved numerous different mechanisms to adapt to the constant invasion by phytopathogenic microorganisms. Over the past few years, it has become increasingly apparent that several families of small basic, cysteine rich antimicrobial peptides may also play a role in plant defense. These families include, lipid transfer proteins (Garcia-Olmedo *et al.*, 1995), plant defensins (Terras *et al.*, 1995), and thionins (Bohlmann and Apel, 1991).

Thionins, were the first antimicrobial peptides to be isolated from plants over 25 years ago (Fernandez de Calaya *et al.*, 1972). Thionins have some toxic effects on bacteria (Fernandez de Calaya *et al.*, 1972), fungi (Bohlmann and Apel, 1987), yeast (Hernandez-Lucas *et al.*, 1974), and animal and plant cells (Reimann-Philippe *et al.*, 1989). Thionins seems to display the conservation of 6-8 cysteine residues. The three-dimensional structure, which has been elucidated for several thionins shows an apparent homology amongst the tertiary structures. Thionin is a compact L-shaped molecule. The long arm of the L is formed by 2  $\alpha$ -helices, the short arm by 2 short antiparallel  $\beta$ -sheets, and the final (+/- 10) C-terminal residues (Bohlmann and Apel, 1991).

Thionins were first found in wheat. These wheat thionins, the first of which was named purothionin, are of low molecular weight (5 kDa), contain 45 amino acids, with 8 cysteine residues, and basic amino acids have been found throughout the molecule (Bohlmann and Apel, 1991). Other Gramineae such as barley, oats, maize and rye have also been investigated and contain similar proteins. The term 'thionin' is used to indicate the close similarity of these proteins. In dicotyledonous plants, related proteins have been found, of

which viscotoxin is the best studied. Viscotoxin was extracted from leaves and stems of mistletoe, and it contains 3 disulfide bonds, as opposed to 4 disulfide bonds in other thionins (Samuelsson, 1974). The crucifer *Crambe abssynica* produces the thionin, crambin (Bohlmann and Apel, 1991). Crambin consists of 46 amino acids and 3 disulfide bonds is very hydrophobic, and has no positive net charge (Bohlmann and Apel, 1991). Crambin lacks notable biological activity when compared with other thionins. Leaf thionins of barley have shown potential significance in defense against pathogens. Leaf thionins isolated in the outer cell wall of the epidermal cell layer of etiolated barley leaves, show great predominance and toxicity to phytopathogenic fungi, and are therefore indicative of a resistance mechanism in barley leaves (Apel *et al.*, 1990).

A novel class of plant peptides displaying similar structural and functional properties to insect and mammalian defensins have recently been characterized. These plant peptides were subsequently called 'plant defensins' (Terras *et al.*, 1995). Plant defensins generally contain 45-54 amino acids, and have a net positive charge. It was observed also that there is limited sequence conservation in plant defensins after comparing 14 representatives spanning 13 different species of 7 different plant families. Conserved residues are confined to the 8 cysteines and 2 glycines at positions 14 and 34, a glutamic acid at position 29, and an aromatic residue at position 11 (Broekaert *et al.*, 1995). Studies on tertiary structure of plant defensins reveals a triple-stranded, antiparallel  $\beta$ -sheet, and an  $\alpha$ -helix lying parallel to the  $\beta$ -sheet (Broekaert *et al.*, 1995). Plant defensins display the cysteine stabilized  $\alpha$ -helix motif similar to insect defensins (Bruix *et al.*, 1993).

Radish seeds have been shown to contain 2 homologous, 5 kDa cysteine rich proteins designated Rs-AFP1 and Rs-AFP2, both of which exert potent antifungal activity *in vitro* (Terras *et al.*, 1992) and are classed as plant defensins. Studies have shown that the 2 cysteine-rich proteins are abundant in the outer layers of the cell, lining different organs of the seed; and are released after disruption of the seed coat at a concentration sufficient to provide a micro-environment around the seed which suppresses fungal growth (Terras *et al.*, 1995).

Further studies have to be carried out to conclude sufficiently that plant defensins and thionins play a role in host defense, in the same manner as these antimicrobial peptides are defense mechanisms in animals. But to date there are several lines of evidence to support this hypothesis of these peptides being important components of the plant defense system.

### **1.3.6 Antimicrobial peptides from bacteria**

From eukaryote to prokaryote, the production of antimicrobial peptides has been noted, although they function in entirely different environments. From the molecular perspective, many facets are similar even if there are systems required to understand antimicrobial peptides from eukaryotic systems better, and vice versa. The general term for antimicrobial peptides produced by bacteria are 'bacteriocins'. Bacteriocins have been grouped into several classes by Klaenhammer (1993) (Table 1). The fourth class of bacteriocins outlined by Klaenhammer (1993), in table 1, are characterized by their complexity and requirement of an associated lipid or carbohydrate moiety for activity (Lewus and Montville, 1992; Jimenez-Diaz *et al.*, 1993). Contrarily, Nes and colleagues (1996) suggest that characterization of such complex bacteriocins as a class on its own may be premature, since these bacteriocins have not been adequately characterized at the biochemical level. Modified bacteriocins, similar to other antimicrobial peptides discussed previously are also produced as prepropeptides which are subsequently cleaved at the prepro leader sequence to release the biologically active peptide (Beck-Sickinger and Jung, 1991). Posttranslational modifications to lantibiotics occur in the propeptide region before cleavage of the leader sequence occurs (Sahl, 1991).

Unmodified bacteriocins require at least 4 genes and in the case of two-peptide bacteriocins, 5 genes, for its production (Nes *et al.*, 1996). These genes are generally located in close proximity to each other. The 4 genes are the bacteriocin structural gene (2 structural genes in two-peptide bacteriocin systems), which codes for the entire prepro form of the bacteriocins; an immunity gene which encodes a protein that serves to protect the producing bacteria against the toxic bacteriocins it produces, thus providing immunity

to the producing organism; membrane associated ABC transporter gene which is involved in transfer of the bacteriocins across the cell membrane; a gene encoding an accessory protein also associated with secretion of bacteriocins (Nes *et al.*, 1996).

The genes required for biosynthesis of lantibiotics include: (1) the structural gene encoding the prepeptide; (2) genes encoding one or more proteins required for immunity; (3) genes encoding a serine protease that is presumed to cleave of the leader peptide of the prelantibiotic; (4) genes encoding an ABC transporter likely to be involved in export of bacteriocins; (5) genes coding for one or two proteins involved in catalysis of dehydration and lanthionine ring formation; (6) the genes of two component regulatory proteins (Sahl *et al.*, 1995) for induction of bacteriocin expression by extracellular signal transmission.

Table 1 Bacterial peptide classes as characterized by Klaenhammer (1993)

<b>Group I</b> Modified peptide bacteriocins (Lantibiotics)	Class I	Known as lantibiotics; 5 kDa; small membrane active peptides containing posttranslationally modified lanthionine residues	
<b>Group II</b> unmodified peptide bacteriocins (Non- lantibiotics)	Class II (a,b,c)	Small heat stable, non-lanthionine containing membrane-active peptides (<10 kDa) characterized by Gly-Gly- 1**+1 Xaa processing site in the bacteriocin precursor	(a) <i>Listeria</i> -active peptides with a consensus sequence in the N-terminus of -Tyr-Gly-Asn-Gly-Val- Xaa-Cys (b) Poration complexes consisting of 2 proteinaceous peptides for activity e.g., lactacin F (c) Thiol-activated complexes requiring reduced cysteine residues for activity e.g., lactococcin B
	Class III	Large heat labile-proteins, >30kDa e.g ; helveticin J	
	Class IV	complex bacteriocins, composed of protein and one or more chemical moieties (lipid or carbohydrates) required for activity e.g., plantaracin S	

Comparison of lantibiotic gene clusters and encoded proteins also show non-homologous genes in some gene clusters e.g., the *nisI* gene of the nisin gene cluster, and the *spaI* gene of the subtilisin cluster, code for lipoproteins involved in immunity (Seizen *et al.*, 1996). Nisin, the first lantibiotic to be characterized is produced by *Lactococcus lactis* strains carrying a transposon-containing genes coding for the nisin precursor and biosynthesis and resistance of nisin (Kaletta and Entian, 1989). Nisin, has been used for the past 30 years and is currently widely used as a food preservative, mainly in dairy products like cheese, and in canned vegetables (Chan *et al.*, 1996).

There have not been many antimicrobial peptides isolated from fungi. Sillucin, a defensin-like peptide, active against Gram-positive bacteria, has been isolated from a thermophilic fungus, *Rhizomucor pusillus* (Hancock *et al.*, 1995).

### **1.3.7 Antimicrobial peptides from other species**

Not many cationic peptides have been characterized in other species, besides mammals, insects, amphibians and chelicerates. Recently the crustacean shore crab, *Carcinus maenas* was reported to produce in its haemocytes, a proline-rich antibacterial peptide with sequence similarity to bactenecin-7, the antimicrobial peptide from bovine neutrophils (Schnapp *et al.*, 1996). Other crustaceans that produce antimicrobial peptides in response to bacterial infection, are the horse shoe crab species including, *Limulus polyphemus* and *Tachypleus tridentatus*. These horse shoe crabs produce 2 classes of antimicrobial peptides, the 17 residue tachyplesins and 18 residue polyphemusins, which are contained within cytoplasmic granules (Hancock *et al.*, 1995). Both classes of peptides contain cysteines which participate in 2 disulfide linkages (Matsuzaki *et al.*, 1997; Hancock *et al.*, 1995). These peptides display activity against bacteria and fungi (Hancock and Lehrer, 1998).

A class of highly potent antibacterial peptides derived from pardaxin, a pore-forming peptide isolated from the Moses sole fish *Pardachirus marmoratus*, have been found (Oren and Shai, 1996). Pardaxin is a 33 amino acid peptide which is structurally similar to



mellitin, comprising 2 helices with a proline hinge between them (Oren and Shai, 1996).

Blood of immune challenged and untreated mussels (*Mytilus edulis*), has been reported to produce antibacterial and antifungal peptides (Charlet *et al.*, 1996). One 34 residue peptide, mytilin, with potent antibacterial activity, and another with apparent characteristics of the insect defensins, were found in these mussels. A novel 6.2 kDa cysteine-rich antifungal peptide was also partially characterized. This may suggest that the separation of arthropods and molluscs occurred after an apparent host defense mechanism of peptides had become apparent (Charlet *et al.*, 1996).

Clavanins which are amphipathic histidine-rich, amidated  $\alpha$ -helical peptides have been isolated from the hemocytes of the tunicate, *Styela clava* (Lee *et al.*, 1997a). These peptides are composed of 23 amino acids, and manifest potent antimicrobial activity at high salinity and acidic pH (Lee *et al.*, 1997b).

It is almost certain with the search for antimicrobial peptides in various types of species being carried out, there will definitely be a greater variety of peptides in the future to support new classifications.

#### **1.4 Structure of cationic peptides**

Basically, all the cationic peptides have 2 distinguishing features, these are: (1) a net positive charge of a minimum of +2 due to the presence of arginine and lysine residues in the peptide sequences; and modified amino acids in the case of lantibiotics; (2) the peptides are folded into a 3-dimensional structure, consisting of a hydrophobic face consisting of non-polar amino acid side chains, and a hydrophilic face of polar and positively charged residues, i.e., amphipathicity. These peptides although similar in the above-mentioned features, shows considerable variation in length, amino acid sequence, and secondary structure. Most peptides possess these general characteristics, and on secondary structure fit into 4 major classes:  $\alpha$ -helices, extended helices with the existence of a dominant

residue throughout the peptide,  $\beta$ -sheet structures stabilized by 2-3 disulfide bridges, and loop structures (Hancock, 1997) (Figure 1).

Some cationic peptides, have been found to differ somewhat from the defined structural classes, sometimes incorporating more than one structural class. Sapecin, the insect defensin, contains 3 disulfide bonds, like animal defensins, but has a considerably different amino acid sequence and the presence of lysines and histidines as positively charged amino acids rather than lysines and arginines (Hanzawa *et al.*, 1990). The solution structure of sapecin contains a flexible loop, an 8 amino acid  $\alpha$ -helix, and 2 extended regions (Hanzawa *et al.*, 1990). This is thought to be due to the different positions of the cysteine disulfide bonds in the animal and insect defensins. Contrary to this hypothesis, scorpion toxins with positions of disulfide bonds similar to animal defensins, have a structure comprising a short triple-stranded  $\beta$ -sheet region, together with a 9 amino acid  $\alpha$ -helix (Bontems *et al.*, 1991).

#### **1.4.1 Structure-Function relationships**

Numerous synthetic peptides have been produced in order to become more acquainted with structure-function relationships of these antimicrobial peptides. A few governing principles about structure and function on the cationic antimicrobial peptides are: (1) a change in amino acid sequence of a peptide influences the activity in a very specific manner, e.g., each position of the 26 amino acid mellitin was individually omitted to generate a series of 24 omission analogs to test for differing bioactivities amongst the analogs (Blondelle and Houghten, 1991). Testing for hemolytic activity of these analogue peptides showed that the activities were nearly the same for all the peptides, except there was increased activity in the analog lacking isoleucine at position 20 (Blondelle and Houghten, 1991). Modellins are synthetic peptides of different lengths and hydrophobicities (Bessalle *et al.*, 1993). These amphipathic peptides were synthesized to be composed of 16-17 amino acids, a hydrophilic lysine composed face, and a highly hydrophobic face due to the tryptophan and phenylalanine residues with high antibacterial

and hemolytic activities (Bessalle *et al.*, 1993). Replacement of tryptophan and phenylalanine with leucine, showed reduced hydrophobicity of the modellins, and a resultant decrease in hemolytic activity was noted, as well as a slightly decreased bioactivity (Bessalle *et al.*, 1993); (2) for  $\alpha$ -helical peptides, changes that increase the tendency to form an  $\alpha$ -helix in aqueous solution tends to increase activity (Oh *et al.*, 1998; Dykes *et al.*, 1998; Blondelle and Houghten, 1991; Cornut *et al.*, 1994); (3) it seems that no relationship exists between the number of positive charges and activity, although the position of specific positive charges is important (Powell *et al.*, 1995; Lee *et al.*, 1997a); (4) chirality of peptide structures have little significance (Wade *et al.*, 1990), and full activity can be achieved with peptides containing either all L- and all D-amino acids in their respective right handed or left-handed helical conformations (Merrifield *et al.*, 1995); (5) there is no absolute relationship between decreased lysis or binding to cells and its correlation with a decreased minimum inhibitory concentration (Hancock, 1995); and, (6) in peptides that contain cysteine disulfide bonds, a reduction of the disulfide bridges destroys activity (Hancock *et al.*, 1995).

Synthetic peptides are now being produced in large numbers and with various modifications of structure in order to produce peptides with improved activity and decreased hemolytic activity among other required features. Length of the peptide may have some influence on cationic peptide bioactivity. There is usually decreased activity in shorter peptides (Hancock and Chapple, 1999; Bessalle *et al.*, 1993).

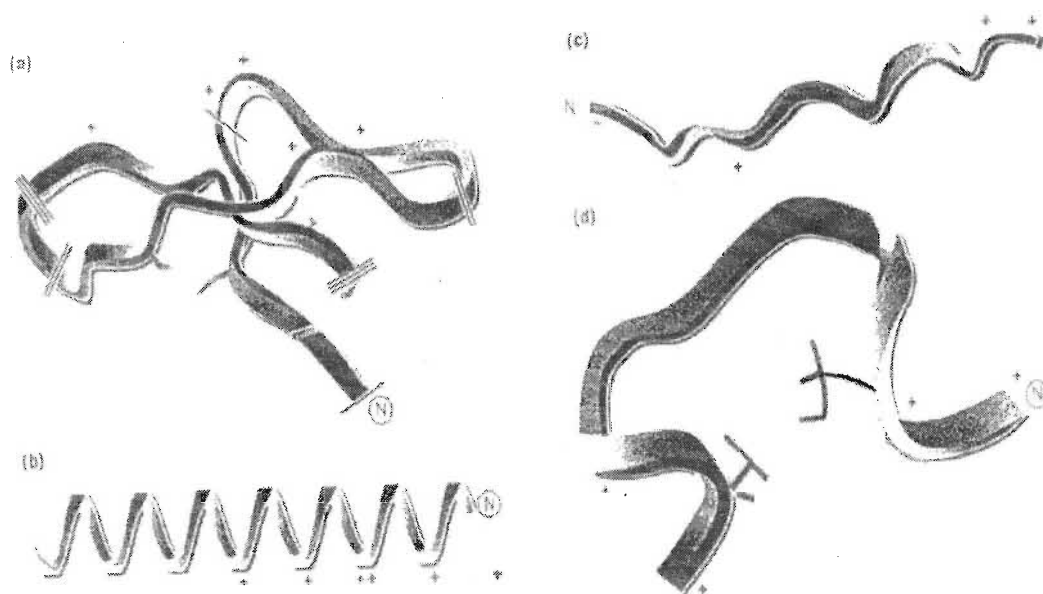
Understanding the general principles that influence structure-activity relationships allows manipulation for enhanced activity. A novel cationic peptide, CP-11, which is based on the structure of the bovine neutrophil peptide, indolicidin, was designed to increase the number of positively charged residues, maintain the 13 amino acid short length, and increase amphipathicity, with respect to these features of indolicidin (Falla and Hancock, 1997). CP-11 and a carboxymethylated derivative CP-11C, demonstrated enhanced activity against Gram negative bacteria, and the yeast *Candida albicans*, a reduced hemolytic activity, and maintained the same activity levels as indolicidin against staphylococci (Falla and Hancock, 1997).

### 1.4.2 Mode of action

There have been numerous studies to determine the mode of action of the cationic antimicrobial peptides, specifically the cecropins, magainins, mellitins and defensins (Hancock and Lehrer, 1998). Currently, the main site of action of these peptides is the cytoplasmic membrane (Falla *et al.*, 1996; Hancock, 1995; Matsuzaki *et al.*, 1997). In Gram positive bacteria the interaction is only with one membrane, i.e., the cytoplasmic membrane. On the other hand, Gram negative bacterial species require, a more involved interaction due to the existence of an outer membrane, and a cytoplasmic membrane.

The predicted mechanism of action based on the model of Christensen *et al* (1988) (Figure 2), states that there is an initial electrostatic interaction between the negatively-charged phospholipid bilayer of the cytoplasmic membrane and the cationic peptide. The bacterial cytoplasmic membrane has a large electric potential, and this influences transition of peptides from an unstructured to structured form. There is an aggregation of peptides in a manner that results in the hydrophobic faces of the peptides being directed toward the interior of the membrane and their hydrophilic faces form the channel pointing inwards. Insertion of these aggregated peptides into the cytoplasmic membrane results in destruction of membrane integrity, and subsequent bacterial cell death, caused by the leakage of cytoplasmic molecules. Factors that favour the formation of channels include, high negatively charged lipid composition of the phospholipid bilayer, large transmembrane potentials maintained by the proton motive force, a lack of cationic lipids and cholesterol (Christensen *et al.*, 1988), which is characteristic of bacterial membranes (Hancock, 1997).

Eukaryotic membranes have cholesterol as part of the lipid composition of the cell's membrane, low membrane potentials and low anionic lipid content, and this could be an explanation for the cationic peptide's specificity for bacterial cell membranes (Boman, 1995). An alternative to the more accepted hypothesis is that the cationic antimicrobial peptides cluster at the cytoplasmic membrane surface and cause a cooperative permeabilisation of the cytoplasmic membrane, also referred to as the 'carpet effect' (Boman, 1995).



**Figure 1** Examples of structures of four classes of cationic peptides. (a)  $\beta$ -strand human defensin-1, (b)  $\alpha$ -helical cecropin mellitin hybrid, (c) extended coil indolicidin, (d) loop structure batenecin. Positive charges denoted by (+), amino termini denoted by (N), and disulfide bridges are present in (a) and (d). In (a) the three  $\beta$ -strands are denoted by pairs of one, two or three lines on the backbone ( Hancock, 1997).

