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THE ISOLATION AND CHARACTERIZATION OF INFLAMMATORY POLYPEPTIDES FROM *STAPHYLOCOCCUS EPIDERMIDIS*

by

Christopher Mehlin

A dissertation submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

University of Washington

1998

Approved by _____
Chairperson of Supervisory Committee

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Program Authorized
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Doctoral Dissertation

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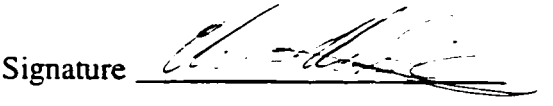
Signature 

Date 7-27-98

University of Washington

Abstract

THE ISOLATION AND CHARACTERIZATION OF INFLAMMATORY POLYPEPTIDES FROM *STAPHYLOCOCCUS EPIDERMIDIS*

by Christopher Mehlin

Chairperson of the Supervisory Committee: Professor Seymour J. Klebanoff,
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Septic shock is the result of an overwhelming immune response to bacterial products, or modulins. Lipopolysaccharide is the primary modulin involved in Gram negative sepsis, but an analogous agent from Gram positive bacteria has not yet been identified. In order to characterize what such a modulin might be, a hospital strain of *S. epidermidis* which had been observed to secrete or shed potent inflammatory agents was examined more closely. Sephadex gel chromatography revealed that the active component in bacterial supernatants had an apparent molecular weight of about 35 kDa. This active material partitioned into the organic layer following hot, aqueous phenol extraction and was found to consist of a complex or aggregate of three polypeptides. These were designated phenol soluble modulins (PSM) alpha, beta, and gamma. The PSMs were isolated by phenol extraction, dialysis and High Pressure Liquid Chromatography and characterized by Edman degradation, mass spectrometry, and genetic sequencing. PSMγ is a 25 amino acid protein identical to the previously characterized *S. epidermidis* delta toxin, while PSMα and PSMβ were 22 and 44 amino acid polypeptides, respectively, with more distant homology to known staphylococcal toxins. PSMα was found to be the most active and least abundant of the three modulins. All three PSMs are highly hydrophobic and lack arginine, cysteine, histidine, proline, and tyrosine, and only the gene for PSMγ is located within the *agr* virulence locus.

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Abbreviations, general

A_{214}	Absorbance at 214nm
C	Celsius
DNA	Deoxyribonucleic Acid
FPLC	Fast Protein Liquid Chromatography
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
IL	Interleukin
IMDM	Iscove's Minimal Defined Medium
kD	Kilodalton
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
LTR	Long Terminal Repeat
LTR_{LUC}	Luciferase Gene Driven by HIV LTR
LUC	Luciferase
MALDI	Matrix Assisted Laser Desorption Ionization
MAP	Mitogen Activated Protein
mM	Millimoles
MS	Mass Spectrometry
MW	Molecular Weight
MWCO	Molecular Weight Cutoff
NaCl	Sodium Chloride (Table Salt)
NF	Nuclear Factor
nm	Nanometers
nM	Nanomolar
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pI	Isoelectric Point
PMA	Phorbol 12-Myristate 13-Acetate
PSM	Phenol Soluble Modulin
PBS	Phosphate Buffered Saline
pBS	Bluescript Plasmid
RLU	Relative Light Units
RPMI	Roswell Park Memorial Institute
RP-HPLC	Reverse Phase High Pressure Liquid Chromatography
SDS	Sodium Dodecyl Sulfate
SEA	Staphylococcal Enterotoxin A
TFA	Trifluoro Acetic Acid
TOF	Time of Flight
TSST	Toxic Shock Syndrome Toxin
uM	Micromolar

Abbreviations, Amino and Nucleic Acids

Amino Acids

A	Alanine
C	Cysteine
D	Aspartic Acid
E	Glutamic Acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

Nucleic Acids

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Introduction

The immune system is responsible for defining the physical individual, for discriminating between oneself and the outside world. It is tempting to regard infection as an invasion and the process of inflammation as a pitched battle between foreign invaders and the defending host immune system. The elucidation of "virulence factors"(1-4) has become fashionable, as their promise is to define the arsenal of the enemy, but virulence is a multifactorial process which is vastly oversimplified when defined in terms of war. Even the distinct line between self and other is misleading, as we live commensally and symbiotically with an abundance of bacteria and viruses -- the absence of some of which would in itself result in disease.(5, 6) A central question is, then, where our immune systems draw a border between allowable, even necessary, colonization and when the presence of foreign micro-organisms warrants mounting a potentially damaging immune response.(7)

Bacterial sepsis is the primary example of immunological overzealousness, as the hypotension and, often, death, of septic patients is precipitated by a massive, disseminated response to what can be a relatively minor, localized infection.(8) This response is triggered by the presence of bacterial products in the bloodstream -- notably lipopolysaccharide (LPS) in the case of gram-negative organisms, which is well known to have potent immunomodulatory capacity.(9, 10) Such agents have come to be called modulins,(11, 12) and literally hundreds have been described (see Table I), although few possess the potency of LPS. Understanding the inflammatory response to Gram-positive bacteria has been hampered somewhat by paradigms established in Gram-negative bacteria. Lipoteichoic acid (LTA), the structural equivalent to LPS in Gram-negative bacteria, does not possess nearly the immunostimulatory capacity of its Gram-negative counterpart. Yet Gram-positive bacteria are known to induce an immune

response at least as strong as Gram-negative bacteria: over half the cases of septic shock are due to Gram-positive infection.(13) In experimental models of sepsis, shock is induced by equal numbers of Gram-positive and negative bacteria.(14-16) While LPS can precipitate shock in these same model systems, LTA is ineffective.(17) Despite the large number of modulins which have been described, a ubiquitous, Gram-positive modulin has remained elusive, and LTA is still frequently referred to as the "Gram-positive LPS."(18)

Peptidoglycan,(19) teichoic acid,(20) proteins,(21) lipids,(22) and DNA(23) from Gram-positive bacteria have all been observed to induce an inflammatory response in various experimental systems. Clinically, the most important of these modulins appears to be a class of proteins known as exotoxins.(8) Bacteria are routinely isolated from infections and assessed for the generation of toxins, and more aggressive therapy is indicated when toxins are found. In the case of Staphylococci, *S. aureus* is known to have the potential to secrete toxins such as toxic shock syndrome toxin (TSST) and staphylococcal exotoxin A (SEA).(24) These strains also induce coagulation of blood and are therefore grouped as "coagulase-positive" bacteria due to the technical simplicity of this test. Traditionally, the coagulase-positive staphylococci are regarded as much more serious in the clinical setting than are coagulase-negative strains.(8) This is despite the large role played by coagulase-negative bacteria in many diseases, including septic shock.(13)

There are at least 32 species of staphylococci, of which about a dozen are commonly associated with humans.(24) Many, especially *S. epidermidis* and *S capitis*, are found as commensal flora on the skin.(25) *S. aureus* is the species generally responsible for staphylococcal disease, but given the appropriate environment (*e.g.* skin damage, immunosuppression, or unsanitary hypodermic injection) commensal

organisms can be spurred into creating serious infections.(26) In any case, it is not clear what bacterial components trigger the septic immune response,(27) and it appears likely that an array of Gram--positive "toxins" may combine with bacterial cell wall components (peptidoglycan and lipoteichoic acid) in a synergistic manner in order to induce the inflammation required for sepsis.(28, 29) Some of the most probable modulins are listed in Table I.