For Gram negative bacterial species it has been proposed that cationic antimicrobial peptides interact with the bacterial outer membrane lipopolysaccharide (LPS), and are taken up by a ‘self- promoted uptake’ pathway (Hancock, 1997; Hancock *et al.*, 1995; Hancock and Lehrer, 1998). The LPS of the outer membrane of Gram negative bacteria is a highly anionic glycolipid. Initiation of the ‘self-promoted uptake’ pathway occurs when the cationic antimicrobial peptide, interacts with the anionic, divalent cation-binding sites on LPS. It has been reported that the addition of 2 positive charges on the carboxyl terminus of a cecropin-mellitin hybrid (CEME), resulted in an enhanced interaction of the peptide with LPS and consequently, the outer membrane (Piers and Hancock, 1994). Direct interactions of other cationic peptides like magainins and defensins with LPS have been demonstrated (Hancock *et al.*, 1995). These interactions seem to be several orders of magnitude higher than the normal divalent cations, demonstrating that this binding is quite

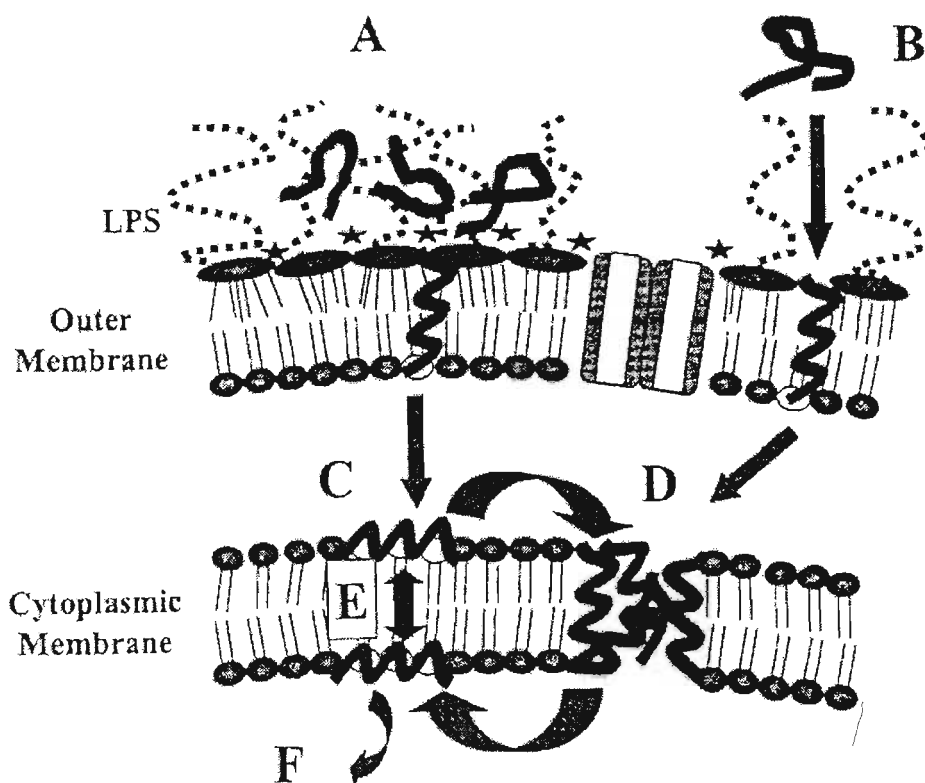
an efficient process. This interaction causes disruption to the normal outer membrane barrier properties by the production of transient 'cracks', which permits the passage of various small molecules, like the cationic antimicrobial peptides, into the cell (Hancock, 1997; Piers and Hancock, 1994) (Figure 2). It is assumed that peptides, that are specific for Gram positive bacteria, lack the ability to access the 'self-promoted uptake' pathway because of the absence of the outer membrane.

Mode of action studies done on lantibiotics show that unlike the amphipathic pore-forming lantibiotics e.g., nisin (Sahl, 1991), the globular lantibiotics appear to be involved in inhibition of enzyme reactions (Brötz *et al.*, 1997). Studies done on the lantibiotic mersacidin, in particular, indicates no impairment of the overall integrity of the cell membrane (Brötz *et al.*, 1998), but instead it exerts bactericidal action by the inhibition of the transglycosylation level of peptidoglycan biosynthesis by the interaction of the Lipid II component of peptidoglycan (Brötz *et al.*, 1998). The lantibiotic actagardine also shows similarity to mersacidin with respect to its mechanism of bactericidal activity (Brötz *et al.*, 1998). Mersacidin and actagardine are of similar size and hydrophobicity, contain 4 intramolecular thioether bridges, and consequently the same globular structure (Brötz *et al.*, 1997). The findings are significant in that there may be different mechanisms of action of different peptides depending on their structures, and that not all antimicrobial peptides have the same mode of action.

## **1.5 Applications of cationic antimicrobial peptides**

### **1.5.1 Antimicrobial efficacy**

Naturally occurring cationic antimicrobial peptides have a wide range of bioactivity levels



**Figure 2** Proposed mechanism of action of cationic antimicrobial peptide with Gram negative outer membrane and the cytoplasmic membrane of bacterial cells. The hypothesized 'self-promoted uptake' pathway of cationic peptide into the cell, occurs when there is an association of the cationic peptide with the negatively charged outer membrane, creating cracks through which the peptide moves across the outer membrane (A); or binding to the divalent cation binding sites on LPS and disrupting the membrane (B). The peptide consequently folds into amphipathic structure (C), once bound to the negatively charged surface of the cytoplasmic membrane. Several peptide molecules insert and span the membrane after forming aggregation complexes (D), or move across the membrane under the influence of the large transmembrane potential (E). (Hancock and Chapple, 1999).

that include a defined to broad spectrum of susceptible microorganisms, and from very potent to weak bioactivity (Hancock and Chapple, 1999). The design of synthetic peptides can be such that these peptide products are non-toxic and can achieve effectively low minimal inhibitory concentrations (MICs) against a broad spectrum of microorganisms. Peptides have the advantage of: (1) being antimicrobicidal at the MIC, and causing rapid killing within 5 minutes at about 4 times the MIC; (2) being unaffected by common clinical resistant mechanisms like methicillin resistant *S. aureus*, thus affording difficult

selection of peptide resistant pathogens; and, (3) showing ability to protect both ,local and systemic infection in animal models (Hancock and Chapple, 1999; Hancock, 1997).

### **1.5.2 Clinical applications**

The pharmaceutical company AMBI, produced ambicin (company name for nisin) and have managed to pass the peptide through phase I clinical trails successfully. Ambicin is being considered as a potential treatment of ulcers caused by *Helicobacter pylori*; and as a replacement treatment for vancomycin to kill of the antibiotic-associated diarrhoeal bacterium *Clostridium difficile*, and vancomycin-resistant enterococci that cause increased risk of patients to lethal systemic infection

(<http://www.businesswire.com/webbox/bw111897/536701.htm> ; Hancock and Chapple, 1999). MSI-78 (Magainin pharmaceuticals), which is also an  $\alpha$ -helical magainin variant peptide, has been passed phase III clinical trials for the treatment of diabetic foot ulcers (Hancock and Lehrer, 1998; <http://www.pslgroup.com/dg/2168e.htm> ).

### **1.5.3 Other applications**

Nisin was introduced by the company Aplin and Baret as nisaplin, in 1957, and is currently being used as a food preservative by over 50 countries. Nisin formulated with 1-propanol has proved to be highly bactericidal against Gram negative pathogens and is now produced as a teat dip to prevent mastitis in cattle (Hancock and Lehrer, 1998). These cationic antimicrobial peptides have also been used in the preservation of cut roses (Florack *et al.*, 1996).

### **1.5.4 Transgenic technology**

It would be feasible to improve the resistance of various species to microbial infection by



attempting to overproduce cationic antimicrobial peptides and synthetic variants thereof by using transgenic technology. This transgenic approach has been attempted several times in plants but with mixed results (Table 2). Transgenic tobacco plants producing the Shiva-1 peptide, a synthetic cecropin B analog, displayed resistance to the bacterial wilt pathogen, *Pseudomonas solanacearum* (Jaynes *et al.*, 1993). SB37 another cecropin B analog showed no increased resistance to bacterial diseases (Cavallarin *et al.*, 1998). The difficulty that seems to be inherent with antimicrobial peptides being introduced into plants to improve their resistance to pathogens, is the peptides susceptibility to intracellular proteases (Cavallarin *et al.*, 1998). Protection of antimicrobial peptides against degradative enzymes within plants would probably require further modeling of the peptide to produce substitution analogs lacking the protease activity site.

Recombinant techniques that have been explored to reduce cost and for easy production methods include, production in insect cells using baculovirus expression systems, yeast expression vectors (Romanos *et al.*, 1992), and in the milk of transgenic mice (Hancock and Lehrer, 1998). However, the exact commercial potential of these procedures has yet to be determined.

Another novel example of transgenic technology has been the introduction of a cecropin A peptide into the endosymbiont bacterium, *Rhodnius prolixus*. This leads to the elimination of *Trypanosoma cruzi* parasite after transformation of the transgenic *R. prolixus* into the disease-transmitting insect (Durvasula *et al.*, 1997).

## 1.6 Expression in yeast

Yeasts are unicellular eukaryotic organisms. These organisms have become attractive systems in the various facets in modern biotechnology. Generally they make effective host organisms for the production of heterologous proteins, specifically eukaryotic proteins. Yeast expression systems on the whole are advantageous in that they possess the ease of genetic manipulation and growth characteristics of prokaryotic organisms, in combination

with the subcellular machinery for complex post-translational modifications (Romanos *et al.*, 1992; Cregg *et al.*, 1993; Gellisen and Hollenberg, 1997). A wide range of yeast species, including *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*, have been used for foreign gene expression. Foreign gene expression in yeast has become an important tool for the production of foreign proteins for research, industrial or medical use (Romanos *et al.*, 1992).

### **1.6.1 Factors influencing foreign gene expression in yeast**

Yeasts being eukaryotic organisms possess similar characteristics to higher eukaryotes and mammalian cells regarding their physiology, protein secretion and basic genetics. Yeasts require simple and accessible nutrition which permits them to be cultured rapidly to a very high cell density (Kukuruzinska *et al.*, 1987). The complete nucleotide sequencing of the yeast genome in 1996 (Goffeau *et al.*, 1996), has facilitated further research that has provided information on various aspects of yeasts including, their growth and cell cycle. The increasing research and information produced on yeast has made it more accessible to easy manipulation. Yeasts possess a highly compartmentalized intracellular organization and with this an elaborate secretory pathway which mediates the secretion and modification of many host proteins. This ability for extracellular secretion of heterologous proteins into culture medium allows the avoidance of toxic accumulated material. Extracellular secretion also affords simple purification procedures for the protein being expressed because not only does the yeast organism secrete very low levels of endogenous proteins (Eckart and Bussineau, 1996), but the yeast expression systems have been genetically manipulated to provide protease-negative mutant host strains for cloning.

Table 2 Transgenic cationic antimicrobial peptides providing disease resistance in plants

Peptide used	Species transformed	Expression levels	Pathogen resisted	Reference
barley $\alpha$ -hordothionin	tobacco	2-60 ng/mg leaf protein	<i>Clavibacter michiganesis</i>	Florack, D (PhD thesis, 1994), cited by Hancock and Lehrer, 1998
barley $\beta$ -hordothionin	tobacco	2-60 ng/mg leaf protein	<i>Clavibacter michiganesis</i>	Florack, D (PhD thesis, 1994), cited by Hancock and Lehrer, 1998
cecropin B	tobacco	not determined	<i>Pseudomonas campestris</i> pv. <i>tabaci</i>	Huang <i>et al.</i> , 1997
tachyplesin I	potato	low expression level	low <i>Erwinia</i> resistance	Allefs <i>et al.</i> , 1996
plant defensin Rs-AFP2	tobacco	0.2-24 $\mu$ g/mg leaf protein	<i>Alternaria longipes</i>	Epple <i>et al.</i> , 1997
Amaranth hevein Ac-AMP2	tobacco	0.6-1.1 $\mu$ g/mg leaf protein	Shows <i>in vitro</i> antifungal resistance only	De Bolle <i>et al.</i> , 1996
Sweet Pepper Knott in Mj-AMP2	tobacco	0.9-1.4 $\mu$ g/mg leaf protein	Shows <i>in vitro</i> antifungal resistance only	Jaynes <i>et al.</i> , 1993
Shiva-1 and SB37 cecropin B analogs	tobacco	up to 1 $\mu$ g/mg leaf protein	bacterial wilt pathogen <i>Pseudomonas solanacearum</i>	Jaynes <i>et al.</i> , 1993
SB37	potato	up to 1 $\mu$ g/mg	bacterial wilt pathogen <i>Pseudomonas solanacearum</i>	Jaynes <i>et al.</i> , 1993
barley $\alpha$ -thionin	tobacco	20 ng/mg leaf protein	<i>Pseudomonas syringae</i> pv <i>syringae</i>	Carmona <i>et al.</i> , 1993

The yeast strain is able to make the proper post-translational modifications on expressed proteins which is imperative to authenticate the mature protein product (Eckart and

Bussineau, 1996; Romanos *et al.*, 1992). Proper post-translational processing of proteins lends to the biological activity, solubility, biodistribution, circulatory half-life and stability of the expressed foreign protein (Eckart and Bussineau, 1996). Also the secretion of properly folded proteins is imperative for the maintenance of biological activity, and is one of the more influential factors favouring yeast as a preferred host for heterologous protein expression (Romanos *et al.*, 1992; Eckart and Bussineau, 1996)

*S. cerevisiae*, commonly known as baker's yeast has achieved GRAS (generally recognized as safe) status, and is therefore a recognized food organism, contributing to it being an ideal system for the production of food and pharmaceuticals (Gellisen *et al.*, 1992; Gellisen and Hollenberg, 1997).

### **1.6.2 Expression and secretion of heterologous proteins in yeast**

A yeast expression system has often been used in the production of heterologous proteins that are unavailable from natural sources, either due to inaccessibility or low purification yields from these natural sources. Also these proteins could be specifically designed proteins with no natural source. This is advantageous in that structural and functional studies can be carried out on the relevant protein with objectives like greater understanding and enhancement of structure and function.

For the expression and secretion of foreign protein in yeast, it is imperative that the appropriate system is chosen, in order to successfully achieve the production of biologically active proteins in high quantities. Many vectors are available for heterologous protein expression in yeast, but the most commonly used vectors are based on the yeast multicopy  $2\mu m$  6.3 kb DNA circle is *S. cerevisiae*. The  $2\mu m$  plasmid contains an autonomous replication sequence that functions as the origin of replication, independent of the yeast chromosome (Romanos *et al.*, 1992). The vectors are generally yeast-*E. coli* shuttle vectors. Yeast expression vectors require effective and appropriate promoters, secretion signal peptides, and transcriptional terminators. For selection of transformants in

yeasts, auxotrophic mutants are used that contain yeast selectable markers, e.g *LEU2*, and *URA3*, which confer auxotrophy for leucine and uracil, respectively. *LEU2-d* and *URA3-d* are truncated promoter variants of *LEU2* and *URA3*, respectively, and are therefore poorly expressed which results in gives higher copy numbers in plasmids expressing the truncated genes (Loison *et al.*, 1989).

Factors contributing to efficient transcription of foreign genes are: (1) cDNAs of the desired protein under yeast promoter control as was shown by the production of leukocyte  $\alpha$ -interferon (Hitzeman *et al.*, 1981); and, (2) yeasts transcriptional terminators which ensure efficient mRNA 3' end formation and consequent maximal expression (Zaret and Sherman, 1982).

The most frequently used signal sequence that directs secretion of heterologous proteins in yeast is the preproregion from the yeast mating factor  $\alpha$  1 (MF $\alpha$ 1) (Romanos *et al.*, 1992). MF $\alpha$ 1 prepro signal sequence is often used with the MF $\alpha$ 1 promoter. MF $\alpha$ 1 codes for the 165 residue prepro- $\alpha$ -factor, which comprise the 19 residue pre signal sequence and a 64 residue proregion. At the carboxyl end of the pro region is a LYS-ARG Kex2 protease cleavage sequence at which point the foreign protein is cleaved from the signal peptide, during its export through the Golgi apparatus (Reichhart *et al.*, 1992).

Studies done on cationic antimicrobial peptides to understand their mode of action and structure-function relationships, requires sufficient amounts of peptide. Often the extraction of naturally occurring antimicrobial peptides from the source is a tedious and difficult process which also generates low yields. Chemical synthesis of antimicrobial peptides can be a practical alternative, but it has also proven to be expensive. For these reasons, there has been a preference for the development of biological expression systems for the facile production of these cationic antimicrobial peptides.

There are several examples of the use of yeast expression vectors for the production of cationic antimicrobial peptides. Insect defensin A, which is a 40 residue peptide, containing 6 cysteines engaged in 3 intramolecular disulfide bonds (Lambert *et al.*, 1989),

has been the first reported cationic antimicrobial peptide to be expressed efficiently, and secreted in a yeast expression system. The defensin A peptide was expressed in frame with the MF $\alpha$ 1 leader sequence under the transcriptional control of the MF $\alpha$ 1 promoter and the phosphoglycerate kinase (PGK) terminator (Reichhart *et al.*, 1992). This study emphasized the importance of the use of an intact signal sequence for the secretion of biologically active peptide, which reinforces the expression of biologically active leech hirudin with the use of the MF $\alpha$ 1 prepro sequence (Loison *et al.*, 1988).

Because the expression of insect defensin A in a yeast expression/secretion system, the radish seed cationic antifungal peptide, Rs-AFP2 has been produced in a similar expression system, and it has also been secreted in a biologically active and correctly processed form (Vilas Alves *et al.*, 1994). Rs-AFP2 contains 4 intramolecular disulfide bonds. This study has also supported the suggestion that the MF $\alpha$ 1 pro sequence is an important requirement for peptide secretion, and it is assumed that this is due to the pro-domain facilitating correct folding of the mature peptide (Vilas Alves *et al.*, 1994). Mutational analysis of Rs-AFP2 was also performed using the polymerase chain reaction-based-site-directed mutagenesis of the *Rs-AFP2* gene and yeast as the expression system. This was based on the same expression system used in the production of native Rs-AFP2. In total 19 correctly processed Rs-AFP2 variants were produced in this way (De Samblanx *et al.*, 1997). The amount of Rs-AFP2 produced heterologously in the yeast system afforded the ability to determine Rs-AFP2 variants with enhanced antifungal activity, and to visualize these variants in three-dimensional representation, in order to perceive structural changes that affected enhanced bioactivity (De Samblanx *et al.*, 1997).

The fruit fly, *Drosophila*, produces drosomycin, a potent 44 residue, cysteine-rich antifungal peptide (Fehlbaum *et al.*, 1994). Drosomycin forms 4 intramolecular disulfide bridges paired identically to those found in plant defensins (Fehlbaum *et al.*, 1994). The 4 disulfide bonds induces the highly compact structure of drosomycin, thus making the molecule resistant to proteases and heat treatment. Isolation of acceptable amounts of drosomycin from the tiny fruit fly has proved difficult (Michaut *et al.*, 1996). A cDNA of drosomycin was therefore expressed in *S. cerevisiae*, and the purified recombinant peptide

displayed the same chromatographic behavior and biological activity as the natural peptide (Michaut *et al.*, 1996). Also, over-expression of the Kex2 protease increased the yield of fully processed drosomycin in the yeast. This successful production of drosomycin in a yeast expression system facilitated determination of an extended activity spectrum including potent activity of drosomycin against agronomically important fungal pathogens; and similarity in disulfide bridge array to another radish seed antifungal peptide. Rs-AFP1. Three dimensional structural analysis of drosomycin was also made possible because there was sufficiently high expression of the peptide in the yeast system (Michaut *et al.*, 1996).

The bacteriocin, pediocin PA-1 was cloned into a laboratory strain of the yeast *S. cerevisiae* Y294 (Schoeman *et al.*, 1999). Pediocin PA-1 has been studied and shown to display food biopreservation qualities (Yousef *et al.*, 1991). The *pedA* gene coding for pediocin PA-1 produced by *Pediococcus acidilactici* was expressed in *S. cerevisiae* Y294 under the control of the *alcohol dehydrogenase I* gene promoter and terminator, and secretion of the pediocin was directed by the Mfa1 secretion signal sequence (Schoeman *et al.*, 1998). The recombinant pediocin PA-1 displayed the same activity spectrum as the naturally produced pediocin, which suggested that there was no alteration of the peptide by the yeast host strain (Schoeman *et al.*, 1999). This study is an example of the use of antimicrobial peptides in creating bactericidal strains of *S. cerevisiae* for use as biopreservatives, rather than using chemical preservation methods, in the production of yeast-fermented food and beverages e.g., wine and bread. The development of the bactericidal yeast strain expressing antimicrobial peptides can be added to the growing list of relevant applications of antimicrobial peptides.