It is worth noting that none of these modulins possess the potency of LPS, and none could account for the response required for the induction of sepsis.(30, 31) It is likely that several components work together in synergy. Peptidoglycan and lipoteichoic acid, for example, are much more potent when combined than when tested singly,(29) and some bacterial toxins enhance the reactivity of macrophages to LPS by more than four orders of magnitude.(32-34)

Macrophages are the primary cells involved in the induction of septic shock,(35) mostly through the production of TNFα.(36) Cells of the monocyte/macrophage lineage are the primary mediators of non-specific immunity against bacteria.(37) Macrophages have the ability to react directly to LPS through the cellular receptor CD14 and a class of receptors known as scavenger receptors: reaction includes mobilization of their own effector machinery as well as the secretion of cytokines which promote the migration and activation of T cells, neutrophils, and more macrophages.(38) In experimental systems, a shocklike state can be induced by TNFα alone.(39)

The Regulation of Monocytic Inflammation and the THP-1 LTR_{LUC} Model

As a primary screen for modulins which activate cells of monocytic lineage, we have been using the THP-1 LTR_{LUC} system developed by Farhad Kazazi, Wes

VanVoorhis, and Seymour Klebanoff (40) This system utilizes a THP-1 (monocytic) cell line which has been transfected with a construct encoding luciferase under the control of the Human Immunodeficiency Virus Long Terminal repeat (HIV-LTR).

All immortal monocytic cell lines are arrested fairly early in their development.(41) The human leukemia cell line THP-1 is probably the most differentiated ("macrophage-like") of the cell lines and thus has become a widely employed experimental model of monocytes/macrophages. Originally isolated by Tsuchiya *et. al.*,(42) the cells have a monocytic morphology, lipid metabolism, surface protein expression and secretion, and a capacity for phagocytosis and pinocytosis. As an immortalized cell line, they are also a good deal easier to work with and more homogenous than primary human cells.

The LTR_{LUC} model employs the Long Terminal Repeat from HIV, a region which includes the viral enhancer and promoter regions. This has been spliced onto luciferase, an easily-assayed, sensitive reporter gene. Luciferase is known for being short-lived,(43) so as a reporter it tends to indicate recent expression. This is in contrast to B-galactosidase(44) and chloramphenicol acetyl transferase (CAT)(43), both of which can linger for days following their production. This assay was originally developed as an assay for early HIV expression. We have found, however, that LTR-mediated promoter activity is not reliably predictive of viral expression. This also has been noted by others(45, 46) and may stem from the presence of multiple regulatory genes within the HIV genome (such as TAT).(47, 48)

The THP-1 LTR_{LUC} model has proven useful, though, as a method for investigating monocyte activation, as the HIV-LTR contains promoter sequences in common with many activation-induced genes in monocytic cells. The primary promoter regions which are of interest are NF-κB, SP-1, and AP-1.(47) Although many other

sites in the HIV-LTR have been identified, these three are likely to play the largest role in basal HIV regulation and monocyte activation.(49-52)

NF-κB is considered to be the primary, central regulator in monocyte activation and is responsible for the regulation of a wide array of inflammatory mediators in macrophages.(53) These include M-CSF, G-CSF, GM-CSF, TNF, IL-1, IL-6, Tissue Factor, IL-2 receptor alpha chain, macrophage chemotactic protein 1, and prostaglandin biosynthesis enzymes. NF-κB is a family of proteins belonging to the REL class of trans-activating proteins.(54) Most commonly, the active form of NF-κB is a p50/p65 dimer which binds its target sequence (5'-GGGPuNNPyPyCC-3') with 10nM affinity.(55) The HIV-LTR contains two NF-κB consensus sequences, and most of the basal transcription of HIV occurs via this system.(56) The regulation of the NF-κB gene complex has been an area of intense study and has proven to be quite complex.(53) NF-κB is held in an inactive form in the cytoplasm of cells by a family of at least six proteins known as I-kB.(57) Activation involves the phosphorylation of I-kB, which is then ubiquitinated and degraded by the proteasome, freeing NF-κB to translocate to the nucleus and bind DNA. One of the primary ways that the initial phosphorylation event occurs is via a MAP kinase cascade, of which at least three serial phosphorylations have been elucidated, starting with MAP kinase kinase kinase and culminating in specific I-kB kinases.(58, 59) Several studies have now demonstrated that these kinases probably function as a large complex in cells.(60-64) Other kinases can also perform this function, such as RNA-dependent protein kinase(65) and certain PKC isoforms.(66) The story of I-kB does not end with its phosphorylation, however, as this phosphorylation is not sufficient for the dissociation of I-kB from NF-κB, and protease inhibitors can block activation of NF-κB.(67, 68) I-kB must also be ubiquitinated,(69) and an oxidation-sensitive step has been found in the events leading up to the

degradation of NF-κB.(70) Antioxidants have been repeatedly shown to prevent the activation of NF-κB.(71, 72) It has been suggested that both TNFα and IL-1 activate NF-κB through a pathway wherein Phospholipase C cleaves phosphatidyl choline, producing diacylglycerol which activates sphingomyelinase to release ceramide from sphingomyelin, activating kinases.(73-75) Reactive oxygen intermediates (ROIs) from mitochondrial sources are thought to be involved in TNF-induced activation of NF-κB in steps prior to phosphorylation events,(76, 77) as PKC can induce the activation of NF-κB without mitochondrial involvement.(53)

The advantages of using an HIV-LTR_{LUC} construct rather than looking at TNF expression directly are threefold. First, luciferase activity is considerably easier to assess than is TNF as it has direct enzymatic activity and linearity over a wider range of concentrations than would be possible with a TNF ELISA. Second, the regulation of TNF is more complex than simple transcriptional regulation, involving proteolytic processing,(78) membrane binding and release,(79) and subsequent inactivation by soluble TNF receptors.(80-83) Third, by looking at a short-lived enzyme one may be confident that the observed activity is due to recent transcription.

The chief transcriptional regulator of the inflammatory response in monocytes is NF-κB, but other factors such as SP-1, AP-1, and NF-IL6 also play a role.(84, 85) Generally, these factors work in combination with NF-κB,(86) often providing a combined response greater than the sum of the parts.(52, 87) NF-κB also serves as the chief early promoter of HIV transcription, so although the HIV-LTR has binding regions for SP-1 and AP-1, it is not unjustly oversimplified to think of the HIV-LTR as an NF-κB-driven promoter.(88-90) While any homogeneous cellular model of inflammation would be founded on bold assumptions, the level of active NF-κB in a monocytic cell line is a reasonable initial screen for inflammatory agents.