### **1.7 The antifungal peptide, ESF1**

Providing disease resistance in crop plants has always been the major goal of plant biotechnology. Resistance to bacterial and/or fungal pathogens will contribute to a superior plant and increased productivity, which explains the heightening trend in the design of potential plant pathogen-resistant gene products. Antimicrobial peptides with their wide

spectrum of activity are a good source on which to base synthetically produced peptide variants.

ESF1 is a designed synthetic peptide, mimicking the charge distribution and amphipathic  $\alpha$ -helical structure of magainin, pGLa (Soravia *et al.*, 1988, cited by Powell *et al.*, 1995). ESF1 differs from pGLa in that it has a different amino acid sequence and is one amino acid shorter. This 20 amino acid, 2.052 kDa ESF1 peptide, is a member of a series of pGLa-based variants, chemically synthesized for the specific purpose of optimizing its desired activity as a gene product prior to cloning it into a plant (Powell *et al.*, 1995). Significantly high activity has been reported for ESF1 against important fungal pathogens like *Fusarium oxysporum* f.sp *lycopersici* (Powell *et al.*, 1995) and *Fusarium oxysporum* f.sp *cubense* race 4 (Dykes *et al.*, 1998), the wilt-causing fungal pathogens in tomato and banana crop plants, respectively. Modifications of the ESF1 peptide's sequence that encouraged the formation of a tighter  $\alpha$ -helical structure seemed to increase the minimum inhibitory activity of the peptide (Dykes *et al.*, 1998). Studies carried out on other ESF1 analogs, show that, elimination of positively charged residues destroys bioactivity, decrease in size of ESF1 length can cause significant reduction in bioactivity, and that altering positive charge density results in modest decreases in bioactivity (Powell *et al.*, 1995). The ESF1 peptide and its analogues have all been chemically synthesized and therefore its stability if it is produced within a biological system, has not been assessed.

In this study we attempt to synthesize, clone and express ESF1 in a heterologous yeast expression system to ascertain if the peptide retains its inhibitory activity in a biological system. Also, the economic feasibility of producing peptides in biological expression systems outweighs chemical production of the peptide, and if so, will therefore also provide an alternative production method when large amounts of peptide are required for analysis.



## **CHAPTER TWO**

### **MATERIALS AND METHODS**

## 2.1 Strains, plasmids and culture conditions

The strains and plasmids used in this study appear in table 3. *E. coli* strains HB101, and JM103 were cultured in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) with vigorous agitation at 37°C. *Carnobacterium mobile* was grown in brain-heart-infusion (BHI) broth (Biolab, Midrand, Gauteng) at 30°C. BHI 0.7% agar was used as the overlaying medium in the spot-on-lawn assays, while BHI agar (Biolab) formed the bottom layer agar in the same assay. Ampicillin (Roche Biochemicals, Midrand, Gauteng) was the supplementing antibiotic added to LB media to a final concentration of 100 µg/ml, to select for *E. coli* ampicillin-resistant plasmid-containing colonies.

The yeast *S. cerevisiae* was cultured in yeast peptone dextrose (YPD) media (Ausubel *et al.*, 1992). Yeast selective media, known as synthetic complete (minimal) media (SCD) containing yeast nitrogen base without amino acids (Difco, Detroit, Michigan), 20% glucose (Associated Chemical Enterprises, Glenvista, Gauteng), and yeast dropout mix (Ausubel *et al.*, 1992) was used in yeast recombinant selection. SCD was used with uracil (Sigma Chemical Company, St. Louis, Missouri), i.e. SCD<sup>ura+</sup> for the selection of non-transformants, and without uracil, i.e. SCD<sup>ura-</sup> for the selection of the yeast recombinants containing the pTG3828 and pTGESF1 hybrid plasmids.

## 2.2 DNA manipulations and transformations

### 2.2.1 DNA isolations

Plasmid DNA was prepared from *E. coli*, using the alkali lysis method (Sambrook *et al.*, 1989) for mini preparations of DNA while for large scale DNA purification, the Nucleobond AX Kit (Macherey-Nagel, Duren, Germany) was used. Yeast DNA was isolated according to the total DNA isolation method outlined by Ausubel *et al* (1992).

Table 3 List of strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i> JM103	( $\Delta$ ( <i>Lac-Pro</i> ), <i>supE</i> , <i>thi</i> , <i>endA</i> , <i>sbcB15</i> , <i>strA</i> , <i>rk</i> <sup>-</sup> , <i>mk</i> <sup>+</sup> /F <i>tra</i> $\Delta$ 36, <i>Pro AB</i> <sup>+</sup> , <i>lacI</i> <sup>q</sup> , <i>lacZ</i> $\Delta$ M15)	Messing and Viera, 1982
<i>E. coli</i> HB101	<i>supE44 hsdS20</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	Sambrook <i>et al.</i> , 1989
<i>S. cerevisiae</i> c13ABYS86	<i>MAT<math>\alpha</math></i> , <i>ura3-<math>\Delta</math>5</i> , <i>leu2-3, -112</i> , <i>his</i> , <i>pral</i> , <i>prb1</i> , <i>prc1</i> , <i>cps1</i>	Shuller and Entian, 1988
<i>C. mobile</i>	indicator organism	this lab
pUC18	<i>amp</i> <sup>r</sup> , <i>lacZ</i> , <i>lacI</i> , 2.69 kb	Sambrook <i>et al.</i> , 1989
pVD4	pBluescript with <i>MFa1</i> promoter sequence, <i>MFa1</i> prepro sequence, and insect defensin A cloned in frame with <i>MFa1</i> pro sequence	from W. F. Broekaert
pTG3828	<i>E. coli</i> / yeast cloning vector	Achstetter <i>et al.</i> , 1992 (from W.F. Broekaert)
pUCESF1	pUC18 containing <i>ESF1</i> gene	this study
pVDESF1	pVD4 containing <i>ESF1</i> gene in place of <i>insect defensin A</i>	this study
pTGESF1	pTG3828 containing <i>MFa1</i> promoter and prepro sequence in frame with <i>ESF1</i> gene	this study

## 2.2.2 Agarose gel electrophoresis

DNA samples were electrophoresed on horizontal agarose gels at 90V for 1.5 hours, and stained with ethidium bromide at a final concentration of 0.5  $\mu$ g/ml, and viewed under UV light. RNA samples were electrophoresed on 1.5% formaldehyde agarose gels ([http://s27w007.pswfs.gov/Protocols/northern\\_blotting.html](http://s27w007.pswfs.gov/Protocols/northern_blotting.html)) , at 60V for 3 hours, and also visualized under UV light with ethidium bromide staining. Low melting temperature agarose (FMC Bioproducts, Rockland, Maine) was used when DNA fragments were required to be recovered after electrophoresis.

### 2.2.3 Recovery of DNA from agarose gels

DNA samples were separated by electrophoresis on 1% low melting temperature agarose and viewed under UV light. The band of interest was excised with a gel cutter. Recovery of DNA from the excised fragment was accomplished using the Nucleospin Extract kit (Macherey-Nagel, Duren, Germany) from the *HindIII/BamHI* digested pVD4 and the *SalI/BamHI* digested pVDESf1. For isolation of the constructed *ESF1* gene from low melting temperature agarose, the Sephaglas Bandprep kit (Amersham-Pharmacia, Johannesburg, Gauteng) was used.

### 2.2.4 Enzymes

All restriction enzymes digests were carried out at 37°C from 3 hours to overnight to ensure complete digestion of DNA. T4 DNA ligase (Promega Corporation, Madison, Wisconsin) reactions were carried out between 16°C to 20°C, according to manufacturer's instructions.

### 2.2.5 Transformation

*E. coli* cells were first made competent by the calcium chloride method (Sambrook *et al.*, 1989), and then the transformation was carried out using the general procedure outlined by Sambrook *et al.* (1989). For transformation in the yeast, *S. cerevisiae*, the high efficiency lithium acetate method was used (Gietz and Shiestl, 1991).

## 2.3 Synthetic *ESF1* gene assembly

The five oligonucleotides comprising the *ESF1* gene: VV1, 5' ATG GCT TCT CGT GCT GCT GGT CTG GCT GCT CG 3'; VV2, 5' TCT GGC TCG TCT GGC TCT GCG TGC

TCT GTA AT3'; VV3, 5' GAC CAG CAG CAC GAG AAG CCA T 3'; VV4, 5' ACG ACG AGC CAG ACG AGC CAG ACG AGC AGC CA 3'; and VV5, 5' CTAGA TTA CAG AGC 3' (the double underlined sequences indicate the *Xba*I restriction enzyme recognition sequence) were added together at a concentration of 100 picomoles each and heated to 95°C for 15 minutes. The oligonucleotide mixture was then allowed to cool to room temperature to facilitate annealing of the complementary oligonucleotides, followed by overnight ligation at 16°C.

## 2.4 Cloning of the assembled *ESF1* gene into pUC18

The assembled *ESF1* gene was cloned into the *Hinc*II (Roche Biochemicals) and *Xba*I (Roche Biochemicals) sites of pUC18. Blue/white colony selection on LB agar supplemented with 100 µg/ml ampicillin, 0.1M IPTG (Roche Biochemicals) and 80 µg/ml X-gal (Roche Biochemicals) was used to select for putative pUC18/*ESF1* recombinant colonies.

## 2.5 Cloning of the *ESF1* gene into pVD4

The pUC universal reverse primer (Roche Biochemicals), and the forward primer VV-GEN1 (5' AAT AAG CTT GGA CAA GAG AAT GGC TTC TCG TGC TGC TGG T 3') were used for to amplify the *ESF1* gene fragment from the pUCESF1 recombinant plasmid. The VV-GEN primer composed of the 3' end of the MFα1 pro sequence as the first 19 nucleotides, and the other 21 nucleotides consisted of the 5' end of the *ESF1* gene sequence. Thirty cycles of PCR was carried out using the following conditions on a Perkin Elmer GeneAmp PCR System 2400 (Perkin Elmer Applied Biosystems, Foster City, California): first 2 amplification cycles consisted of denaturation at 94°C, annealing at 37°C, and elongation at 72°C; while the next 25 amplification cycles consisted of denaturation at 94°C, annealing at 55°C, and elongation at 72°C. The PCR amplification product and the pVD4 vector were restricted with *Hind*III (Roche Biochemicals) and

Table 4 Components of the PCR reaction mixture for amplification of the *ESF1* gene from the recombinant pUC/ESF1 vector

Volumes in $\mu$ l								
Reagents	[Final]	1.5mM MgCl <sub>2</sub>	2mM MgCl <sub>2</sub>	3mM MgCl <sub>2</sub>	3.5mM MgCl <sub>2</sub>	4mM MgCl <sub>2</sub>	1.5mM MgCl <sub>2</sub> no template DNA	1.5mM MgCl <sub>2</sub> pMal-p2 DNA
10xPCR reaction buffer	1x	10	10	10	10	10	10	10
25mM MgCl <sub>2</sub>	1.5mM to 4mM	6	8	12	14	16	6	6
PCR nucleotide mix	200 $\mu$ M each	2	2	2	2	2	2	2
pUC universal reverse primer	300nM	1	1	1	1	1	1	1
VV-GEN1 forward primer	300nM	1	1	1	1	1	1	1
pUCESF1 DNA	< 1 $\mu$ g	0.5	0.5	0.5	0.5	0.5	-	-
pMal-p2 DNA	< 1 $\mu$ g	-	-	-	-	-	-	0.5
Sterile millipore H <sub>2</sub> O	—	79	77	73	71	69	79.5	79

Volumes in $\mu$ l								
Reagents	[Final]	1.5mM MgCl <sub>2</sub>	2mM MgCl <sub>2</sub>	3mM MgCl <sub>2</sub>	3.5mM MgCl <sub>2</sub>	4mM MgCl <sub>2</sub>	1.5mM MgCl <sub>2</sub> no template DNA	1.5mM MgCl <sub>2</sub> pMal-p2 DNA
Taq polymerase 1U/ $\mu$ l	0.5U	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total Volume	100	100	100	100	100	100	100	100

*Bam*HI (Roche Biochemicals), and were ligated with T4 DNA ligase. Following transformation, putative pVD4 recombinants containing the *ESF1* gene in place of the *insect defensin A* gene were selected on LB agar supplemented with ampicillin.

## **2.6 Cloning of the *ESF1/MFα1* expression cassette into pTG3828**

The yeast/*E. coli* shuttle vector, pTG3828, was cleaved with *Sal*I (Roche Biochemicals) and *Bgl*II (Roche Biochemicals) and ligated to the *Sal*I/*Bam*HI digested pVDESF1 fragment containing the *ESF1/MFα1* gene expression cassette. Yeast colonies transformed with pTG3828 containing the *ESF1/MFα1* expression cassette were selected on SCD<sup>ura-</sup> medium.

## **2.7 Verification of recombinant plasmids**

### **2.7.1 Southern Hybridization**

Southern hybridization was carried out using the general method of Sambrook *et al.* (1989). A DIG 3' end-labeling kit (Roche Biochemicals) was used to digoxigenin label oligonucleotide probes. Detection of the hybridized probe was accomplished with the DIG Detection kit (Roche Biochemicals). Both 3' end-labeling and detection were done according to manufacturer's instructions.

To determine the pVDESF1 clones by Southern hybridization, a hybridization temperature of 55°C was used with digoxigenin-labeled VV3 as probe. Digoxigenin-labeled probe proMF (5' ACA GCA CAA ATA ACG GG 3') was used at a hybridization temperature of 42°C for detection of pTGESF1, isolated from *E. coli* and yeast transformants, and *ESF1* cDNA produced by reverse transcriptase PCR from pTGESF1, isolated from yeast transformants. The probe proMF hybridizes to part of the *MFα1* pro



sequence.

### 2.7.2 Restriction analysis

Restriction digest of pUCESF1 putative recombinants with *Pst*I (Roche Biochemicals) and *Bam*HI was carried out to ascertain the insertion of the 63 bp *ESF1* gene in pUC18. Restriction digest by *Kpn*I of pVDESF1 recombinants was carried out to determine replacement of *insect defensin A* by the *ESF1* gene in pVD4 (figure 5). A *Sal*I and *Cla*I (Roche Biochemicals) restriction digest of pTGESF1 recombinants was also done to verify the presence of the *ESF1/MFa1* gene expression cassette in pTG3828 (figure 8).

### 2.7.3 Polymerase chain reaction (PCR)

To determine the presence of the *ESF1/MFa1* gene expression cassette in pTGESF1, amplification of part of the *MFa1 pro* sequence and *ESF1* was carried out. The proMF forward primer and PGKrev reverse primer (5' GCA ACA CCT GGC AAT TC 3'), which anneals to the 5' end of the PGK terminator sequence were used. The PCR profile for 25 cycles was as follows: denaturation at 94°C, annealing at 37°C, and elongation at 72°C.

### 2.7.4 DNA Sequencing

DNA sequencing of recombinant plasmid DNA was performed by the dideoxy chain termination method outlined by Sanger *et al.* (1977), using the Sequenase™ Version 2.0 DNA sequencing kit (USB-Amersham Life Sciences, Cleveland, Ohio).

## **2.8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

A 12 % non-denaturing acrylamide gel (30% T; 1% C) was used to separate the restricted DNA fragments of the pUCESF1 *Pst*I/*Bam*HI digestion. The gel was electrophoresed at 100V for 45 minutes, stained with ethidium bromide and visualized under UV light.

Table 5 Components of the PCR reaction mixture for amplification of the *ESF1* gene from the recombinant vector pTGESF1.

Volumes in $\mu$ l							
Reagents	[Final]	1.5mM MgCl <sub>2</sub>	2.5mM MgCl <sub>2</sub>	3.5mM MgCl <sub>2</sub>	4.5mM MgCl <sub>2</sub>	1.5mM MgCl <sub>2</sub> pTGESF1	1.5mM MgCl <sub>2</sub> no template DNA
10xPCR reaction buffer	1x	10	10	10	10	10	10
25mM MgCl <sub>2</sub>	1.5mM to 4mM	6	10	14	18	6	6
PCR nucleotide mix	200 $\mu$ M each	2	2	2	2	2	2
proMF forward primer	300nM	1	1	1	1	1	1
pGK-rev reverse primer	300nM	1	1	1	1	1	1
A5 yeast transforma nt DNA	< 1 $\mu$ g	0.5	0.5	0.5	0.5	-	-
pTGESF1 DNA	< 1 $\mu$ g	-	-	-	-	0.5	-
Taq polymerase 1U/ $\mu$ l	0.5U	0.5	0.5	0.5	0.5	0.5	0.5
Sterile millipore H <sub>2</sub> O	—	79	75	71	67	79	79.5
Total Volume	100	100	100	100	100	100	100

## 2.9 Reverse Transcriptase PCR (RT-PCR)

Yeast total RNA was extracted from the recombinant logarithmic phase *S. cerevisiae* culture by treating the cells with Zymolyase (ICN Biomedicals Incorporated, Aurora, Ohio) to form spheroplasts, followed by lysing the cells by vortexing them in the presence of acid-washed glass beads, and then repeating a phenol/chloroform extraction to purify the RNA (Ausubel *et al.*, 1992). An amount of 0.25  $\mu$ g of VV3 oligonucleotide DNA was added to 0.5  $\mu$ g yeast total RNA, and the nucleic acids were denatured at 95°C for 10 minutes. To this mixture, AMV reverse transcriptase (Promega Corporation) was added and the reverse transcription reaction was carried out at 42°C for 1 hour. Lithium chloride was then added to a final concentration of 2M, along with 100% ethanol and the mixture was incubated at 70°C for another hour, to precipitate the DNA. The precipitated DNA was centrifuged, washed in 70% ethanol and resuspended in 10  $\mu$ l of sterile distilled water. A 20  $\mu$ l PCR reaction using VV3 oligonucleotide as the reverse primer and proMF as the forward primer was performed to amplify the 5' region of the *ESF1* gene and part of the *MFa1 pro* sequence from the cDNA. The PCR conditions were as follows for 25 cycles: denaturation at 94 °C, annealing at 37°C and elongation at 72°C.

## 2.10 Purification of ESF1 peptide from the yeast pTGESF1 transformant culture supernatant

A 10ml overnight culture of the selected pTGESF1 yeast transformant was used to inoculate 500ml of SCD<sup>ura-</sup> yeast selective media. After 48 hours incubation at 30°C with vigorous shaking, the culture was centrifuged at 12000×g for 30 minutes at 4°C. Purification was carried out on a Perkin Elmer series 200 high pressure liquid chromatography pump (Perkin Elmer Applied Biosystems). The supernatant containing the ESF1 peptide was acidified to pH 3 with glacial acetic acid and applied onto a preparative Bondapak-C<sub>18</sub> (Waters RCM 25×10) HPLC column (Millipore, Bedford, Massachusettes) equilibrated with acidified water. Stepwise removal of unwanted media

components and proteins was conducted by carrying out 2,10 minute elution steps at 10% and 30 %

*Table 6* Components of the PCR reaction mixture for amplification of the *ESF1* gene from cDNA of the *ESF1* gene sequence from the vector pTGESF1 isolated from yeast transformant A5.

Reagents	[Final]	pTG3828 cDNA	pTGESF1 cDNA	positive control pVDESF1 DNA
10xPCR reaction buffer containing MgCl <sub>2</sub>	1X PCR reaction buffer with 1.5mM MgCl <sub>2</sub>	2μl	2μl	2μl
proMF forward primer	300nM	1μl	1μl	1μl
VV3 reverse primer	300nM	1μl	1μl	1μl
PCR nucleotide mix	200μM each	0.4μl	0.4μl	0.4μl
sterile millipore H <sub>2</sub> O	-	5.35μl	5.35μl	15.35μl
template cDNA from RT reaction	< 0.75μg	10μl	10μl	1μl
Taq polymerase	0.25U	0.25μl	0.25μl	0.25μl
Total volume	20μl	20μl	20μl	20μl

acetonitrile in acidified water, and a linear gradient 30-40% acetonitrile elution over 5 minutes, all at a flowrate of 4ml/min, until no more proteins were detected. Synthetic ESF1 peptide applied to a C<sub>18</sub> analytical Brownlee column (Applied Biosystems, Foster City, California) eluted at 41.883% acetonitrile in acidified water (figure 13), following a linear elution gradient at 10-60% acetonitrile in acidified water, over 50 minutes at a flow rate of 1ml/min. Therefore, the 40-50% acetonitrile in acidified water elution gradient carried out over 30 minutes at a flow rate of 4ml/min was collected, freeze dried, and reconstituted in 0.2ml 0.1% trifluoroacetic acid (TFA). This 0.2ml fraction was then applied on the C<sub>18</sub> analytical Brownlee column and the 41-44% acetonitrile in acidified water fraction was collected, following the same linear acetonitrile gradient used in purification of the synthetic ESF1 peptide above.

## 2.11 Bioassays

The spot-on-lawn assay (Hastings *et al.*, 1991) was used to determine presence and activity of ESF1 peptide. *C. mobile* was used as the indicator organism since ESF1 was reported to have a low MIC of 2.5 nM against the bacterium (Dykes *et al.*, 1998).

## 2.12 Tricine SDS-PAGE

A separating layer (10% T, 3% C), spacer layer (10% T, 3% C) and stacking layer (4% T, 3% C) tricine polyacrylamide gel (Schagger and von Jagow, 1987) was used to detect the presence of ESF1 peptide in the analytical column, lyophilized, purified fraction. Electrophoresis was performed at 30V for 2.5 hours. The gel was first fixed in 10% formaldehyde for 1 hour, and then washed for a further 1 hour with 5 changes of distilled water. After washing, the gel was stained in Coomassie Brilliant Blue R-250 (Sigma) for 1 hour and left in destain solution (7% acetic acid, 5% methanol) overnight. A low range protein marker (Promega Corporation) was also electrophoresed on the gel to determine the approximate sizes of the protein bands in the gel.

### **2.13 Electroblothing of protein onto polyvinylidene fluoride (PVDF) membrane**

Electroblothing of the proteins on the tricine gel was performed using the Mini Transblot Cell (Biorad, Hercules, California) electroblothing apparatus for 60 minutes at 90V in electrotransfer buffer (10mM 3- cyclohexylamino- 1- propanesulfonic acid (CAPS), 10% methanol, 0.1mM thioglycolic acid, pH 11 ). The method used was described by Matsudaira (1987) with minor modifications. A 0.2 micron Transblot Transfer Medium (Biorad) PVDF membrane was the blotting membrane of choice since its small pore size facilitates efficient transfer of small peptides like ESF1.

### **2.14 Amino acid sequencing**

N-terminal amino acid sequence analysis of an excised band from the PVDF membrane was analyzed on a Perkin Elmer Applied Biosystems Procise 491 (Applied Biosystems) automated protein sequencer using the pulsed liquid PVDF peptide method (Hewick *et al.*, 1981) at the University of Natal Molecular Biology Unit.

### **2.15 Electro Spray Mass Spectrometry (ES-MS)**

Mass spectrometric analysis of 20 $\mu$ l of the crude preparative column fraction of the pTGESF1 transformed yeast culture supernatant was conducted on a model API III quadruple mass spectrometer equipped with an Ionspray source (Sciex, Thornhill, Canada) at the Center for Mass Spectrometry.

## **2.16 Purification of antibodies produced in chickens against the synthetic ESF1 peptide**

### **2.16.1 Immunization of chickens**

Laying hens (Amber-link) received an initial injection intramuscularly at 2 sites in their large breast muscles with 200 $\mu$ g ESF1 peptide. The peptide was suspended in phosphate-buffered saline (PBS; 1ml), and emulsified with Freund's complete adjuvant (Sigma) in a 1:1 (v/v) ratio. After the initial injection the hens received a further 3 injections at the same dose in Freund's incomplete adjuvant, fortnightly, in the same manner. Eggs of immunized chickens were collected daily over the immunization period, labeled and stored at 4°C, until processed for isolation of IgY antibodies.

### **2.16.2 Isolation of IgY antibodies from chicken egg yolks**

The method of Polson and von Wechmar (1980), was used to extract IgY from the egg yolks of immunized chickens. Eggs collected on the fifth day of every week during immunization were used in the IgY isolation procedure. The concentration of IgY was measured at an absorbance of 280 nm, and protein content calculated as described by Coetzer (1985). An enzyme-linked immunosorbent assay (ELISA) was performed to determine the presence of anti-ESF1 antibodies. Synthetic ESF1 peptide at a concentration of 1 $\mu$ g/ml in PBS was used to coat the multititre plate (Nunc maxisorb, Amersham- Pharmacia), overnight at 4°C. A synthetic leucocin A peptide (Hastings *et al.*, 1991) at the same concentration was used to coat a duplicate plate as a control for the specificity of anti-ESF1 antibodies. Dilutions of the antibodies purified from eggs collected at different intervals during the immunization program, as well as, non-immune IgY at concentrations of 500  $\mu$ g/ml, 100  $\mu$ g/ml, 50  $\mu$ g/ml, and 10  $\mu$ g/ml, were allowed to incubate in the antigen-coated microtitre plates. Incubation was performed for 2 hours at 37°C. Binding of the primary antibodies was visualized by incubation



with rabbit anti-IgY-HRPO conjugate (Jackson Laboratories, USA), and ABTS (Roche Biochemicals) as outlined by Coetzer *et al* (1991).

### **2.16.3 Purification of anti-ESF1 antibody**

To purify anti-ESF1 antibodies, affinity chromatography was employed. Approximately 4mg of synthetic ESF1 peptide was immobilized on a 2ml aminolink column using the Immunopure Ag/Ab Immobilization Kit (Pierce, Rockford, Illinois) as per the manufacturer's instructions. A volume of 125ml of isolated IgY was circulated through the aminolink-peptide column overnight at a flow rate of 10ml/h. Following this the affinity purification protocol was performed as per manufacturer's instructions. Elution of the antibody was achieved using 0.1M glycine-HCl, pH 3, and the absorbance was measured at 280nm to determine in which fractions the antibody eluted from the column. The fractions with the highest protein concentrations were pooled and another ELISA performed to ascertain the presence of anti-ESF1 antibody in the Immunoaffinity-purified fractions.

### **2.17 Western dot blot analysis of pTGESF1 transformant culture supernatant**

Synthetic ESF1 peptide, C<sub>18</sub> preparative and analytical HPLC column freeze dried fractions, and leucocin A peptide were spotted on to Hybond-C nylon membrane (Amersham Life Sciences, Cleveland, Ohio), and left to air dry for 30 minutes. The available sites on the membrane were blocked for 1 hour with 5% non-fat milk in Tris buffered saline (TBS) and washed 3 times for 5 minutes each with TBS. Next, the membrane was incubated for 2 hours with the affinity purified anti-ESF1 antibody at a concentration of 25 µg/ml in 0.5% BSA-TBS. The membrane was washed again with TBS (3 times, 5 minutes), and incubated for another 1 hour in alkaline-phosphatase linked rabbit anti-chicken IgG (Sigma) in 0.5% BSA-TBS. This antibody cross-reacts with chicken IgY. Following the final TBS wash (3 times, 5 minutes) the membrane

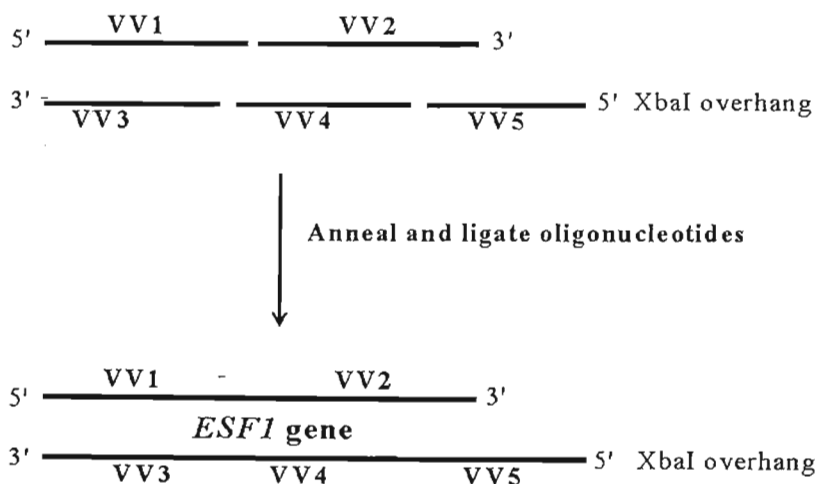
was allowed to react in the dark with the BCIP/NBT substrate (Sigma) dissolved in distilled water, until dots appeared on the membrane against a lightly stained background. The dot blots were stored between sheets of filter paper in the dark until photographed.

# **CHAPTER THREE**

## **RESULTS AND DISCUSSION**

### 3.1 Assembly of *ESF1* gene sequence and its cloning into pUC18

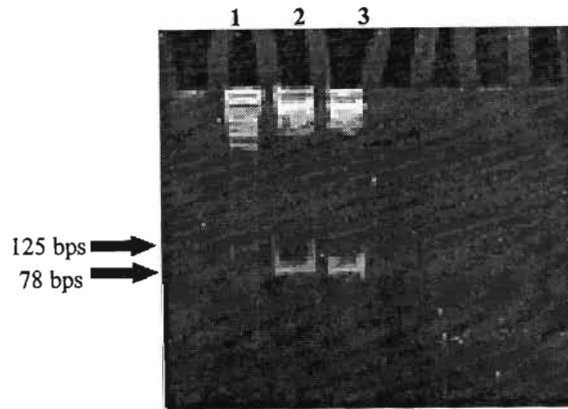
The VV1 and VV2 oligonucleotides comprised the 5' to 3' strand of the *ESF1* gene, while the VV3, VV4 and VV5 oligonucleotides comprised the 3' to 5' strand of the *ESF1* gene (figure 3). VV5 was designed with a *Xba*I overhang at the 3' end, of the gene to facilitate easy cloning into pUC18. The intention of cloning into pUC18 was to make the *ESF1* gene sequence easily accessible for manipulation in further cloning experiments since pUC18 is a high copy number plasmid and therefore ensures provision of numerous copies of the *ESF1* gene sequence. Polyacrylamide gel electrophoresis of *Bam*HI/*Pst*I restricted putative pUCESF1 recombinants verified the insertion of the constructed *ESF1* gene fragment due to the presence of an expected 78 bp restriction product on the agarose gel (figure 4).



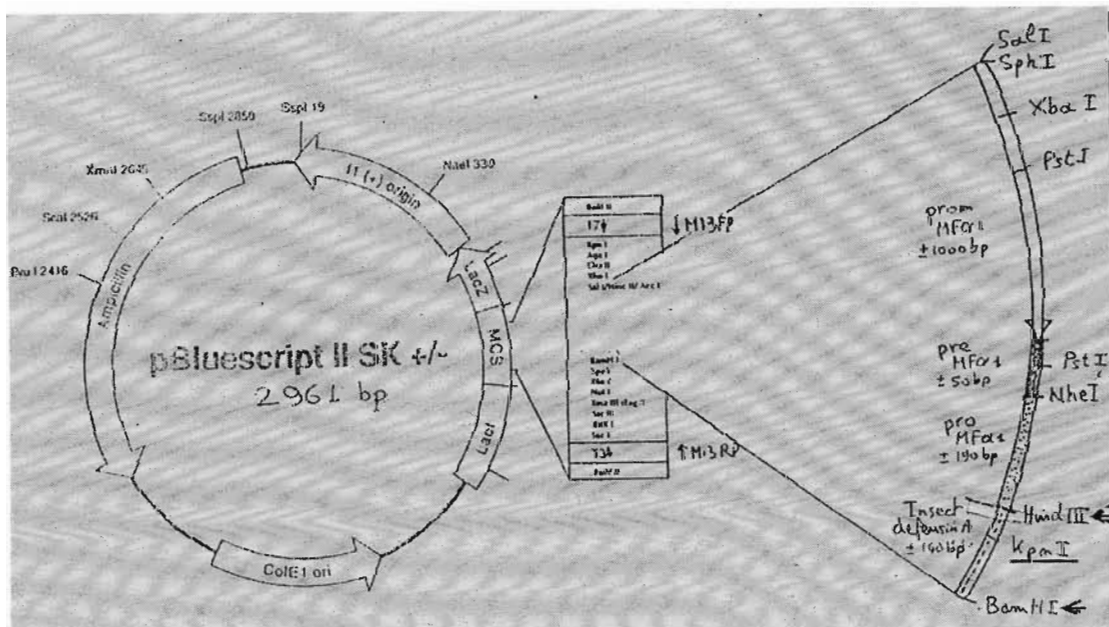
**Figure 3** Construction of the *ESF1* gene. The five oligonucleotides VV1, VV2, VV3, VV4 and VV5 were designed to comprise the *ESF1* gene sequence. Annealing allows the joining of the complementary oligonucleotides VV1 and VV2 of the 5' to 3' strand to VV3, VV4 and VV5 of the 3' to 5' strand, while ligation facilitates joining of the VV1 to VV2, VV3 to VV4, and VV4 to VV5 oligonucleotides.

### 3.2 Construction of the pVDESf1 recombinant vector

For the efficient expression of the *ESF1* gene and secretion of ESF1 peptide, the Mfa1 promoter that drives transcription of the gene and the Mfa1 prepro sequence which directs secretion of the peptide is required. This Mfa1 promoter and prepro sequences are

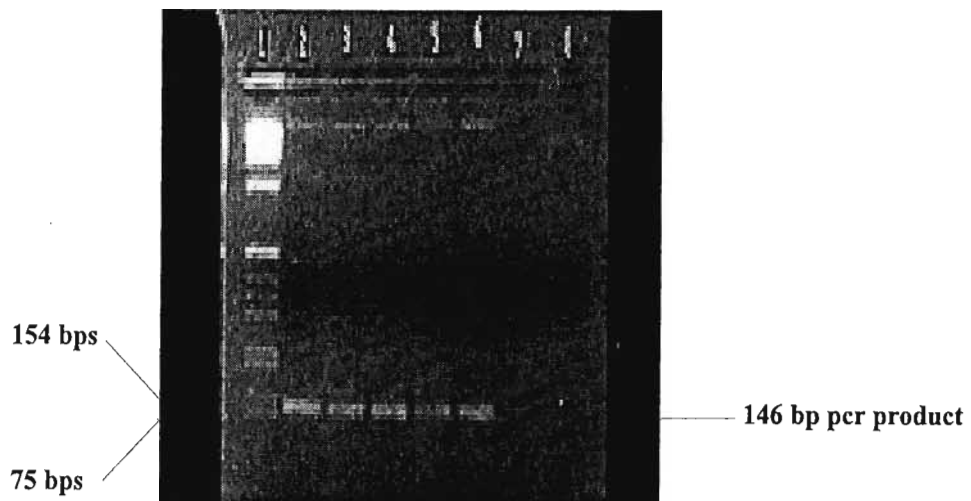


**Figure 4** 12.5% Polyacrylamide gel of putative pUC/ESF1 clones restricted with *Bam*HI/ *Pst*I to give a 78 bp fragment containing the *ESF1* gene. Lanes: (1) *Eco*RI/ *Hind*III digested  $\lambda$ -DNA; (2) pUC/ESF1 transformant 1; (3) pUC/ESF1 transformant 2.



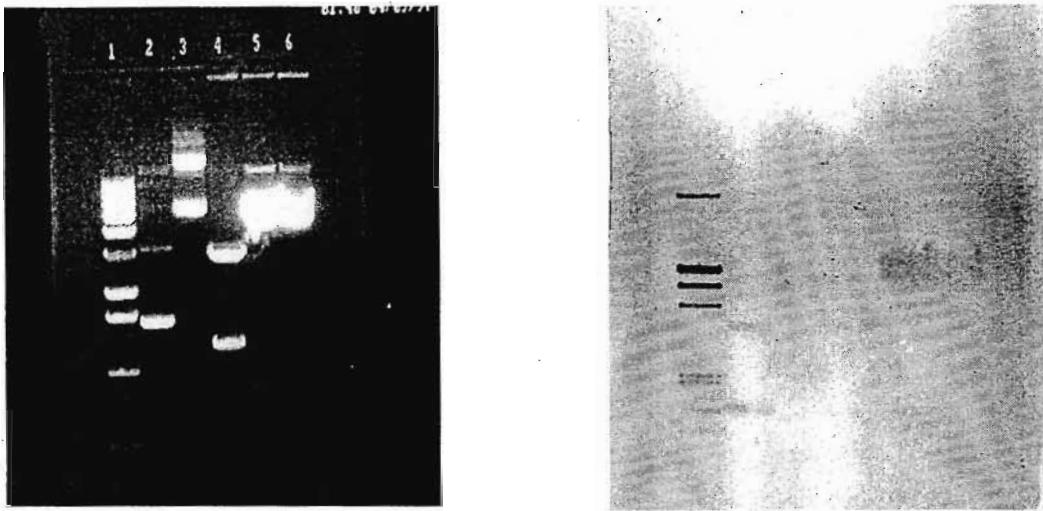
**Figure 5** pBluescript-based vector pVD4. The vector has been assembled from a basic pBluescript vector by the introduction of the MFA1 promoter and prepro leader sequences to form an expression cassette, with the *insect defensin A* sequence cloned in frame with the expression cassette. This will allow expression and secretion of the cloned peptide in yeast. The underlined *Kpn*I site contained within the *insect defensin A* sequence is not present in the *ESF1* sequence. This allows determination of the probable replacement of *insect defensin A* by the *ESF1* gene during restriction analysis of putative pVDESF1 clones. The arrows indicate the site of insertion for the *ESF1* gene (W.F Broekaert, personal communication).

contained as an expression cassette within the pBluescript-based vector, pVD4 (figure 5). pVD4 also contains the *insect defensin A* sequence cloned in frame to the pro sequence. The expression cassette has a *Hind*III site engineered 5' to the Kex2 protease cleavage site. This cleavage site causes separation of the ESF1 peptide from the prepro peptide once processed by the Kex2 protease. The *ESF1* gene was cloned into the *Hind*III/*Bam*HI sites of pVD4 therefore effectively replacing the *insect defensin A* gene to lie in frame with the pro sequence. PCR of the pUCESF1 recombinant produced the expected 146 bp fragment (figure 6), which after restriction with *Bam*HI/*Hind*III was cloned into the pVD4 vector at the compatible sites. *Kpn*I restriction of putative pVDESF1 recombinants should not produce a 140 bp restriction fragment since the *ESF1* gene does not contain a *Kpn*I site like the *insect defensin A* gene contains.

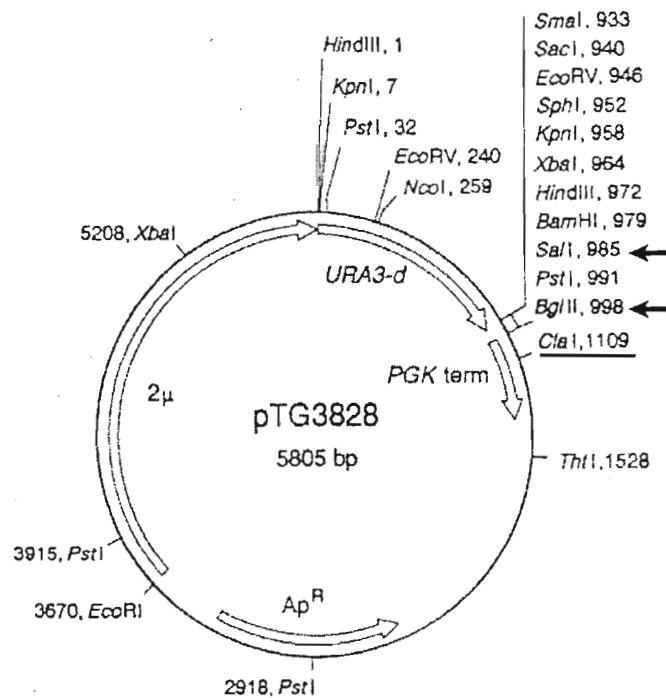


**Figure 6** Agarose gel (3%) of PCR amplification of *ESF1* gene from pUCESF1 clone. Lanes: (1) 1018 bp fragment and its multimers plus pBR322 fragments; (2) pUCESF1, 1.5mM MgCl<sub>2</sub>; (3) pUCESF1, 2mM MgCl<sub>2</sub>; (4) pUCESF1, 3mM MgCl<sub>2</sub>; (5) pUCESF1, 3.5 mM MgCl<sub>2</sub>; (6) pUCESF1, 4mM MgCl<sub>2</sub>; (7) no template, 1.5mM MgCl<sub>2</sub>; (8) pMAL-p2, 1.5mM MgCl<sub>2</sub>

This aided in determining whether the replacement of the insect defensin A sequence by ESF1 had taken place (figure 7). Southern hybridization (figure 7) verified the presence of *ESF1* in the pVDESF1 recombinant DNA.