Staphylococcus epidermidis strain UW-3

It had been demonstrated previously that 31 strains of staphylococci (9 *S. aureus*, 2 *S. capitis*, 11 *S. epidermidis*, 2 *S. hemolyticus*, 4 *S. hominis*, 1 *S. saprophyticus*, and 2 *S. warneri*) all stimulated the THP-1 LTR_{LUC} system to some extent, although the degree of stimulation varied widely.(40) For those experiments, the bacteria were grown in standard broth, collected by centrifugation, and vigorously vortexed with PBS to remove loosely-associated material. These PBS rinsings, re-centrifuged to remove bacteria, were the "extract" used for stimulating the THP-1 cells. The study described in this thesis was undertaken to determine what the activating factor is, and a strain of *Staphylococci* was selected (UW-3) which provided a high level of stimulation without producing much bacterial slime. A mixture of bacterial wall components and sugars, slime is thought to be important for adhesion and immuno-evasion,(91-97) but as the level of slime production was not correlated with the level of LTR_{LUC} stimulation (data not shown), this low-slime strain was chosen in order to simplify the isolation of the active factor(s). In another study, supernatants from 64 strains of Gram positive bacteria (not limited to staphylococci) were found to stumulate cytokine expression from whole human blood.(98)

Table I. **Gram-Positive Modulins**

Modulin	Host Cells Responding T-cells	Macrophages	Staphylococcal source *Aureus*	*Epidermidis*	References
Peptidoglycan		X	X	X	(99)
Lipoteichoic Acid		X	X	X	(20)
Enterotoxins A-E	X		X		(21, 100)
Exfoliative toxins			X		(101, 102)
Hemolysins a-d		X	X	X	(103)
TSST-1	X		X		(104)
Coagulase			X		(105)
Muramyl Dipeptide		X	X	X	(106-108)
Protein A			X		(109)
Bacterial DNA		X	X	X	(23)

The modulins most likely to be involved in Gram-Positive septic shock.

Chapter One

The Development of a High-Throughput Assay for HIV-LTR Mediated Stimulation

Introduction

Many systems have been described to measure transcription from the HIV-LTR using a wide array of cells and reporter genes. Luciferase, when used as a reporter gene, has the advantages of exquisite sensitivity, linearity over a wide range of concentrations, and a brief cellular half-life. Assessing luciferase activity from cells has historically been labor-intensive, however, involving multiple centrifugation, washing, and lysis steps. These steps are especially problematic when dealing with small sample volumes or a large number of samples. Typically, cells are incubated with stimulation, harvested by centrifugation, washed, lysed and subsequently clarified by centrifugation. My attempts to adapt this protocol to a 96-well format resulted in wide variability between duplicates within an experiment (data not shown). It seemed likely that this variability was due to inconsistencies in handling the small volumes of cells and media required by this format, especially given the relatively large number of manipulations required for each well. To address this possibility, I explored the use of filter-bottomed plates.

It had been previously observed that the THP-1 LTR_{LUC} cells utilized in this assay had a peak activity which varied greatly from day to day (Seymour Klebanoff, unpublished observations). This variation was multi-factorial and was influenced by the

particular cellular substrain employed and passage history, among other things. In order to be a useful measure of activity, therefore, a standard curve and sample dilutions were required in every assay, conditions which rapidly multiplied the number of samples which needed to be processed. The ability to handle a large number of samples on a single day can compensate for variability which occurs between days, but it is necessary to minimize as much as possible the variability within an individual experiment.

Materials and Methods

Cells and Culture Conditions

The cells employed in this study were a monocytic cell line, THP-1, which had been stably transfected with a construct encoding luciferase under the control of the HIV-LTR as previously described.(40) Cells were routinely grown in RPMI 1640 medium with 25mM Hepes, 2mM l-glutamine, 50 U/ml penicillin, 50 ug/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, Bio-Whittaker, Walkersville, MD). The activation assay was conducted in the same buffer without FBS. The cells were plated in 96-well filter plates with a .22uM pore size, hydrophilic, low protein binding membrane (Multiscreen, Millipore, Bedford, MA). The cells were plated in a final volume of 250ul per well with $1x10^6$ cells/ml in each well; this volume includes 25ul of PBS (phosphate buffered saline) which contained the stimuli of interest.

Harvesting and Analysis

Cells were cultured for 6 hours in a 37° humidified incubator at 5% CO_2. The cells were harvested by low-speed centrifugation (150g for 3 min.) onto the filters,

rinsed with 100ul PBS and another centrifugation (7 min.), and lysed with 33ul lysis buffer (Promega). The plate containing lysis buffer was shaken at room temperature for 10 minutes and the lysis buffer was collected by placing the filter plate over a 96-well vinyl plate (Costar, Corning, NY) and centrifuging for 10 min. 25ul of this filtered lysis buffer was then utilized to measure light production in a luminometer (Monolight 1500, Analytical Luminescence Laboratory, San Diego) using a luciferase assay kit (Promega, Madison, WI).

Stimuli

Stimulation was either from a supernatant of *S. epidermidis* UW-3 grown in IMDM (Iscove's Minimum Defined Medium)(Bio-Whittaker) with 1% glucose and filter sterilized or from PMA (Sigma, St. Louis, MO). PMA was stored at 1 mg/ml in ethanol at -70° and diluted just prior to use.

Results

Figure 1 shows the dose-response curve of THP-1 cells to a *S. epidermidis* UW-3 culture supernatant. The assay was done in quadruplicate and shows a useful range of about one log, above and below which the response tends to flatten. As shown by the standard deviation for each triplicate point, the filter plate modification does not appear to induce well-to-well variability.

Figure 2 shows the response of THP-1 cells to 1ug/ml PMA in a series of nineteen separate experiments. Each experiment was run in quadruplicate (except numbers 1, 2, 3, and 6 which were repeated five times and number 18 repeated three times), and these replicates were found to be remarkably reproducible within a particular

experiment. Day-to-day variation in cellular responsiveness is apparent in looking across the graph, however. This variability did not correlate reliably with the background level of luciferase activity or the length of time since the last passage of cells (data not shown).

Discussion

The low variability of replicate samples within an experiment indicates that the high-throughput method described here does not in itself generate a significant amount of error. Samples within an experiment, therefore, are directly comparable to each other.