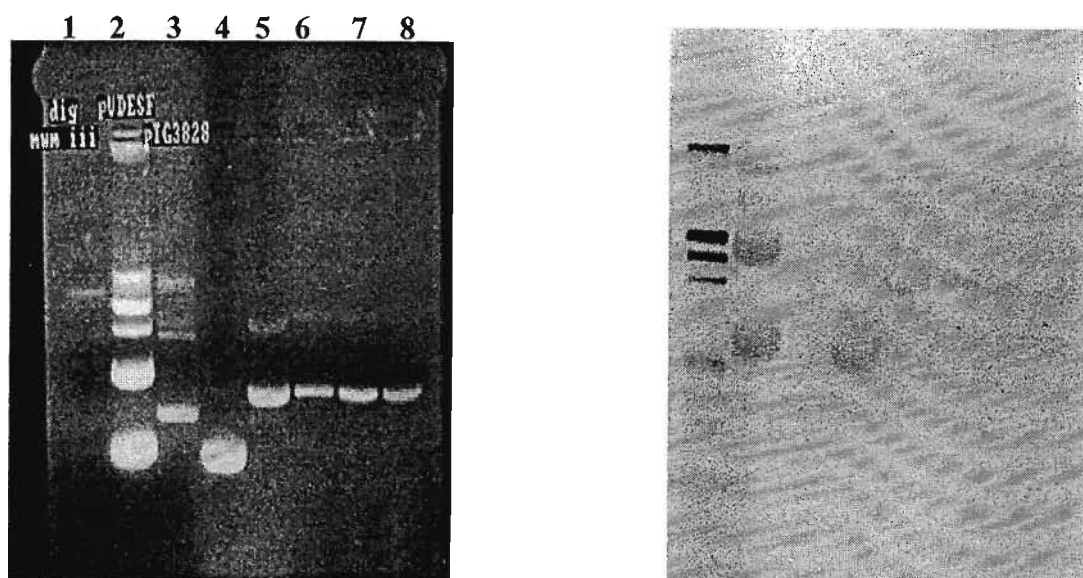
**Figure 7** Agarose gel (left) and corresponding Southern blot (right) of pVD4/ESF1 (*KpnI*) putative hybrids. Lanes: (1) 1018 bp fragment and its multimers plus pBR322 fragments; (2) pVD4; (3) pVD4 (*KpnI*); (4) transformant 6b; (5) transformant 7b; and (6) transformant 8b. Digoxigenin-labeled probe VV3, which hybridizes to *ESF1* was used at a hybridization temperature of 55°C.



**Figure 8** Yeast/*E. coli* shuttle vector pTG3828. The multiple cloning site contains the *SalI* and *BglII* sites indicated by arrows at which the *MFa1/ESF1* gene expression cassette can be cloned. The expression cassette will then be flanked at the 3' end by the PGK transcriptional terminator thus providing stability to the *MFa1* prepro leader and *ESF1* mRNA strand. The underlined *ClaI* site will lie 3' to the inserted expression cassette, and following cleavage of pTGESF1 DNA with *SalI* and *ClaI*, an approximate 1,3 kb fragment should be produced (Achstetter *et al.*, 1992).

### 3.3 pTG3828 containing the *Mfa1/ESF1* gene expression cassette isolated from *E. coli* transformants

pTG3828 is a yeast /*E. coli* shuttle vector (figure 8). This shuttle vector consists of the efficient yeast phosphoglyceratekinase (PGK) transcriptional terminator to produce more stable mRNA transcripts; an ampicillin resistance gene for selection of the plasmid in an *E. coli* host strain; multiple cloning site to facilitate the insertion of a promoter and coding sequence; and the *URA3-d* gene for maintenance of a high plasmid copy number (Loison *et al.*, 1989). The 1296 bp *Mfa1/ESF1* expression cassette from the pVDESf1 recombinant plasmid was cloned into the pTG3828 vector. DNA isolated from transformants was verified as pTGESf1 recombinants by Southern hybridization (figure 9).



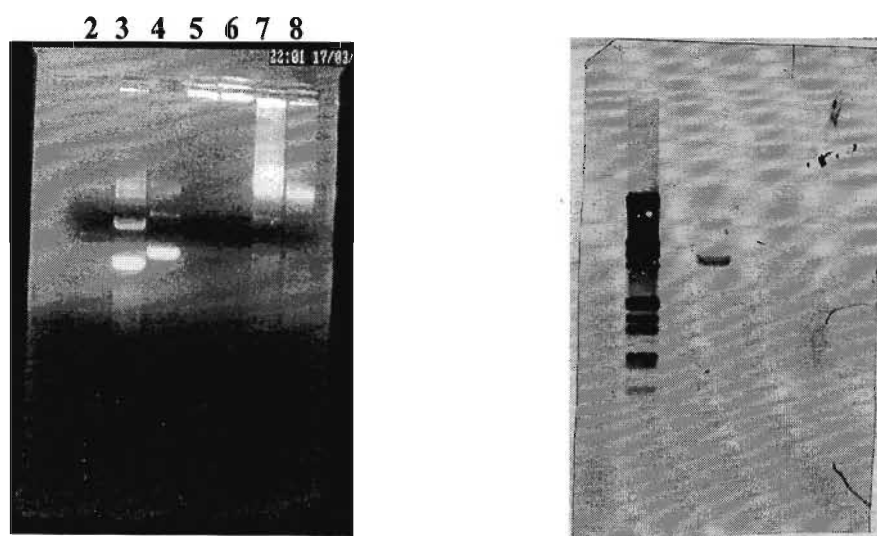
**Figure 9** Agarose gel (left) and corresponding Southern blot of putative pTGESf1 recombinants. Lanes: (1) digoxigenin-labeled *EcoRI/HindIII* cleaved  $\lambda$ -DNA; (2) pVDESf1; (3) pTG3828; (4, 5, 6, 7, 8) putative pTGESf1 clones. Digoxigenin-labeled probe, proMF, hybridizing to the *Mfa1 pro* sequence, was used at a hybridization temperature of 42°C for Southern hybridization.

### 3.4 pTG3828 containing the *Mfa1/ESF1* gene expression cassette isolated from *Saccharomyces cerevisiae* c13ABYS86 transformants

DNA of the yeast transformants was isolated but very low amounts were visible in the



agarose gel (figure 10), which was also indicated by faint detection of the pTGESF1 recombinant after Southern hybridization (figure 10). PCR amplification of part of the *MFa1/ESF1* gene expression cassette did result in the expected amplification product of 216 bp, indicating the presence of pTGESF1 (figure 11). Isolation of DNA from the same yeast transformants was carried out in triplicate resulting in similar low yields of recombinant plasmid DNA as was shown by agarose gel electrophoresis, and DNA concentration analyses (results not shown). It is probable that the recombinant plasmid exists in low copy number within the *S. cerevisiae* c13ABYS86 transformation strain.

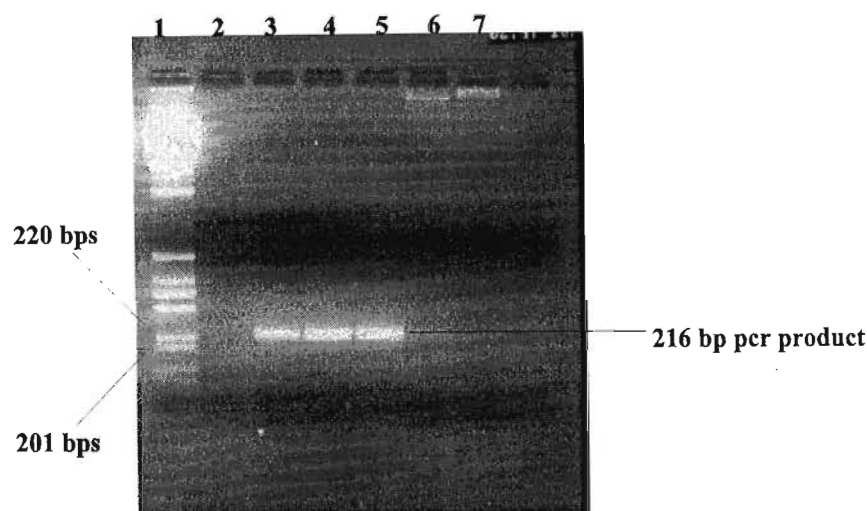


**Figure 10** Agarose gel (left) and corresponding Southern blot (right) of yeast pTGESF1 transformants. Lanes: (2) digoxigenin-labeled *EcoRI/HindIII* cleaved  $\lambda$ -DNA; (3) pTG3828 (*E. coli*); (4) pTGESF1 (*E. coli*); (5, 6, 7) pTGESF1 transformants (yeast); and (8) pTG3828 (yeast). Digoxigenin-labeled probe proMF, hybridizing to the *MFa1 pro* sequence was used as a probe at a hybridization temperature of 42°C in Southern hybridization

### 3.5 Transcription of the *MFa1 prepro* sequence and *ESF1* gene

RT-PCR shows that transcription of the *MFa1 prepro* sequence and *ESF1* gene does occur (figure 12). Southern hybridization (figure 12b) verified that the amplification

product

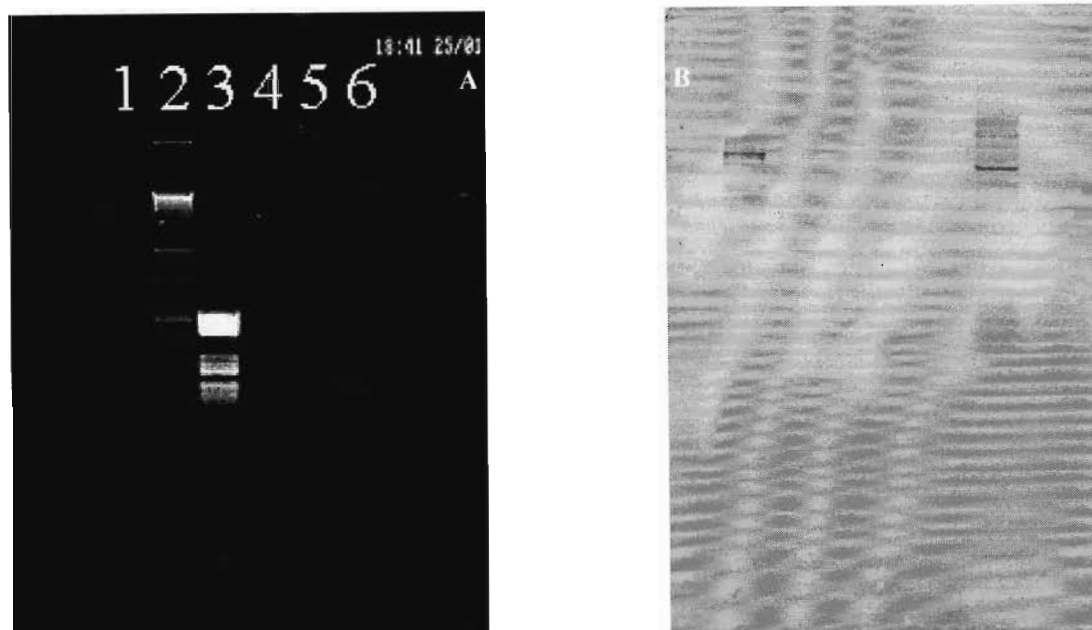


**Figure 11** Agarose gel (3%) of PCR amplification of DNA isolated from pTGESF1 transformant. Lanes : (1) 1018 bp fragment and its multimers plus pBR322 fragments; (2) transformant A5 DNA, 1.5mM MgCl<sub>2</sub>; (3) A5 DNA, 2.5mM MgCl<sub>2</sub>; (4) A5 DNA, 3.5mM MgCl<sub>2</sub>; (5) A5 DNA, 4.5mM MgCl<sub>2</sub>; (6) pTGESF1 positive control DNA, 1.5mM MgCl<sub>2</sub>; (7) no template

obtained after PCR does contain the *ESF1* gene sequence. Reverse transcriptase transcription of yeast total mRNA revealed no product following agarose gel electrophoresis (results not shown), therefore PCR was carried out to amplify any cDNA that was produced after the transcription reaction. The need for PCR amplification to detect the presence of cDNA suggests again the probable presence of a small amount of recombinant DNA available for transcription and subsequent translation.

### 3.6 Purification and detection of ESF1 peptide in pTGESF1 transformant culture supernatant

The elution profile from the preparative HPLC column (figure 14a) determined a peak at the approximate retention time of synthetic ESF1 peptide (figure 13), which is absent in the pTG3828 transformed culture supernatant (figure 14b). No clear peak was visible therefore a 41 to 44% acetonitrile in acidified water fraction (figure 15) from the analytical column



**Figure 12** RT-PCR of yeast total mRNA (A) and Southern blot (B) of the gel indicating mRNA transcription of the *ESF1* gene. Lanes: (1) Digoxigenin-labeled *EcoRI/HindIII* cleaved  $\lambda$ -DNA; (2) 1018 bp fragment and its multimers plus pBR322 fragments; (3) pBR322 (*HaeIII* digested); (4) RT-PCR of pTGESF1 transformed yeast mRNA; (5) RT-PCR of pTG3828 transformed yeast mRNA; and (6) PCR of pVDESF1. Amplification products are noted for the reverse transcribed pTGESF1 transformed yeast mRNA and the positive control pVDESF1 DNA (contains recombinant MF $\alpha$ 1/*ESF1* expression cassette).

was collected because it was at about 41.8% acetonitrile in acidified water that synthetic *ESF1* eluted (figure 13). Spotting 5  $\mu$ l of the freeze dried HPLC analytical column fraction showed a zone of inhibition in a lawn of *C. mobile* seeded BHI agar (figure 16). Activity was noted only after HPLC analytical column purification which is an approximate 10 000 times concentrate of the supernatant which suggests a very small quantity of *ESF1* peptide present in the supernatant of the pTGESF1 yeast transformant culture.

Tricine SDS-PAGE (figure 17) of a 10  $\mu$ l fraction eluted from the analytical column and freeze dried, showed an approximate 2 kDa protein whose sequence analysis revealed the presence of amino acid residues present within *ESF1*. Too many N-terminal amino acids were visible in each amino acid sequencing cycle to determine a proper sequence. It is possible that there are small protein fragments of around 2 kDa present in the fraction that



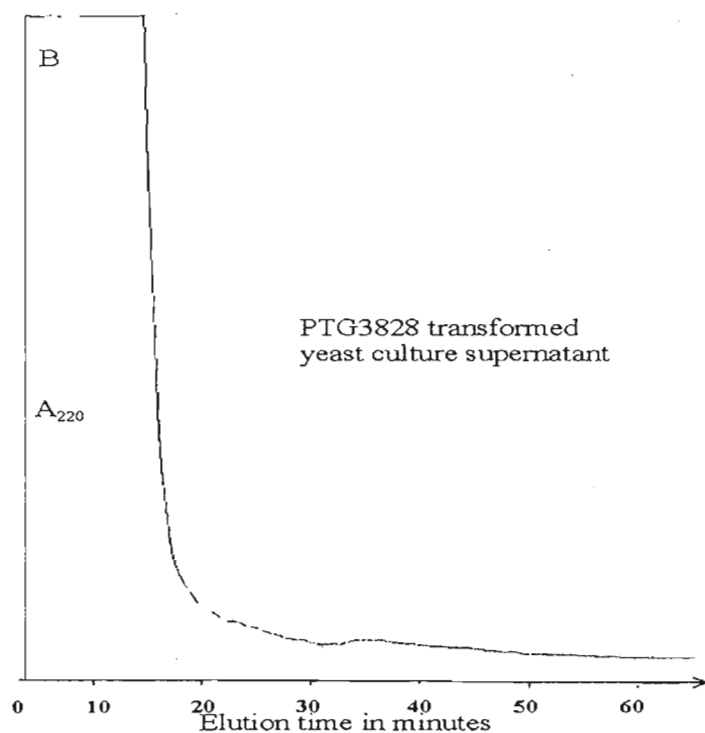
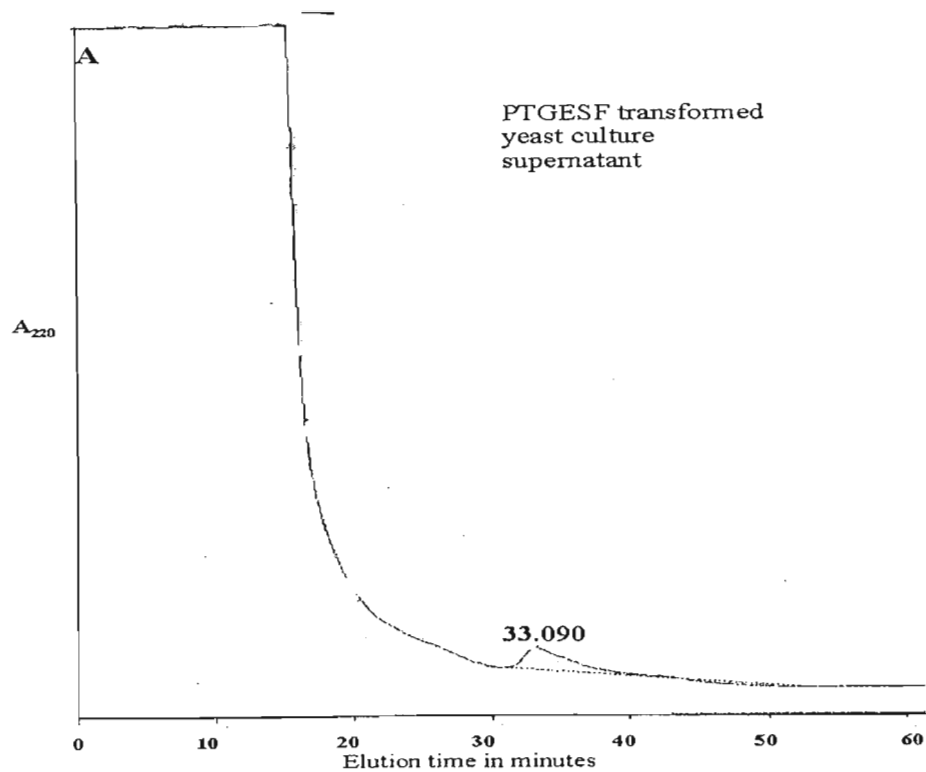
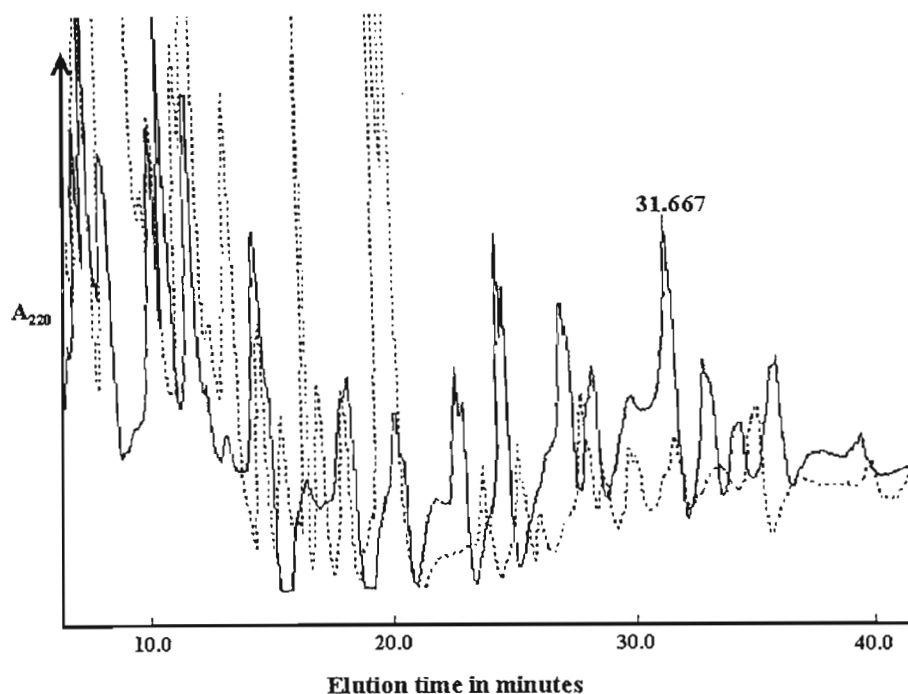
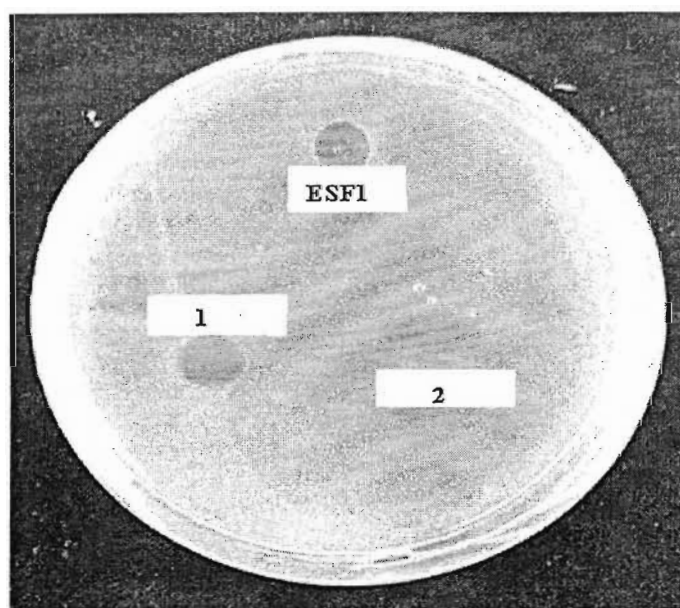


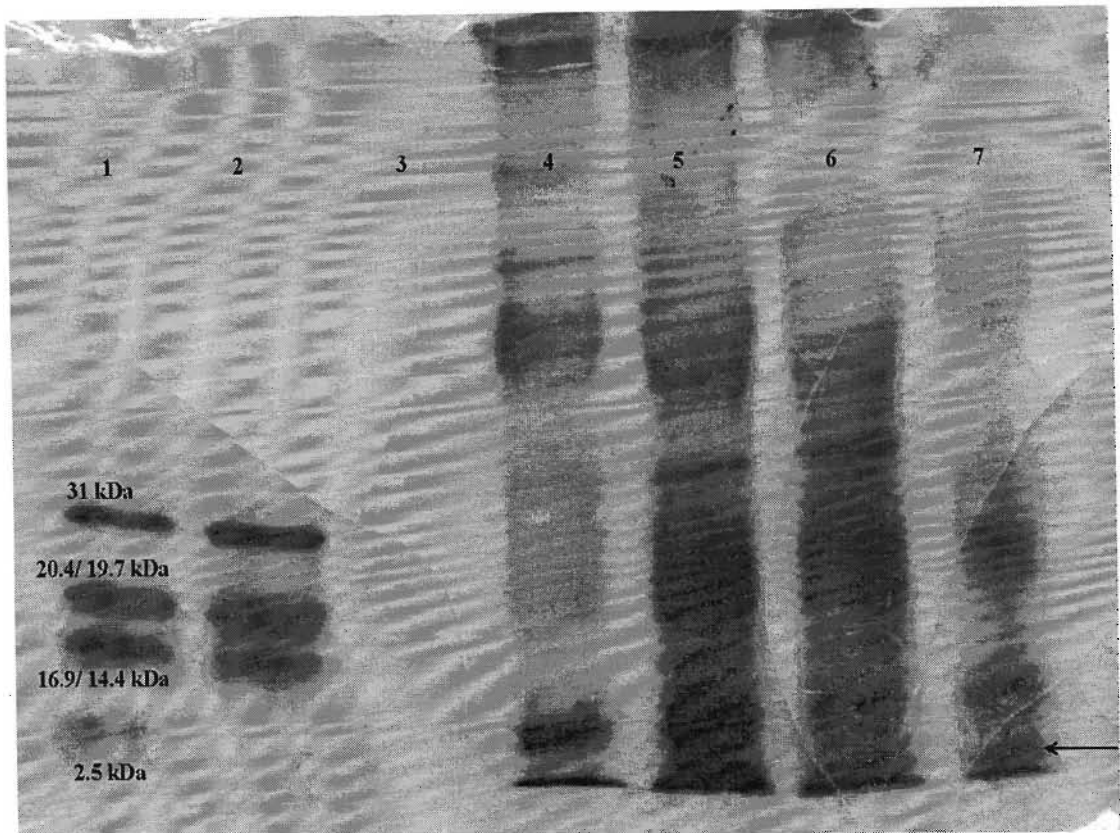
Figure 14 Elution profile of pTGESF1 (A) and pTG3828 (B) transformed culture supernatant using Bondapak- $C_{18}$  preparative HPLC column, monitored by absorbance at 220nm, using acetonitrile in acidified water as the solvent. Eluent at 30-40 minutes retention time was collected since the visible protein from the recombinant yeast culture supernatant (A) has a retention time of 33.090 minutes, which correlates with the retention time 31.883 for synthetic ESF1 peptide from a  $C_{18}$  analytical HPLC column.



**Figure 15** Elution profile of pTGESF1 (solid line) and pTG3828 (dotted line) transformed yeast culture supernatant using the C<sub>18</sub> analytical HPLC column monitored at A<sub>220</sub>, using acetonitrile/ acidified water as solvent. A fraction with 31-34 minutes retention time was collected, lyophilized and activity against *Carnobacterium mobile* was displayed. The peak at the retention time of 31.667 minutes is thought to represent ESF1 peptide.

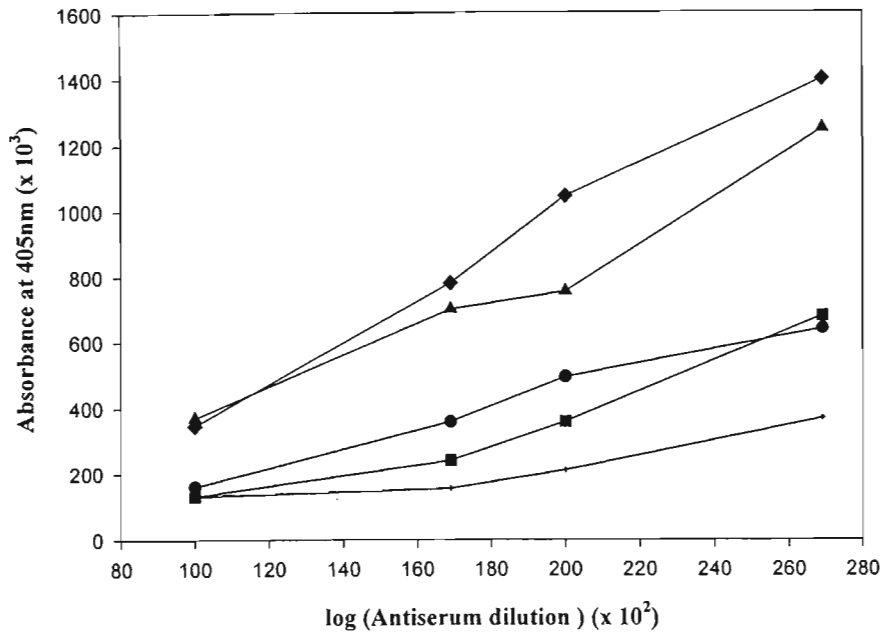


**Figure 16** Spot-on-lawn assay of HPLC analytical column, lyophilized fractions of pTGESF1 and pTG3828 transformed yeast culture supernatants. 5 $\mu$ l of pTGESF1 (1) and pTG3828 (2) culture preparations were spotted on brain-heart-infusion agar seeded with the indicator organism, *Carnobacterium mobile*. Only the pTGESF1 culture supernatant shows activity against the indicator bacterium.



**Figure 17** Tricine (10% C; 3% T) SDS-PAGE of HPLC analytical column, lyophilized fractions of both pTGESF1 and pTG3828 transformed yeast cultures. The arrow indicates the position of the 2.052 kDa ESF1 peptide, and this band was blotted to PVDF membrane and subjected to amino acid sequencing. Lanes : (1) Promega low range protein marker, (2) Promega low range protein marker, (3) empty, (4) pTG3828 yeast culture supernatant (10 $\mu$ l), (5) and (6) pTGESF1 transformed yeast culture supernatant (20 $\mu$ l), (7) pTGESF1 transformed yeast culture supernatant (10 $\mu$ l).

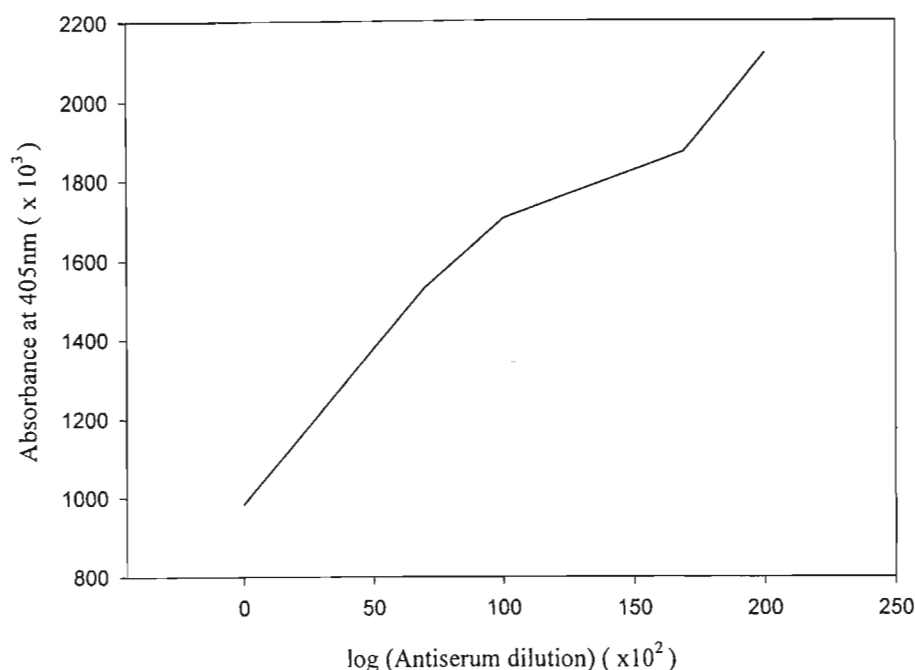
The absorbance values for a concentration of 1  $\mu$ g/ml purified anti-ESF1 antibody (figure 19) is similar to absorbance values obtained for a 100  $\mu$ g/ml ESF1 antibody concentration from total IgY isolations (figure 18) from the eggs of the ninth week. These results were interpreted as an approximate 100 fold increase in the sensitivity of immunoaffinity purified anti-ESF1 antibody to ESF1 peptide compared with the antibody sensitivity for the total IgY antibody isolated using ELISA. The immunoaffintiy purification concentrated only the ESF1 peptide specific IgY antibodies. Consequently, there is removal of non-specific antibodies, and the specificity of anti-ESF1 antibody is enhanced.



**Figure 18** Progress of immunization with ESF1 peptide as determined by ELISA. ESF1 peptide was coated at 1  $\mu\text{g/ml}$  to microtitre plates and incubated with antibody dilutions of 500  $\mu\text{g/ml}$ , 100  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$  and 10  $\mu\text{g/ml}$ , collected after 4 (●), 6 (■), 8 (▲), and 9 (◆), and non-immune IgY (+). Binding was visualized by incubation with rabbit anti-chicken HRPO-linked secondary antibody as described by Coetzer et al. (1991). Each point is a mean at  $A_{405}$  of duplicate samples.

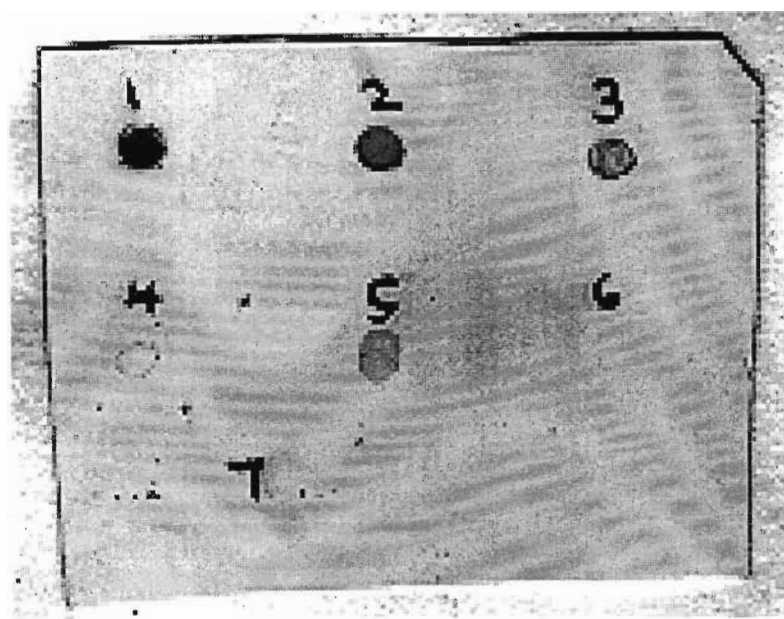
Western dot blot analysis of the analytical column freeze dried fraction shows positively that there is ESF1 peptide present in the pTGESF1 culture supernatant (figure 20). The leucocin A (Hastings *et al.*, 1991) control shows no binding to anti-ESF1 antibody and therefore provides evidence of the specificity of anti-ESF1 antibody. There is, although, a visible antibody binding to the preparative column freeze dried pTG3828 yeast culture supernatant fraction. The reason for this is that the preparative column fraction still contains media components such as caramelized sugar which is present as a brown residue with a high concentration of insignificant proteins, which binds the antibody non-specifically.





**Figure 19** Representation of different immunoaffinity purified ESF1 IgY concentrations with ESF1 peptide as determined by ELISA. ESF1 peptide was coated at 1  $\mu\text{g/ml}$  to microtitre plates and incubated with antibody dilutions of 100  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$  and 10  $\mu\text{g/ml}$ , and 1  $\mu\text{g/ml}$  collected during immunoaffinity purification. Binding was visualized by incubation with rabbit anti-chicken HRPO-linked secondary antibody as described by Coetzer *et al.* (1991).

The method for purification of ESF1 peptide on the HPLC analytical column removes these contaminating media and protein components and concentrates the ESF1 peptide thus permitting a pure antibody-peptide antigen immunogenic reaction. Figure 20 shows that the brightness of the dot at position 2 (preparative column pTGESF1 culture supernatant fraction) is greater than that at position 3 (preparative column pT3828 culture supernatant fraction), which is due to binding of antibody to other media and

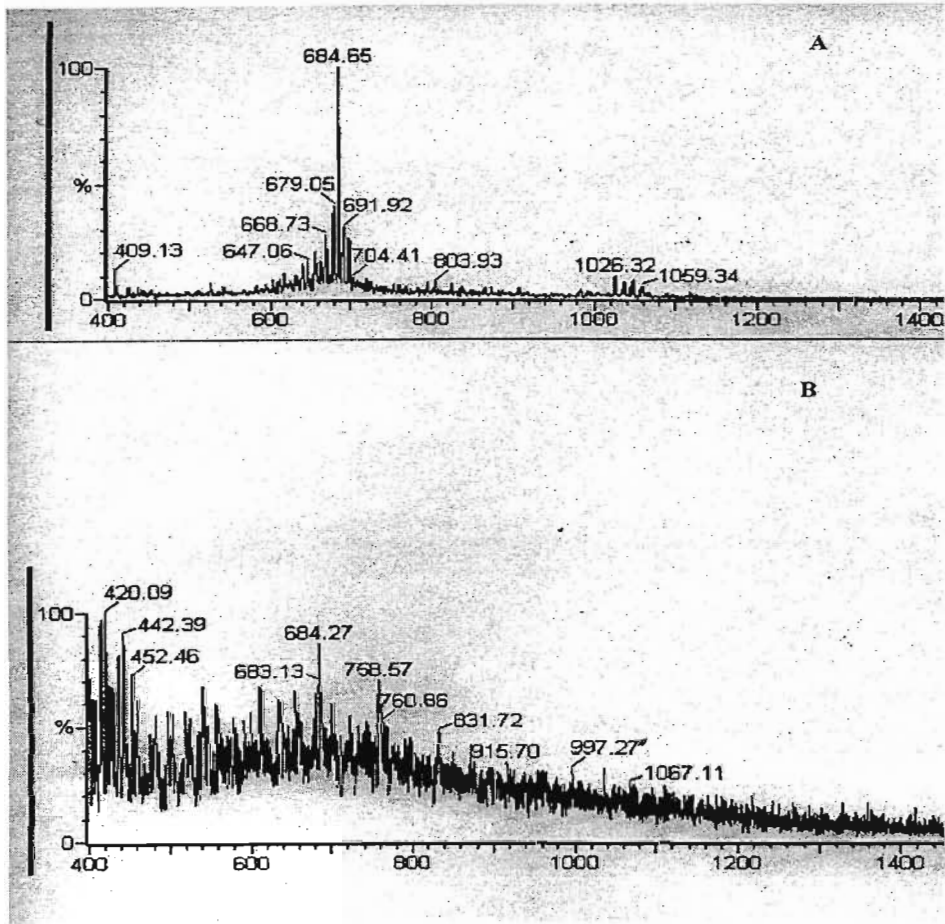


**Figure 20** Western dot blot of synthetic ESF1 peptide (1 $\mu$ g), leucocin A peptide (1 $\mu$ g), and preparative and analytical HPLC column lyophilized fractions of pTG3828 and pTGESF yeast culture supernatants. (1) ESF1 peptide. (2) pTGESF1 culture supernatant, preparative column fraction. (3) pTG3828 culture supernatant, preparative column fraction. (4) leucocin A peptide. (5) pTGESF1 culture supernatant, analytical column fraction. (6) pTG3828 culture supernatant, analytical column fraction. (7) empty

contaminating high concentration protein components, and to ESF1 peptide, while there is only a dot visible at position 5 (analytical column pTGESF1 culture supernatant fraction) and none visible at position 6 (analytical column pT3828 culture supernatant fraction), which is due to the presence of concentrated ESF1 peptide, and no other impurities resulting in a greater anti-ESF1 antibody/ ESF1 peptide antigen immunogenic reaction.

Mass spectrometry analysis of synthetic ESF1 peptide and a 20 $\mu$ l preparative column freeze dried fraction reveal the presence of a peak at a 684 mass to charge (m/z) ratio for both samples (figure 21). This 684 m/z ratio equates to 2052 Da, which is the approximate size of ESF1 peptide, and thus verifies the presence of EFS1 peptide in the recombinant yeast supernatant. The peak profile of the preparative column fraction still shows a large number of impurities, which contributes to the reasoning as to why there is

a non-specific reaction with anti-ESF1 antibody.



**Figure 21** Electro spray mass spectrometry of synthetic ESF1 peptide and a Bondapak-C<sub>18</sub> preparative column HPLC preparation of pTGESF1 yeast culture supernatant. The 684.27 (m/z) ration in (B, HPLC ESF1 preparation) corresponds to the 684.65 (m/z) ratio in (A, synthetic ESF1 peptide). 684 (m/z) is calculated to be 2052 Da, which reflects the ESF1 peptides mass, and this shows that ESF1 peptide is contained within preparative column fraction of the pTGESF culture supernatant.