The high interexperiment variability was observed using traditional techniques as well and likely reflects an inherent quality of the THP-1 LTR_{LUC} model. This shortcoming can be dealt with in several ways. First, a standard curve can be run with every assay; samples within that assay can be compared to the standard curve and expressed as a percentage of the positive control. This is the preferred method, as it normalizes the data to a standard reference and allows for direct comparison of different experiments. Second, non-parametric statistics can be used (*e.g.* Mann-Whitney, Wilcoxson Rank-Sum) to derive statistical significance from a group of experiments with widely differing maxima.(110) This is still likely to require many duplicate experiments. Third, multiple experiments can be run on the same day. Using cells from different flasks and entirely separate solutions, one could greatly reduce the inter-experiment variability. This last approach begs the question of how separate a separate experiment need be (*i.e.* the difference between replicates and duplicates). It could convincingly be argued that the stated experimental error should reflect the actual

variability inherent in the experiment (and that therefore the experiments need to be done on different days). It is also the case, however, that one's experimental technique should minimize variability which is due to causes other than the one being tested. A reasonable compromise would be to run an experiment in duplicate or triplicate and compare several such experiments done on different days to see if the results are consistent. In actual practice, the approach taken varied depending upon the circumstances. Thus, fractions from a chromatographic column were typically serially diluted and compared to a standard in order to assess the percentage recovered in any particular fraction. It is more sensible to run a second column and evaluate a second set of fractions than it does to re-assay the original fractions. In this case, one is more concerned with where the activity elutes and the percentage recovered. If one wishes to compare the activity of two agents, however, it would be desirable to have a statistical value associated with the comparison. Ideally, such statistics would be based upon entirely different experiments done on different days. For the sake of statistical significance, it would be tempting to run many experiments on the same day as the groupings would be a lot tighter. Realistically, if one is concerned with simply eliminating artifact from one's observations, it does make sense to design experiments so as to minimize the effects of the day-to-day variability. Such an approach would be valid only if the data could be supported by experiments run on different days, thereby excluding artifacts due solely to the peculiarities of the particular day on which the experiment was performed. As this assay was used primarily to judge the percentage of recovery, I found it most practical to simply run a standard curve with every experiment and normalize the data points relative to this curve.

The assay described herein allows for the high-throughput assessment of samples, with a capacity of hundreds per day. The reproducibility of replicate samples

on any given day is quite good, although the variability in day-to-day responsiveness of the THP-1 LTR_{LUC} cells remains unresolved. For the measurement of luciferase production induced by *S. epidermidis* UW-3, the useful range of the assay is over about a tenfold dilution. The high capacity of this assay allows for a large number of sample dilutions and for the generation of a standard curve within every experiment, largely compensating for the variability inherent in the system. It is this large volume which makes it possible to contemplate purifying the active factor from the *S. epidermidis* supernatant, as chromatographic steps rapidly generate many samples which need to be assessed.

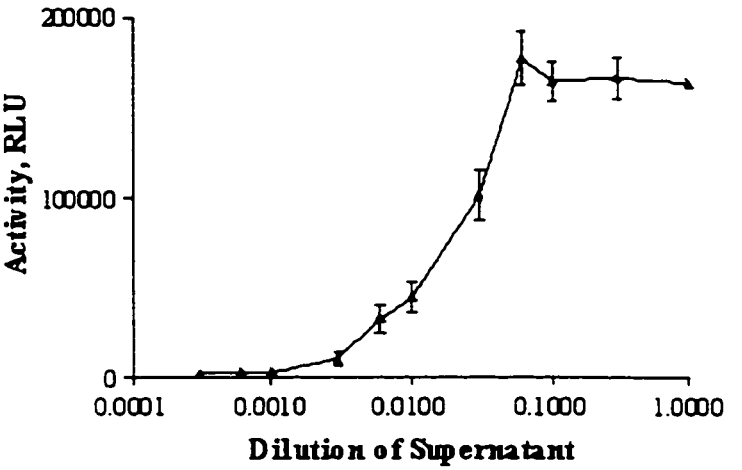


Figure 1. Luciferase activity of LTR_{LUC} system with dilutions of *S. epidermidis* UW-3 supernatant. Shown is one representative experiment done in quadruplicate; error bars represent the standard deviation. A dilution of one indicates that 25ul of the supernatant was used in a total assay volume of 250ul.

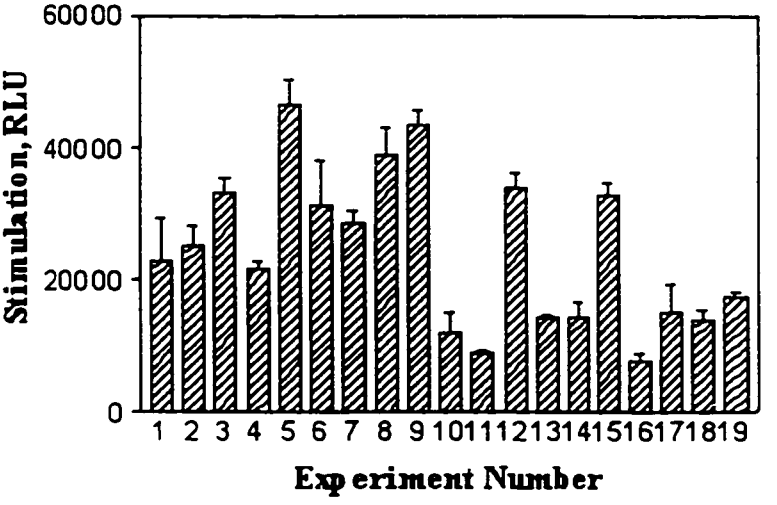


Figure 2. Reproducibility of the LTR_{LUC} assay was determined as described in the text. Values shown above are for nineteen consecutive experiments.

Chapter Two

S. epidermidis UW-3 Induces HIV-LTR Mediated Transcription Via an Aggregate of Polypeptides

Introduction

It has been previously shown that supernatants from staphylococcal bacteria induce HIV-LTR mediated transcription in THP-1 cells.(40) The work in this thesis was undertaken to define what the nature of this stimulus is. Lipoteichoic acid(111) and peptidoglycan(112) have both been shown to induce similar activities, although the doses required were typically very high. The purpose of these initial investigations was to determine the basic nature of the stimulating product (*e.g.* protein, carbohydrate) and to determine whether it is distinct from peptidoglycan and lipoteichoic acid.

Materials and Methods

Growth of Organisms, Harvesting Supernatant

S. epidermidis strain UW-3 was obtained from the University of Washington hospital (Seattle, WA) and grown overnight with shaking at 37° C. The bacteria were grown in IMDM (with Hepes and L-glutamine from BioWhittaker) which had been brought to 1% glucose and filter sterilized through a .2uM membrane (ZapCap, Schleicher and Schuell, Keene, NH). Following overnight growth, the media was clarified by centrifugation and membrane filtration. For initial dialysis experiments, 1 ml of supernatant was dialyzed against four liters of distilled water, PBS

(BioWhittaker), .4M NaCl, or glucose-supplemented IMDM (as above) using cellulose dialysis tubing with either a 12 kD (Sigma, St Louis, MO) or a 25 kD (Spectrum, Houston, TX) molecular weight cutoff. Dialysis was done overnight at 4° C with three changes of buffer, and the recovery of activity was determined by comparing dilutions of the retentate to a standard curve made from dilutions of undialyzed material in the THP-1 LTR_{LUC} assay.