There was a low yield of ESF1 peptide which may have been related to the low DNA concentrations isolated from yeast transformants and low concentrations of ESF1 peptide purified from the transformed yeast culture supernatant. Frequent variation in the

productivity of different transformants has been noted when using 2 $\mu$ m vectors, due to an unexplained variation in copy number between different transformants (Loison *et al.*, 1989; Purvis *et al.*, 1987). For this reason numerous transformants were screened for the recombinant pTGESF1 plasmid. All transformants seemed to contain approximately the same low amounts of recombinant plasmid DNA as can be seen in figure 10, indicated by the faint DNA bands. Repeated DNA purification of the same yeast transformants gave approximately the same DNA concentrations and even lower DNA concentrations with further subculturing. There might be a possible reduction in copy number with further subculturing, and therefore decreased expression of ESF1 peptide. This could be attributed to an unstable inheritance of the recombinant plasmid vector through each generation resulting from subculturing of the recombinant yeast culture. The possibility of protease degradation of ESF1 peptide is unlikely, because the *S. cerevisiae* c13ABYS86 strain carries the *prb* and *prc* genetic markers which are deficient in the major vacuolar proteases.

The *ESF1* gene sequence was designed for optimal expression in *E. coli*, although analysis of the codons shows a high percentage correlation with yeast codon usage when compared with yeast codon usage tables in literature (Bennetzen and Hall, 1982; <http://www.gcg.com/techsupport/data/yeast-high.> ) Eckart and Bussineau (1996) commented that codon usage can play a role in regulating gene expression, and in the production of large quantities of high quality heterologous protein. The *ESF1* gene sequence contains a high percentage similarity but not an optimal codon usage. This sub-optimal codon usage could be a factor that may have contributed to the inefficient production of large quantities of peptide from yeast transformants.

It is also uncertain whether there may have been a rate-limiting cleavage of the LYS-ARG dipeptide by the Kex2 protease which was reported in the expression of drosomycin in the same pTG3828 vector and *S. cerevisiae* c13ABYS86 strain (Michaut *et al.*, 1996). Mature drosomycin was being produced at a low level (25%), with various N-terminally extended drosomycin peptide products (Michaut *et al.*, 1996). Michaut *et al.* (1996), also reported from 25% to 90% increase in production of completely

processed drosomycin after coexpressing the Kex2 endoprotease with drosomycin, by inserting a full length *KEX2* gene next to the *drosomycin* gene sequence. Another example of a Kex2 protease processing problem and its effects on concomitant secretion is described in the production of insulin precursor, wherein efficiency of expression was improved by inserting a removable spacer peptide between the  $\alpha$ -factor-leader and the insulin precursor genes (Kjeldsen *et al.*, 1996). Amino acid analysis of the PVDF blotted band (figure 17), thought to be ESF1 revealed amino acids also present in the MF $\alpha$ 1 pro sequence, and this may suggest incomplete processing of the ESF1 peptide, and resultant low production and yield of ESF1 peptide after HPLC purification.

# **CHAPTER FOUR**

## **CONCLUSION**

This study has shown that it is possible to produce and secrete biologically active ESF1 peptide in yeast. The yeast expression system has correctly processed the ESF1 peptide such that it retains its biological activity. Although, the yeast expression system may not be ideal for the mass production of heterologous peptides, it can be used to determine whether synthetically designed antimicrobial peptides will retain their activity when produced in a eukaryotic biological system. This has significance when synthetic peptides with enhanced activity are designed with the lack of knowledge of how they would react in a biological system. The numerous synthetic peptides that are presently being designed leads also to the design of an equally large number of antimicrobial peptide gene sequences; and if these peptides are stably produced with intact biological activity in a biological system, they may provide the organism e.g., crop plant in which they are produced with intrinsic resistance to the appropriate microbial pathogen that they are active against.

In our experience, we were able to get better yields from freshly transformed cultures. Unstable inheritance of the recombinant plasmid could be a contributing factor to an increasingly lower copy number after subculturing of the recombinant yeast culture, and this may decrease the production levels. A precise determination of copy number would have to be done to corroborate this. There was, however, a distinct correlation between the yields of plasmid obtained and the number of passages the culture had been through. Whether this relates to copy number or some other phenomenon, needs to be determined to gain a better understanding for the optimization of production.

Production of secreted antimicrobial peptides by recombinant yeast systems may still require optimization through increasing peptide expression levels. This could be achieved by improving the strength and regulation of the promoters used to drive expression, exploring more efficient yeast transformation methods, overexpressing signal peptide proteases to ensure proper processing of mature foreign proteins, and optimizing codon usage.

## **CHAPTER FIVE**

### **REFERENCES**



- Achstetter, T., Nguyen-Juilleret, M., Findeli, A., Merkamm, M., and Lemoine, Y.** 1992. A new signal peptide useful for secretion of heterologous proteins from yeast and its applications for synthesis of hirudin. *Gene*. **110**: 25-31.
- Allefs, J.J.H., De-Jong, E.R., Florack, D.E.A., Hoogendoorn, C., and Stiekema, W.J.** 1996. *Erwinia* soft rot resistance of potato cultivars expressing antimicrobial peptide tachyplesin I. *Mol. Breed.* **2**: 97-105.
- Ando, K., Okada, M., and Natori, S.** 1987. Purification of sarcotoxin II antibacterial proteins of *Sarcophaga peregrina* (flesh fly) larvae. *Biochem.* **26**: 226-230.
- Ando, K., and Natori, S.** 1988. Inhibitory effect of sarcotoxin IIA, an antibacterial protein of *Sarcophaga peregrina*, on growth of *Escherichia coli*. *J. Biochem.* **103**: 735-739.
- Apel, K., Bohlmann, H., and Reimann-Philippe, U.** 1990. Leaf thionins, a novel class of putative defense factors. *Physiol. Plant.* **80**: 315-321.
- Argiolas, A., and Pisano, J.J.** 1985. Bombolitins, a new class of mast cell degranulating peptides from the venom of the bumblebee *Megabombus pennsylvanicus*. *J. Biol. Chem.* **260**: 1437-1444.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K.** 1992. *Short Protocols in Molecular Biology*. 2<sup>nd</sup> Edn., John Wiley and Sons, New York.
- Bals, R., Goldman, M.J., and Wilson, J.M.** 1998. Mouse beta-defensin is a salt sensitive antimicrobial peptide present in epithelia of the lung and urogenital tract. *Infect. Immun.* **66**: 1225-1232.
- Beck-Sickinger, A.G., and Jung, G.** 1991. Synthesis and conformational analysis of

lantibiotic leader-pro and pre- peptides. p218-230. In Jung, G., and Sahl, H.G.(ed). Nisin and novel lantibiotics. ESCOM Leiden. The Netherlands.

**Bennetzen, J.L., and Hall, B.D.** 1982. Codon selection in yeast. *J. Biol. Chem.* **257**: 3026-3031.

**Bessalle, R., Gorea, A., Shalit, I., Metzger, J.W., Dass, C., Desidario, D.M., and Fridkin, M.** 1993. Structure-function studies of amphiphilic antibacterial peptides. *J. Med. Chem.* **36**:1203-1209.

**Bevins, C.L., and Zasloff, M.** 1990. Peptides from frog skin. *Annu. Rev. Biochem.* **59**: 395-402.

**Blondelle, S., and Houghten, R.A.** 1991. Hemolytic and antimicrobial activities of the twenty-four individual omission analogs of mellitin. *Biochem.* **30**: 4671-4678.

**Bohlmann, H., and Apel, K.** 1991. Thionins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1991. **42**: 227-240.

**Bohlmann, H., and Apel, K.** 1987. Isolation and characterization of cDNAs coding for leaf specific thionins closely related to the endosperm-specific hordothionin of barley (*Hordeum vulgare* L.). *Mol. Gen. Genet.* **207**: 446-454.

**Boman, H.G.** 1995. Peptide antibiotics and their role in innate immunity. *Annu. Rev. Immunol.* **13**: 61-92.

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

**Bontems, F., Roumestand, C., Gilguin, B., Menez, A., and Toma, F.** 1991. Refined structure of charybdotoxin-common motifs in scorpion toxins and insect defensins.

Science. **254**: 1521-1523.

**Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Osborn, R.W.** 1995. Plant defensins: Novel antimicrobial peptides as components of the host defense system. *Plant Physiol.* **108**: 1353-1358.

**Brötz, H., Bierbaum, G., Leopold, K., Reynolds, P.E., and Sahl, H.G.** 1998. The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. *Antimicrob. Agents Chemother.* **42**: 154-160.

**Brötz, H., Bierbaum, G., Reynolds, P.E., and Sahl, H.G.** 1997. The lantibiotic mersacidin inhibits peptidoglycan biosynthesis at the level of transglycosylation. *Eur. J. Biochem.* **246**: 193-199.

**Bruix, M., Jimenez, M.A., Santoro, J., Gonzalez, C., Colilla, F.J., Mendez, E., and Rico, M.** 1993. Solution structure of gamma 1-H and gamma 1-P thionins from barley and wheat endosperm determined by <sup>1</sup>H-NMR: nine structural motifs common to toxic arthropod proteins. *Biochem.* **132**: 715-724.

**Bulet, P., Cociancich, S., Dimarcq, J.L., Lambert, J., Reichhart, J.M., Hoffmann, D., Hetru, C., and Hoffmann, J.A.** 1991. Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. *J. Biol. Chem.* **266**: 24520-24525.

**Bulet, P., Dimarcq, J.L., Hetru, C., Lagueax, M., Charlet, M., Hegy, G., Van Dorsselaer, A., and Hoffmann, J.A.** 1993. A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution. *J. Biol. Chem.* **268**: 14893-14897.

**Carmona, M. J., Molina, A., Fernandez, J.A., Lopezfando, J. J., and Garcia - Olmedo, F.** 1993. Expression of the alpha-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. *Plant J.* **3**: 457-462.

**Casteels, P., Ampe, C., Jacobs, F., Vaeck, M., and Tempst, P.** 1989. Apidaecins: antibacterial peptides from honeybees. *EMBO. J.* **8**: 2387-2391.

**Casteels, P., Ampe, C., Jacobs, F., and Tempst, P.** 1993. Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*). *J. Biol. Chem.* **268**: 7044-7054.

**Casteels, P., and Tempst, P.** 1994. Apidaecin-type peptide antibiotics function through a non-pore-forming mechanism involving stereospecificity. *Biochem. Biophys. Res. Comm.* **189**: 339-345.

**Casteels, P., Ampe, C., Riviere, L., Van Damme, J., Elicone, C., Fleming, M., Jacobs, F., and Tempst, P.** 1990. Isolation and characterization of abaecin, a major antibacterial response peptide in the honey bee (*Apis mellifera*). *Eur. J. Biochem.* **187**: 381-386.

**Cavallarin, L., Andreu, D., and Segundo, B.S.** 1998. Cecropin A derived peptides are potent inhibitors of fungal plant pathogens. *Mol. Plant Microbe Interact.* **11**: 218-227.

**Chan, W.C., Leyland, M., Clark, J., Dodd, H.M., Lian, L.Y., Gasson, M.J., Bycroft, B.W., and Roberts, G.C.K.** 1996. Structure-activity relationships in the peptide antibiotic nisin: antibacterial activity of fragments of nisin. *FEBS Lett.* **390**:129-132.

**Charlet, M., Cherynsh, S., Phillipe, H. Hetru, C., Hoffman, J.A., and Bulet, P.** 1996. Innate immunity: Isolation of several cysteine rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. *J. Biol. Chem.* **271**: 21808-21813.

**Charpentier, S., Amich, M., Mesler, J., Vouille, V., Le Caer, J. P., Nicolas, P., and Delfour, A.** 1998. Structure, synthesis and molecular cloning of Demaseptins B, a family of frog skin peptide antibiotics. *J. Biol. Chem.* **273**: 14690-14697.

- Chernysh, S., Cociancich, S., Briand, J.P., Hetru, C., and Bulet, P.** 1996. The inducible antibacterial peptides of the hemipteran insect *Palomena prasina*: Identification of a unique family of proline-rich peptides and of a novel insect defensin. *J. Insect. Physiol.* **42**: 81-89.
- Christensen, B., Fink, J., Merrifield, R.B., and Mauzerall, D.** 1988. Channel-forming properties of cecropin and related model compounds incorporated into planar lipid membranes. *Proc. Natl. Acad. Sci. USA.* **85**: 5072-5076.
- Coetzer, T.H.T.** 1985. Preparation and characterization of antibodies against mouse IgG (all classes). Internal Report, Bioclones, (Pty.) Ltd. Immunology group, Stellenbosch.
- Coetzer, T.H.T., Elliot, E., Fortgens, P.H., Pike, R.N., and Dennison, C.** 1991. Anti-peptide antibodies to cathepsins B, L, and D and type IV collagenase. *J. Immunol. Methods.* **136**: 199-210.
- Cociancich, S., Dupont, A., Hegy, G., Lanot, R., Holder, F., Hetru, C., Hoffmann, J.A., and Bulet, P.** 1994. Novel inducible antibacterial peptides from a hemipteran insect, the sap-sucking bug *Pyrrhocoris apterus*. *Biochem. J.* **300**: 567-575.
- Cornut, I., Klaus, B., Jean-Louis, D., and Jean, D.** 1994. The amphipathic alpha helix concept. Application of the de nova design of ideally amphipathic Leu, Lys peptides with hemolytic activity higher than that of mellitin. *FEBS Lett.* **349**: 29-33.
- Cregg, J.M., Vedrick, T.S., and Raschke, W.C.** 1993. Recent advances in the expression of foreign genes in *Pichia pastoris*. *BT.* **11**: 905-910.
- Dahrer, K.A., Lehrer, R.I., Ganz, T., and Kronenberg, M.** 1988. Isolation and characterization of human defensin cDNA clones. *Proc. Natl. Acad. Sci. USA.* **85**: 7327-7331.

- De Bolle, M.F.C., Osborn, R.W., Goderis, I.J., Noe, L., Acland, D., Hart, C.A., Torrekens, S., van Leuven, F., and Broekaert, W.F.** 1996. Antimicrobial peptides from *Mirabilis jalapa* and *Amaranthus caudatus*: expression, processing, localization and biological activity in transgenic tobacco. *Plant Mol. Biol.* **31**: 993-1008.
- De Samblanx, G.W., Goderis, I.J., Thevissen, K., Raemaekers, R., Fant, F., Borremans, F., Acland, D.P., Osborn, R.W., Patel, S., and Broekaert, W.F.** 1997. Mutational analysis of a plant defensin from radish (*Raphanus sativus* L) reveal 2 adjacent sites important for antifungal activity. *J. Biol. Chem.* **272**: 1171-1179.
- Diamond, G., Zasloff, M., Eck, H., Brasseur, M., Maloy, W.L., and Bevins, C.L.** 1991. Tracheal antimicrobial peptide, a cysteine-rich peptide from the mammalian tracheal mucosa: peptide isolation and cloning of cDNA. *Proc. Natl. Acad. Sci USA.* **88**: 3952-3956.
- Durvasula, R.V., Gumbs, A., Panackal, A., Kruglov, O., Aksoy, S., Merrifield, R.B., Richards, F.F., and Beard, C.B.** 1997. Prevention of insect-borne diseases: An approach using transgenic symbiotic bacteria. *Proc. Natl. Acad. Sci. USA.* **94**: 3274-378.
- Dykes, G.A., Aimoto, S., and Hastings, J.W.** 1998. Modification of a synthetic antimicrobial peptide (ESF1) for improved inhibitory activity. *Biochem. Biophys. Res. Comm.* **248**: 268-272.
- Eckart, M.R., and Bussineau, C.M.** 1996. Quality and authenticity of heterologous proteins synthesized in yeast. *Current. Opin. Biotech.* **7**: 525-530.
- Epple, P., Apel, K., and Bohlmann, H.** 1997. Over expression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. *Plant Cell.* **9**: 509-520.
- Falla, T.J., Karunaratne, D.N., and Hancock, R.E.W.** 1996. Mode of action of

antimicrobial peptide indolicidin. J. Biol. Chem. **271**: 19298-19308.

**Falla, T.J., and Hancock, R.E.W.** 1997. Improved activity of a synthetic indolicidin analog. Antimic. Agents. Chemother. **41**: 771-775.

**Fehlbaum, P., Bulet, P., Cherysh, S., Briand, J., Roussel, J., Letellier, L., Hetru C., and Hoffman, J. A.** 1996. Structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides. Proc. Natl. Acad. Sci. USA. **93**: 1221-1225.

**Fehlbaum, P., Bulet, P., Michaut, L., Lageueux, M., Broekaert, W.F., Hetru, C., and Hoffmann, J.A.** 1994. Insect immunity: Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. J. Biol. Chem. **269**: 33159-33163.

**Fernandez de Calaya, R., Gonzalez-Pascual, B., Garcia-Olmedo, F., and Carbonero, P.** 1972. Susceptibility of phytopathogenic bacteria to wheat purothionins *in vitro*. Appl. Microbiol. **23**: 998-1000.

**Fleury, Y., Vouille, V., Beren, L., Amiche, M., Wroblewski, H., Delfour, A., and Nicolas, P.** 1998. Synthesis, antimicrobial activity and gene structure of a novel member of the dermaseptin B family. Biochim. Biophys. Acta. **1396**: 228-236.

**Florack, D.E.A., Stiekema, W.J., and Bosch, D.** 1996. Toxicity of peptides to bacteria present in the vase of cut roses. Postharvest Biol. Technol. **8**: 285-291.

**Fujii, G., Selsted, M.E., Eisenberg, D.** 1993. Defensin promote fusion and lysis of negatively charged membranes. Protein. Sci. **2**: 1302-1312.

**Fujiwara, S., Imai, J., Fujiwara, M., Yaeshima, T., Kawashima, T., and Kobayashi, K.** 1990. A potent antibacterial protein in royal jelly. J. Biol. Chem. **265**: 11333-11337.

**Gallagher, D.S. Jr., Ryan, A.M., Diamond, G., Bevins. C.L., and Womack, J.E.** 1995. Somatic cell mapping of beta-defensin genes to cattle syntenic group U25 and fluorescence in situ localization to chromosome 27. *Mamm. Genome.* **6**: 554-556.

**Ganz, T., and Lehrer, R.I.** 1995. Defensins. *Pharmaco. Therap.* **66**: 191-205

**Garcia-Olmedo, F., Molina, A., Segura, A., and Moreno, M.** 1995. The defensive role of nonspecific lipid-transfer proteins in plants. *Trends Microbiol.* **3**:72-74.

**Gellisen, G., Melber, K., Janowicz, Z.A., Dahlems, U., Weydemann, U., Piontek, M., Strasser, A.W.M., and Hollenberg, C.P.** 1992. Heterologous gene expression in yeasts. *Antonie van Leeuwenhoek. Int. J. Gen. Mol. Microbiol.* **62**: 79-93.

**Gellisen, G., and Hollenberg, C.P.** 1997. Applications of yeasts in gene expression studies: a comparison of *Saccharomyces cerevisiae*, *Hansenula polymorpha*, and *Kluyveromyces lactis* - a review. *Gene.* **190**: 87-97.

**Gennaro, R., Skerlavaj, B., and Romeo, D.** 1989. Purification, composition, and activity of two batenecins, antibacterial peptides of bovine neutrophils. *J. Biol. Chem.* **57**: 3142-3146.

**Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Philippsen, P., Tettelin, H., and Oliver, S.G.** 1996. Life with 6000 genes. *Science.* **274**: 546-567.

**Hancock, R.E.W.** 1997. Peptide antibiotics. *Lancet.* **349**: 418-422.

**Hancock, R.E.W., Falla, T., and Brown, M.** 1995. Cationic bactericidal peptides. *Adv. Microb. Physiol.* **37**: 135-175.



**Hancock, R.E.W., and Chapple, D.S.** 1999. Peptide antibiotics. *Antimic. Chemother.* **43**: 1317-1323.

**Hancock, R.E.W., and Lehrer, R.** 1998. Cationic peptides: a new source of antibiotics. *TIBTECH.* **16**: 82-88.

**Hanzawa, H., Shimada, I., Kuzuhara, T., Komano, H., Kohda, D., Inagak, F., Natori, S., and Arata, Y.** 1990. H-1 Nuclear magnetic resonance study of the solution conformation of an antibacterial protein, sapecin. *FEBS Lett.* **269**: 413-420.