Phenol Extraction

Large-scale (10 liter) preparations of material were made by growing the bacteria and collecting the supernatant as above. This was then concentrated using a tangential-flow ultrafiltration cartridge with a 10 kD pore size (Millipore, Bedford, MA) to a volume of about 350 ml. Further concentration was done by centrifugal ultrafiltration (Centriprep 10, Amicon, Beverly, MA) to about 20 ml. This material was dialyzed extensively against 0.4M NaCl using 25kD MWCO tubing and then against distilled water with 12 kD MWCO tubing. 50 ml of buffer-saturated phenol was added (Gibco/BRL, Grand Island, NY) and enough 1M NaOAc was added to bring the aqueous portion to 0.1M in sodium acetate. The mixture was vortexed and incubated at 65° C with agitation for sixty minutes. The two layers were then separated by centrifugation and the aqueous layer was extracted twice more in a similar manner with 25ml of phenol and thirty minutes of incubation each time. The phenol extractions were pooled and then dialyzed extensively against distilled water in 12 kDa MWCO tubing. The off-white precipitate which formed during this dialysis was resuspended by vigorous stirring, and this suspension was then lyophilized and stored frozen at -20° C. At every step of this process, aliquots containing 0.1% of the volume of material were

lyophilized and frozen for determination of activity, and mass recovery was determined by lyophilizing 1.0% of the material in a weighed glass test tube.

Small-scale phenol extractions were done using lyophilized, double-dialyzed culture supernatant (as above -- not phenol extracted). A total of 180ug of this material was tumbled at 65° C in 500 ul .1M NaOAc (pH 4.7) with 500ul of phenol. The layers were separated by centrifugation, and 100ul of both the phenol and aqueous layers were gathered for analysis. These samples were added to 1.9 ml of PBS and dialyzed against 4 liters of PBS with 12 kD MWCO dialysis tubing. The recovery of activity in the phenol layer and the aqueous layer could thus be directly compared.

Gel Chromatography

Gel chromatography was performed on a Pharmacia (Piscataway, NJ) Fast Protein Liquid Chromatography setup with a HR 10/30 column packed with Superose 12 resin. The column was run in PBS at .4 ml/min., and was monitored at 280nm. For the determination of molecular weight, the elution time of the peak of activity was compared to standards of known size, and the numerical value was calculated based on the principle that the retention time (past void volume) is inversely proportional to the $\log_{10}$ of the molecular weight (as per manufacturer's instructions). The molecular weight was not affected by phenol extraction, and the numerical results are the mean of three experiments ± standard deviation.

Proteolysis

Proteases immobilized on agarose beads were obtained from Sigma and were rinsed thoroughly with PBS prior to use. 20ug of phenol-extracted material was incubated with tumbling in PBS with and without proteases. The proteases were

removed by low-speed (325g) centrifugation after three hours, and the supernatant was frozen at -20° C and later assayed in the THP-1 LTR_{LUC} system.

Determination of the V-8 protease

200ug of dialyzed, lyophilized culture supernatant was run on a 12.5% SDS-PAGE gel.(113) The gel was allowed to run until the dye front reached the bottom, and the resultant single band was electroblotted onto PVDF membrane (Immobilon P, Millipore, Bedford, MA) and submitted for Edman degradation.

The V-8 protease activity was assayed directly through the use of a specifiic substrate: Carbobenzoxy-Phe-Leu-Glu-4-nitrilide (Boehringer Mannheim, Mannheim, Germany). The activity of culture supernatant was compared to the V-8 protease available commercially (Sigma) for the determination of the level of activity present. Activity could be readily assessed by monitoring the absorbance at 405 nm (measuring liberated nitroaniline).

Cibacron Blue Chromatography

Cibacron Blue Resin (Sigma) was rinsed with PBS and dialyzed, lyophilized culture supernatant was applied in the same buffer at 4° C. The material was allowed to bind the column for half an hour, and then the column was rinsed with 22 ml of PBS at .1 ml/min. Elution was performed by switching to 6M urea, and the eluate was dialyzed overnight prior to assay.

High molecular weight SDS-PAGE gels were made in the method of Schagger (114) and had a stacking gel of 12% and a resolving gel of 16%. Gels were fixed wtih 20% TCA and stained with Coomassie Blue.

Results

S. epidermidis active factor has a molecular weight of about 35 kDa

As shown in Table II, it was determined that the active component of *S. epidermidis* culture supernatants is retained by dialysis tubing. This was consistent with what we had observed with microfiltration, in that the active component was retained by 30 kDa cutoff membranes (data not shown). The crude *S. epidermidis* material appeared by Superose gel chromatography to have a mass of about 34,500 +/- 6000 kDa (Figure 3). The retention of the active material by dialysis tubing with a 25 kDa cutoff was utilized as a primary purification step. The largest component of the defined media used to grow the bacteria was 3 kDa, so all the media componenets could be removed as well as the smaller bacterial products. As dialysis with 25 kDa MWCO tubing had to be done in .4M NaCl to retain activity, a two-step protocol was developed: dialysis into .4 M NaCl with 25 kD MWCO tubing was followed by dialysis in 12 kD MWCO tubing into distilled water. This material could then be lyophilized and frozen or further purified by chemical or chromatographic means.

S. epidermidis UW-3 activity is extractable into phenol

Hot phenol extraction has traditionally been used to remove protein contaminants from preparations of LPS and lipoteichoic acid.(115, 116) It was found, however, that *S. epidermidis* activity completely partitioned into the phenol layer; this activity could be measured after dialyzing away the phenol. This observation led to calling the material Phenol Soluble Modulin, or PSM. In five separate, small scale experiments, the recovery of activity following phenol extraction was 71.1 ± 29.4% compared to unextracted control samples. A fair amount of precipitate was observed when dialyzing

away the phenol, and it is likely that the variability in recovery is due to insoluble material becoming adsorbed onto the dialysis bags.

The results of two large scale (10 liter) preparations are shown in Table III. The bacterial supernatant was concentrated by ultrafiltration, dialyzed with the two step process discussed above, extracted into hot phenol, dialyzed again, and stored lyophilized and frozen. A large amount of precipitate formed as the phenol was being dialyzed away, and this material was driven into aqueous solution by vigorous stirring prior to lyophilization.

Following phenol extraction, the PSM was subjected to MALDI-TOF mass spectrometry. This revealed only low molecular weight components, as shown in Figure 4.

S. epidermidis activity is sensitive to proteolysis

Enzymatic methods were used to determine whether the activating factor from *S. epidermidis* is a protein. Active material was incubated with proteases immobilized on agarose beads, allowing removal of the proteases by low-speed centrifugation and the supernatant assessed for activity. Several proteases were found to disrupt the activity of the material under these conditions (Figure 5). In contrast, mannosidase, lipoprotein lipase, lysozyme, lysostaphin, and N-glycanase did not affect activity (data not shown).