**Hara, S., and Yamakawa, M.** 1995. Moricin, a novel type of antibacterial peptide isolated from the silkworm, *Bombyx mori*. *J. Biol. Chem.* **270**: 29923-29927.

**Harder, J., Bartels, J., Christopher, E., and Shroeder, J.M.** 1997. A peptide antibiotic from human skin. *Nature.* **387**: 861-863.

**Harwig, S., Swiderek, K., Lee, T., and Lehrer, R.** 1995. Determination of disulfide bridges in protegrin-2, an antimicrobial peptide from porcine leukocytes. *J. Pept. Sci.* **3**: 207-215.

**Harwig, S.S.L., Waring, A., Yang, H.J., Cho, Y., Tan, L., and Lehrer, I.** 1996. Intramolecular disulfide bonds enhance the antimicrobial and lytic activities of protegrins at physiological sodium chloride concentrations. *Eur. J. Biochem.* **240**: 352-357.

**Hastings, J.W., Sailer, M., Johnson, K., Roy, K.L., Vederas, J.C., and Stiles, M.E.** 1991. Characterization of Leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum* UAL187. *J. Bacteriol.* **173**: 7491-7500.

**Hernandez-Lucas, C., Fernandez de Calaya, R., and Carbonero, P.** 1974. Inhibition of brewer's yeast by wheat purothionins. *Appl. Microbiol.* **28**: 165-168.

- Hewick, R.M., Hunkapiller, M.W., Hood, L.E., and Dreyer W.J.** 1981. A gas-liquid solid phase peptide and protein sequenator. *J.Biol. Chem.* **256**: 7990-7997.
- Hitzeman, R.A., Hagie, F.F., Levine, H.L., Goeddel, D.W., Ammerer, G., and Hall,B.D.** 1981. Expression of human gene for interferon in yeast. *Nature.* **293**: 717-723.
- Hoffmann, J.A., and Hetru, C.** 1992. Insect defensins: inducible antibacterial peptides. *Immunol. Today.* **13**: 411-415.
- Hoffmann, J.A., and Hoffmann, D.** 1990. The inducible antibacterial peptides of dipteran insects. *Res. Immuno.* **141**: 910-918.
- Holak, T.A., Engstrom, A., Kraulis, P.J., Lindeberg, G., Bennich, H., and Jones, H.A.** 1988. The solution comformation of the antibacterial peptide Cecropin A: a nuclear magnetic resonance and dynamical stimulated annealing study. *Biochem.* **27**: 7620-7629.
- Huang, Y., Di, M., Owens, L.D., Mcbeath, J,H, and Nordeen, R.O.**1997. Expression of an engineered cecropin gene cassette in transgenic tobacco plants confers disease resistance to *Pseudomonas syringae* pv. *tabaci*. *Mol. Plant Path.* **87**: 494-499.
- Hultmark, D., Steiner, H., Rasmuson, T., and Boman, H.G.** 1980. Insect immunity: Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. *Eur. J. Biochem.* **106**: 7-16.
- Inouye, S.** 1994. NAD(P)H-flavin oxidoreductase from the bioluminescent bacterium, *Vibrio fischeri* ATCC 7744, is a flavoprotein. *FEBS Lett.* **347**: 163-168.
- Jaynes, J.M., Nagpala, P., Destefanobeltran, L., Huang, J.H., Kim, J.H., Denny, T., and Cetiner, S.** 1993. Expression of a Cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas-Solanacearum*. *Plant Sci.* **89**: 43-53.

- Jimenez-Diaz, R., Rios-Sanchez, R.M., Desmazeaud, M., Ruiz-Barba, J.L., and Piard, J.C.** 1993. Plantaricin S and T, 2 bacteriocins produced by *Lactobacillus plantarum* LPC010. *Appl. Environ Microb* .**59**: 1416-1424.
- Juvvadi, P., Vunnam, S., and Merrifield, R.B.** 1996. Synthetic mellitin, its enantio, retro, and retroenantio isomers, and selected chimeric analogs: their antibacterial, hemolytic, and lipid bilayer action. *J. Chem. Soc.* **118**: 8989-8997.
- Kagan, B.L., Selsted, M.E., Ganz, T., and Lehrer, R.I.** 1990. Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA.* **87**: 210-214.
- Kaletta, C., and Entian, K.D.** 1989. Nisin, a peptide antibiotic: cloning and sequencing of the *nisA* gene and posttranslational processing of its peptide product. *J. Bacteriol.* **171**:1597-1601.
- Kjeldsen, T., Brandt, J., Andersen, A.S., Egel-Matani, M., Hach, M., Petterson, A.F., and Vad, K.** 1996. A removable spacer peptide in an  $\alpha$ -factor-leader/insulin precursor fusion protein improves processing and concomitant yield of the insulin precursor in *Saccharomyces cerevisiae*. *Gene.* **170**: 107-112.
- Klaenhammer, T.R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* **12**: 39-86.
- Kokrayakov, V.N., Harwig, S.S.L., Panyutich, E.A., Shevchenko, A.A., Aleshino, G.M., Shamova, O.V., Korneva, H.A., and Lehrer, R.I.** 1993. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. *FEBS Lett.* **327**: 231-236.
- Krisanaprakornkit, S., Weinberg, A., Perez, C.N., and Dale, B.A.** 1998. Expression of the peptide antibiotic human  $\beta$ -defensin 1 in cultured gingival epithelial cells and

gingival tissue. Infect. Immun. **66**: 4222-4228.

**Kukuruzinska, M.A., Bergh, M.L.E., and Jackson, B.J.** 1987. Protein glycosylation in yeast. Annu. Rev. Biochem. **56**: 915-944.

**Lambert, J., Keppi, E., Dimarcq, J.L., Wicker, C., Reichhart, J.M., Dunbar, B., Lepage, P., Van Dorsselaer, A., Hoffmann, J., Fothergill, J., and Hoffmann, D.** 1989. Insect immunity: Isolation from immune blood of the dipteran *Phormia terranovae* of two insect antibacterial peptides with sequence homology to rabbit lung macrophage bactericidal peptide. Proc. Natl. Acad. Sci. USA. **86**: 262-266.

**Lee, D.G., Park, J.H., Shin, S.H., Lee, S.G., Lee, K.M., Kim, K.L., and Hahm, K.S.** 1997a. design of novel analogue peptides with potent fungicidal but low hemolytic activity based on the cecropin A-mellitin hybrid structure. Biochem. Mol. Biol. Int. **43**: 489-498.

**Lee, R.H., Cho, Y., and Lehrer, R.I.** 1997b. Effects of pH and salinity on the antimicrobial properties of clavanins. Infect. Immun. **65**: 2898-2903.

**Lee, S., Kurata, S., and Natori S.** 1995. Molecular cloning of cDNA for Sapecin B, an antibacterial protein of *Sarcophaga*, and its detection in larval brain. FEBS Lett. **368**: 485-487.

**Lee, J.Y., Boman, A.S., Sun, C.X., Andersson, M., Jomvall, H., and Mutt, V.** 1989. Antibacterial peptides from pig intestine: isolation of a mammalian cecropin. Proc. Natl. Acad. Sci. USA. **86**: 9159-9162.

**Lepage, P., Bitsch, F., Roeklin, D., Keppi, E., Dimarcq, J.L., Reichhart, J.M., Hoffmann, J.A., Roitsch, C., and Van Dorsselaer, A.** 1991. Determination of disulfide bridges in natural and recombinant insect defensin A. Eur. J. Biochem. **196**: 735-742.

- Lewus, C.B., and Montville, T.J.** 1992. Further characterization of bacteriocins plantaricin BN, bavaricin MN, and pediocin A. *Food Biotech.* **6**: 153-174.
- Loison, G., Findeli, A., Bernard, S., Nguyen-Juilleret, M., Marquet, M., Riehl-Bellon, N., Carvallo, D., Guerra-Santos, L., Brown, S.W., Courtney, M., Roitsch, C., and Lemoine, Y.** 1988. Expression and secretion in *Saccharomyces cerevisiae* of biologically active leech hirudin. *Biotechnology.* **6**: 72-77.
- Loison, G., Vidal, A., Findeli, A., Roitsch, C., Ballou, J.M., and Lemoine, Y.** 1989. High level of expression of a protective antigen of Schistosomes in *Saccharomyces cerevisiae*. **5**: 497-507.
- Mak, P., Wojcik, K., Thogerson, I.B., and Dublin, A.** 1996. Isolation, antimicrobial activities and primary structures of hamster neutrophil defensins. *Infect. Immun.* **64**: 4444-4449.
- Matsudaira, P.** 1987. Sequence from picomole quantities of protein electroblotting onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**: 10035-10038.
- Matsuzaki, K., Yoneyama, S., Fujii, N., Miyajima, K., Yamada, K.I. Kirino, Y., and Anzai, K.** 1997. Membrane permeabilization mechanisms of a cyclic antimicrobial peptide, Tachyplesin I, and its linear analog. *Biochemistry. Wash.* **36**: 9799-9806.
- McCray, P.B. Jr., and Bentley, L.** 1997. Human airway epithelia express a  $\beta$ -defensin. *Am. J. Respir. Cell. Mol. Biol.* **16**: 343-349.
- Merrifield, R.B., Juvvadi, P., Andreu, D., Ubach, J., Boman, A., and Boman, H.G.** 1995. Retro and retroenantiomer analogs of cecropin-mellitin hybrids. *Proc. Natl. Acad. Si. USA.* **92**: 3449-3453.
- Messing, J., and Viera, J.** 1982. A new pair of M13 vectors for selecting either DNA

strand of double digest restriction fragments. *Gene*. **19**: 269-276.

**Michaut, L., Fehlbaum, P., Moniatte, M., Van Dorsselaer, A., Reichhart, J.M., and Bulet, P.** 1996. Determination of the disulfide array of the first inducible antifungal peptide from insect :drosomycin from *Drosophila melanogaster*. *FEBS Lett.* **395**:6-10.

**Miyanoshita, A., Hara, S., Sugiyama, M., Asaoka, A., Taniai, K., Yukuhiro, F., and Yamakawa, M.** 1996. Isolation and characterization of a new member of the insect defensin family from a beetle, *Allomyrina dichotoma*. *Biochem. Biophys. Res. Comm.* **220**: 526-531.

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

**Mor, A., and Nicolas, P.** 1994. Isolation and structure of novel defense peptides from frog skin. *Eur. J. Biochem.* **219**: 145-154.

**Nes, I.F., Diep, D.B., Havarstein, L.S., Eijsink, V., and Holo, H.** 1996. Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie van Leeuwenhoek.* **70**: 113-128.

**Nicolas, P., and Mor, A.** 1995. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. *Annu. Rev. Microbiol* **49**: 277-304.

**Nissen-Meyer, J., and Nes, I.F.** 1997. Ribosomally synthesized antimicrobial peptides and their function, structure, biogenesis, and mechanism of action. *Arch. Microbiol.* **167**: 67-77.

**Oh, J.E., Hong, S.Y., and Lee, K.H.** 1998. Structure-activity relationship study: short antimicrobial peptides. *J. Pept. Res.* **53**: 41-46.

**Okada, M., and Natori, S.** 1985. Ionophore activity of sarcotoxin I, a bactericidal

protein of *Sarcophaga peregrina*. Biochem. J. **229**: 453-458.

**Oren, Z., and Shai, Y.** 1996. A class of highly potent antibacterial peptides derived from pardaxin, a pore-forming peptide isolated from the Moses sole fish *Pardachirus marmoratus*. Eur. J. Biochem. **237**: 303-310.

**Piers, K.L., and Hancock, R.E.W.** 1994. The interaction of a recombinant cecropin/mellitin hybrid peptide with the outer membrane of *Pseudomonas aeruginosa*. Mol. Microbiol. **12**: 951-958.

**Polson, A., and von Wechmar, B.** 1980. Isolation of viral IgY antibodies from yolks of immunized hens. Immuno. Commun. **9**: 475-493.

**Powell, W.A., Catranis, C.M., and Maynard, C.A.** 1995. Synthetic antimicrobial peptide design. Mol. Plant Microbe Interact. **8**: 792-794.

**Purvis, I.J., Bettany, A.J.E., Loughlin, L., and Brown, A.J.P.** 1987. Translation and stability of an *Escherichia coli*  $\beta$ -galactosidase mRNA expressed under the control of pyruvate kinase sequences in *Saccharomyces cerevisiae*. Nucleic Acids Res. **15**: 7963-7974.

**Qu, X.D., Harwig, S.S.L., Oren, A., Shafer, W.M., and Lehrer, R.I.** 1996. Susceptibility of *Neisseria gonorrhoeae* to protegrins. Infect. Immun. **64**: 1240-1245.

**Reichhart, J.M., Essrich, M., Dimarcq, J.L., Hoffmann, D., Hoffmann, J.A., and Lagueux, M.** 1989. Insect immunity. Isolation of cDNA clones corresponding to dipterecin, an inducible antibacterial peptide from *Phormia terranova* (Diptera). Transcriptional profiles during immunization. Eur. J. Biochem. **182**: 423-427.

**Reichhart, J., Petit, I., Legrain, M., Dimarcq, J., Keppi, E., Lecocq, J., Hoffmann, J., and Achstetter, T.** 1992. Expression and secretion in yeast of active insect defensin,

an inducible peptide from the flesh fly *Phormia terranova*. Invert. Reprod. Devel. **21**:15-24.

**Reimann-Philippe, U., Sharader, G., Martinoia, E., Barkholt, V. and Apel, K.** 1989. Intracellular thionins of barley. A second group of leaf thionins closely related to but distinct from cell wall-bound thionins. J. Biol. Chem. **264**: 8978-8974.

**Romanos, M.A., Scorer, C.A., and Clarke, J.J.** 1992. Foreign gene expression in yeast: a review. Yeast. **8**: 423-488.

**Romeo, D., Skerlavaj, B., Bolognesi, M., and Gennaro, R.** 1988. Structural and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. J. Biol. Chem. **263**: 9573-9575.

**Russell, J.P., Diamond, G., Tarver, A.P., Scanlin, T.F., and Bevins, C.** 1996. Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumor necrosis factor alpha. Infect. Immun. **64**: 1565-1568.

**Sahl, H.G.** 1991. Pore formation in bacterial membranes by cationic lantibiotics. p347-358. In Jung, G., and Sahl, H.G. (ed.), Nisin and novel lantibiotics. ESCOM. Leiden. The Netherlands.

**Sahl, H.G., Jack, R.W., and Bierbaum, G.** 1995. Biosynthesis and biological activities of lantibiotics with unique post-translational modification. Eur. J. Biochem. **230**: 827-853.

**Sambrook, J., Fritsch, E.F., and Maniatis, T.** 1989. Molecular Cloning: A laboratory manual. 2<sup>nd</sup> Edn., Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

**Sammuelsson, G.** 1974. Mistletoe toxins. Syst. Zool. **22**: 566-569.



**Schagger, H., and von Jagow, G.** 1987. Tricine- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1-100 kDa. *An. Biochem.* **166**: 368-379.

**Schnapp, D., Kemp, G.D., and Smith, V.J.** 1996. Purification and characterization of a proline rich antibacterial peptide, with sequence similarity to bactenecin-7, from the hemocytes of the shore crab, *Carcinus maenas*. *Eur. J. Biochem.* **240**: 532-539.

**Schoeman, H., Vivier, M.A., du Toit, M., Dicks, L.M.T., and Pretorius, I.S.** 1999. The development of bactericidal yeast strains by expressing the *Pediococcus acidilactici* pediocin gene (*ped A*) in *Saccharomyces cerevisiae*. *Yeast.* **15**: 647-656.

**Seizen, R.J., Kuipers, O.P., and De Vos, W.M.** 1996 Comparison of lantibiotic gene clusters and encoded proteins. *Antonie van Leeuwenhoek.* **69**: 171-184.

**Selsted, M.E., Tang, Y., Morris, W.L., Macguire, P.A., Navotny, M.J. Smith, W., Henschen, A.H., and Culler, J.S.** 1993. Purification, primary structures and antibacterial activities of beta defensins, a new family of antimicrobial peptides from bovine neutrophils. *J. Biol. Chem.* **268**: 6641-6648.

**Selsted, M.E. and Harwig, S.S.L.** 1989. Determination of the disulfide array in the human defensin HNP-2. A covalently cyclized peptide. *J. Biol. Chem.* **264**: 4003-4007.

**Shai, Y.** 1995. Molecular recognition between membrane-spanning polypeptides. *TIBS.* **20**: 460-464.

**Shuller, H.J., and Entian, K.D.** 1988. Molecular characterization of yeast regulatory gene CAT3 necessary for glucose depression and nuclear localization of its product. *Gene.* **67**: 247-257.

**Simmaco, M., Mignogna, G., Barra, D., and Bassa, F.** 1994. Antimicrobial peptides

from frog skin secretions of *Rana esculenta*. J. Biol. Chem. **269**:11956-11961.

**Sipos, D., Andersson, M., and Ehrenberg, A.** 1992. The structure of mammalian antibacterial peptide cecropin P1 in solution, determined by proton-NMR. Eur. J. Biochem. **209**:163-169.

**Subbalakshmi, C., Krishnakumari, V., Nagaraj, R., and Sitaram, N.** 1996. Requirements for antibacterial and hemolytic activities in the bovine neutrophil derived 13-residue peptide indolicidin. FEBS Lett. **395**: 48-52.

**Taguchi, S., Nakagawa, K., Maeno, M., and Momose, H.** 1994. *In vivo* monitoring system for structure-function relationship analysis of the antibacterial peptide apidaecin. Appl. Environ. Microbiol. **60**: 3566-3572.

**Terras, F.R.G., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuven, F., Vanderleyden, J., Cammue, B.P.A., and Broekaert, W.F.** 1995. Small cysteine-rich antifungal proteins from raddish: their role in host defense. Plant Cell. **7**: 573-588.

**Terras, F.R.G., Schoofs, H.M.E, De Bolle, M.F.C., Van Leuven, F., Rees, S.B., Vanderleyden, J., Cammue, B.P.A., and Broekaert, W.F.** 1992. Analysis of 2 novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. J. Biol. Chem. **267**: 15301-15309.

**Vilas Alves, A.L., De Samblanx, G.W., Terras, F.R.G., Cammue, B.P.A., and Broekaert, W.F.** 1994. Expression of functional *Raphanus sativus* antifungal protein in yeast. FEBS Lett. **348**: 228-232.

**Wade, D., Boman, A., Wahlin, B., Drain, C.M., Andreu, D., Boman, H.G., and Merrifield, R.B.** 1990. All D-amino acid containing channel-forming antibiotic peptides. Proc. Natl. Acad. Sci. USA. **87**: 4761-4765.

**Westerhoff, H., Zasloff, M., Rosner, J., Hendler, R., De Waal A., Vaz Games, A., Jongsma, A., Riethorst, A., and Juretic, D.** 1995. Functional synergism of the magainins pGLa and magainin-2 in *Escherichia coli*, tumor cells and liposomes. Eur. J. Biochem. **228**: 257-265.

**Yasin, B., Lehrer, R.I., Harwig, S.S.L., and Wagar, E.A.** 1996. Protegrins: structural requirements for inactivating elementary bodies of *Chlamydia trachomatis*. Infect. Immun. **64**: 4863-4866.

**Yousef, A.E., Luchansky, J.B., Degnan, A.J., and Doyle, M.P.** 1991. Behaviour of listeria monocytogenes in wiener exudates in the presence of *Pediococcus acidilactici* or Pediocin AcH during storage at 4 or 25°C. Appl. Environ. Microbiol. **57**: 1461-1467.

**Zaret, K.S., and Sherman, F.** 1982. DNA sequence required for efficient transcription termination in yeast. Cell. **28**: 563-573.

**Zasloff, M.** 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA. **84**: 5449-5453.

**Zenno, S., Saigo, K., Kanoh, H., and Inouye, S.** 1994. Identification of the gene encoding the major NAD(P)H-flavin oxidoreductase of the bioluminescent bacterium *Vibrio fischeri* ATCC 7744. J. Bacteriol. **176**: 3536-3543.