Crude, dialyzed S. epidermidis supernatant contains the V-8 protease of S. aureus

Dialyzed *S. epidermidis* supernatant was filtered through a 100kDa MWCO membrane and run on an SDS-PAGE gel (Figure 6 -- 10-10-94 gel). The profile of the material was remarkably simple -- only one band was seen, migrating with an apparent molecular weight of about 24kDa, and this band was only seen when the gel was

overloaded. This band was electroeluted and blotted onto PVDF membrane and submitted for Edman degradation. The results are shown in Table IV. Using an indicator substrate specific for this enzyme, it was found that approximately .075 U/mg of V-8 protease activity was retained in dialyzed, lyophilized UW-3 supernatant. V-8 protease is highly specific, cleaving on the carboxy-terminal side of aspartatic and glutamic acid, and purified preparations of this enzyme are routinely employed for use in protein sequencing. Commercially available V-8 protease had no effect in the THP-1 LTR_{LUC} assay, however (data not shown).

Cibacron Blue Chromatography

It was noted that the active material stuck to reactive blue dye resin, and the possibility of using this as a preparative chromatographic procedure was explored. Cibacron Blue is a broadly specific dye resin which has been used to purify molecules as diverse as adenylate cyclase, glutathione reductase, and albumin.(117) The material was applied in distilled water, the column was rinsed with distilled water, and the active material was eluted with 6M urea, which was subsequently dialyzed away. The average activity recovered, compared to material similarly incubated and dialyzed but not applied to the column, was 97.1 +/- 2.1 % for three experiments. The rinses contained activity equal to 26.1 +/- 7.4 % for these same experiments. The incubation and dialysis steps alone resulted in a loss of about a third of the activity compared to material kept lyophilized and frozen (data not shown). Following dialysis, the eluate was found by tricine-PAGE to contain only proteins of a low molecular weight, as shown in Figure 7. As these masses are below the resolving power of even this high-percentage gel, the current had to be stopped before the dye front reached the bottom of the gel in order to see anything at all.

Discussion

The dialysis and gel chromatography experiments were consistent with a mass of about 35kDa. As the material was sensitive to proteolysis and soluble in phenol, it was expected that it was a protein of about 35kDa. Following centrifugation and dialysis of *S. epidermidis* UW-3 extracts, there was only one protein near this size range by SDS-PAGE, and nothing of this size was seen by mass spectrometry. Electroblotting and microsequencing revealed that this protein was homologous to the V8 protease of *S. aureus*, and its presence was confirmed with the use of an assay specific for this protease. Commercially available preparations of this enzyme, however, failed to induce luciferase expression in the HIV LTR_{LUC} model system. Although it was surprising that this protease did not show up in mass spectrometry, this may be due to the fact that it was present in minute amounts. This hypothesis is supported by the low level of V8 proteolytic activity observed and the need to overload SDS PAGE gels for electrophoretic visualization.

The active material was found to bind Cibacron Blue resin, and this characteristic was used as a rough purification step. Harsh conditions (6M urea) were needed to elute the bound activity, and it was found that slow gradients produced a broad band of activity (data not shown). Batch preparation provided material with excellent retention of activity, and visualization of this material on a variety of high-percentage PAGE gels revealed that the active material consisted of components with a molecular weight under 6 kDa. It was of some concern that the combined activity of the flow through and the eluate had more total activity than an unseparated control, but given that the flow through contains at least one protease, it may well be that material bound to the column is protected from the action of this protease. This is consistent with the observation that

the incubated positive control had activity which dropped about a third compared to material stored lyophilized and frozen.

The conditions used for phenol extraction mirror those normally used to remove protein contaminants from preparations of lipoteichoic acid. The observation that the active portion partitions into the phenol layer lends further weight to the hypothesis that the active material is not LTA. Although the protease sensitivity, solubility in phenol, and staining with Coomassie Blue demonstrate the presence of protein, it may well be that other elements, such as lipids or carbohydrates, are also present as parts of this complex. Following phenol extraction, mass spectrometry showed the presence only of low molecular weight components. The most likely explanation for the lack of a V-8 protease signal is that there is a very low amount of this protease in PSM, an explanation which is bolstered by the low level of protease activity in *S. epidermidis* supernatants. Given the sensitivity of PSM to proteolysis, it is probably fortunate that only a small amount of this protease is present in extracted material.

Table II. Dialysis Under Several Conditions

Dialyzed Into	% Recovery, MWCO 12kD	25kD
Water	79	40
Media	76	71
PBS	79	53
.4M NaCl	99	81

S. epidermidis UW-3 supernatant was dialyzed overnight at 4° C and the retentate was assessed for the ability to stimulate luciferase production in the THP-1 LTR_{LUC} assay. Activity recovered was in relation to a standard curve made from dilutions of undialyzed supernatant. Data shown is the mean of two experiments and is representative of observations from several other experiments.

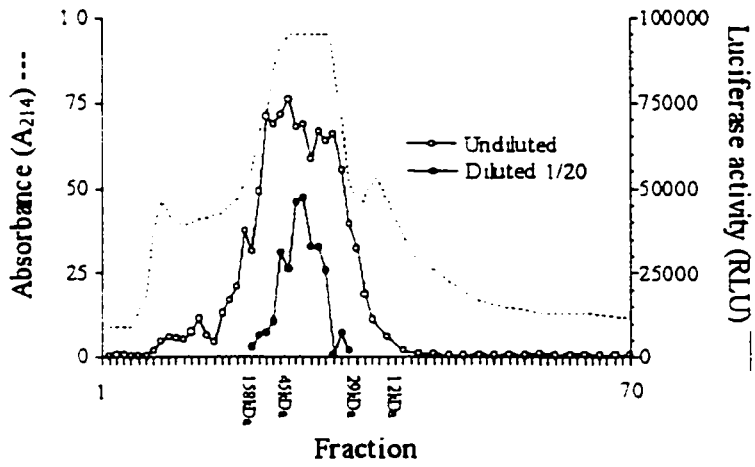


Figure 3. Superose gel chromatography of concentrated supernatant from *S. epidermidis* UW-3 culture. The dotted line shows the absorbance at 280nm, and the solid lines show the activity of the material (neat and at a 1/20 dilution) in the THP-1 LTR_{LUC} assay system. The elution points of size standards are indicated along the X axis.

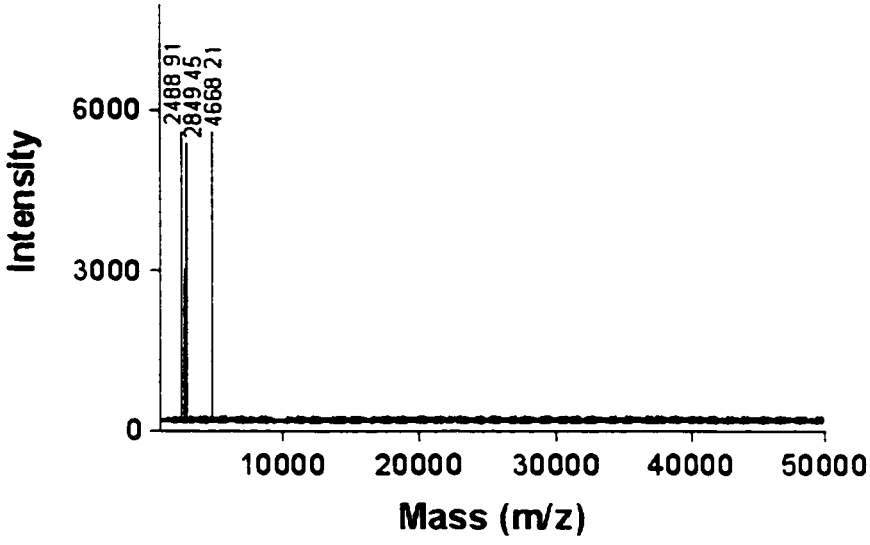


Figure 4. MALDI-TOF mass spectrum of PSM. The three largest peaks are labeled with their corresponding masses.

Table III. 10 Liter Preparations of Phenol Soluble Material

Preparation Stage	Percentage Recovery (Weight) Prep A	Prep B
Initial	100 (236.2g)	100 (213.7g)
After Concentration and Dialysis	124 +/- 30	234 +/- 42
After Phenol, Dialysis	190 +/- 64 (74.8 mg)	87 +/- 60 (230 mg)

S epidermidis strain UW-3 was grown and isolated as described in Materials and Methods. One milliliter of the initial supernatant was lyophilized and frozen and a standard curve was generated based upon serial dilutions of this material. 1/10,000 of the volume at each step was lyophilized and frozen for comparison to this standard in the THP-1 LTR_{LUC} assay system, and the results shown above are three such assays +/- S.D.

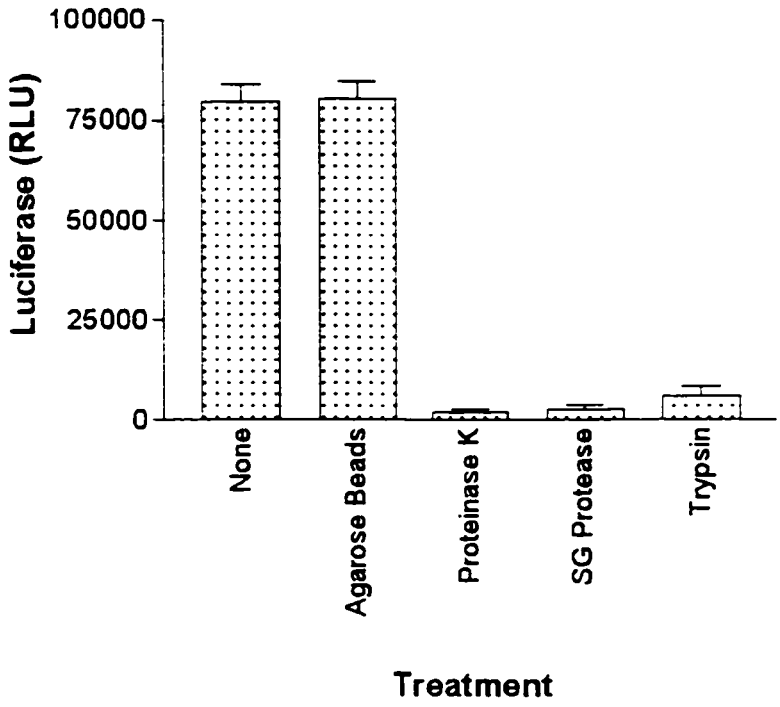


Figure 5. *S. epidermidis* UW-3 phenol extract is sensitive to proteolysis. PSM was incubated with the proteases shown, immobilized on agarose beads. SG refers to *Streptomyces* griseus. Shown is the mean (± S.D.) of three experiments.

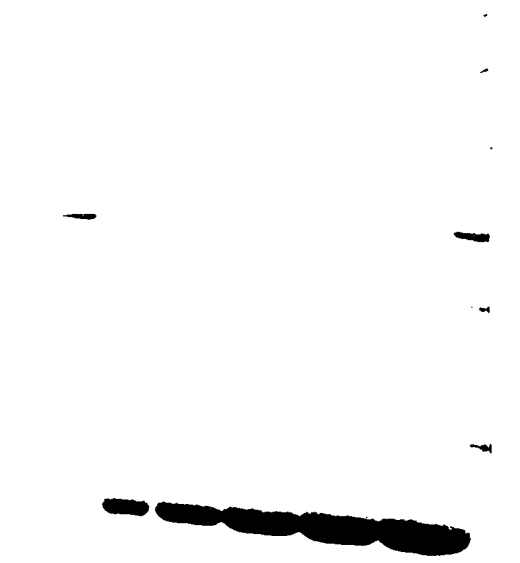


Figure 6. SDS-PAGE chromatography of dialyzed, concentrated *S. epidermidis* UW-3 supernatant in increasing concentrations from left to right. Also shown are 10 kDa markers. This 12.5% gel was run to completion and stained with Coomassie Blue.

Table IV. Comparison of UW-3 SDS-PAGE Band to the V-8 Protease

Our Sequence:	V I L P N N N R H Q I F N T T Q
V-8 Protease:	- - - - - - - D - - - - - T D - - N

Comparison of elecroblotted *S. epidermidis* UW-3 SDS-PAGE band with that previously found for the V-8 protease of *S. aureus*.(118) Dashes represent identical amino acids, and overall homology is at least 81%.

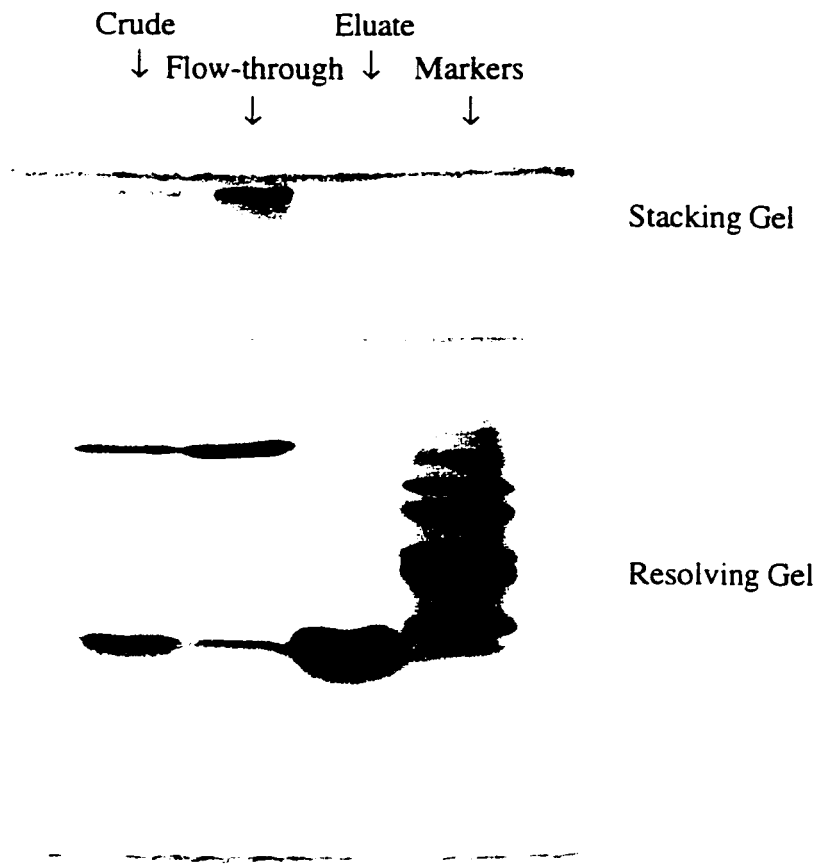


Figure 7. High-percentage tricine gel of *S. epidermidis* UW-3 dialyzed, concentrated supernatant following Cibacron Blue chromatography. Shown are crude material, flow-through (rinsing), and eluate (removed in 6M urea). Samples were concentrated before application. The stacking gel is 12% and the resolving gel is 16% acrylamide, and the dye front was not allowed to run off the bottom prior to staining with Coomassie Blue.

Chapter Three: Chemical Analysis

Introduction

It had been observed that the activating factor from *S. epidermidis* supernatants was likely to be an aggregate of smaller peptides, and a chromatographic approach was desired which would separate them. Although the activating complex bound quite strongly to Cibacron Blue resin, this matrix required harsh conditions to elute, necessitated extensive dialysis, and did not separate the low molecular weight components. Although proteins of high molecular weight were removed, these appeared to be only minor constituents of the bacterial supernatant. This matrix was therefore abandoned in favor of ion-exchange and reverse-phase (hydrophobic) chromatography.

Any chromatographic system which is based upon adsorption and elution of sample components is essentially a competition between a solid-phase matrix and a liquid eluent.(119) Typically the eluent gradually approaches the conditions of the solid matrix (e.g. it contains more salt in ion-exchange or more organic solvent in reverse phase columns) in a gradient-wise fashion, and the individual components elute as the liquid hits a threshold level of competition. The hope is that each component will have a different threshold and therefore elute at different points on the gradient. Unfortunately, the threshold level at which a given component elutes is not precisely defined, resulting in peak broadening and tailing as the component diffuses through the column. One remedy for this is to run the columns at higher pressures, allowing for a greater exposure of the sample components to the liquid phase while limiting the amount of time for diffusion. This approach is very effective and is limited only by technical

constraints: as the pressures get higher, it becomes more complex to apply large amounts of sample and more difficult to design solid matrices which will not compress. The end result is that adsorption chromatography is almost always a trade-off between resolution and sample size.(119)

Reverse-phase High Pressure Liquid Chromatography (RP-HPLC) has become a mainstay of the chromatographic field for the separation of peptides as the resolution attainable by this method is higher than any other standard approach.(120, 121) This resolution comes at a cost, however: sample sizes are small and the harsh conditions typically destroy protein secondary structure. HPLC is run at pressures around 1000 p.s.i., and modern column packings maintain a surface area roughly the size of a football field within a stainless steel column smaller than a pencil. Although a variety of eluents and matrices are used, by far the most common combination is an eluent of aqueous acetonitrile acidified with trifluoroacetic acid (TFA) on a column packed with derivatized silica.(122) This derivatization is generally in the form of carbon chains of four (C4) or eighteen (C18) atoms in length. Although industrial use of massive HPLC columns is not uncommon, practical constraints of a typical benchtop machine limit one to a sample size of about 100ug. The disadvantage of this small sample size has been compensated for by the development of reliable microscale Edman degradation which can provide useful protein sequence information from picomoles of sample.(123)

Ion exchange chromatography is a technique which can separate proteins which differ in only one or two residues. (120) In addition, the columns can be run under aqueous conditions, thereby preventing the misfolding and inactivation of proteins which can occur in organic solvents. Modifiers such as detergents can be included to inhibit aggregation. Using medium-pressure equipment, such as the Pharmacia Fast Protein Liquid Chromatography (FPLC) device employed here, one can typically run

about 100mg of material. While the resolution of such a system is not as good as that of a high-pressure setup, the ability to separate comparatively large amounts of material while retaining biological activity is a decided advantage.

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) is a gentle mass spectrometry technique which has recently become the method of choice for determining the molecular weight of proteins.(124-127) Older ionization techniques tended to have a destructive effect on the sample, so the resulting data would represent fragments of the protein rather than the full molecule. Electrospray mass spectrometry is also a "gentle" ionization technique, but the resultant spectrum can be difficult to interpret as it reflects an array of mass-to-charge (m/z) ratios. MALDI-MS, on the other hand, generally gives a good parent ion mass peak (i.e. where there is only one charge on the molecule) in the range of 500-200,000 daltons.

Materials and Methods

High Pressure Liquid Chromatography

HPLC was performed on an LKB (now Pharmacia, Piscataway, NJ) 2150 machine equipped with a Vydak .46 x 15 cm C4 column and a flow cell detector measuring absorbance at 214nm. All solvents were HPLC grade from Aldrich and were degassed by sparging with helium prior to use. For the rapid HPLC procedure, a ten minute gradient was run from 36-100% 1-propanol, and the aqueous portion was buffered with 2.5mM ammonium acetate, pH6.7. 100ug of dialyzed, lyophilized PSM was injected in 20 ul 20% 1-propanol, and fractions were collected, lyophilized, and frozen. Slow HPLC was done with a gradient from 28-48% 1-propanol with the same buffer over one hour; material applied was either crude PSM or pooled, peak1 material

from several rapid HPLCs. Some HPLCs were also done using a gradient in acetonitrile containing .05% TFA, as mentioned in the text.

For assay, the lyophilized material was dissolved in PBS with vigorous vortexing, and dilutions of these fractions were compared to a standard curve made from material which was similarly processed (in terms of lyophilization and solvent exposure) but not subjected to the HPLC column. Phosphorous assay was done by a modified Fiske-Subborrow procedure (Chen et al, 1956). SDS-PAGE, silver and Coomassie staining were done with standard techniques(128), and Alcian Blue pretreatment (for carbohydrates) was done as previously described.(129)

Anion Exchange Chromatography

Anion exchange Chromatography was performed on a Fast Protein Liquid Chromatography apparatus (Pharmacia) with a Mono-Q (quaternary amine) column and a buffer of 20mM Tris (pH7.9) with .1% octyl-b-D-thioglucopyranoside (a dialyzable detergent). Following a gradient to 2M NaCl and a flow rate of 1ml/min., one milliliter fractions were collected and dialyzed in 12 kD MWCO tubing against distilled water; dilutions of these fractions were then compared to a standard curve made from a sample spiked with crude, phenol-extracted PSM in the THP-1 LTR_{LUC} assay (as detailed in Chapter One). Areas under FPLC curves were determined by photocopying the output and cutting out and weighing the areas of interest.

Protein Sequencing, Mass Spectrometry, and Amino Acid Analysis

Edman degradation and mass spectrometry were performed by the University of Washington Biochemistry Core Facility. Edman degradation was done on an Applied Biosystems 470A sequencer with an on-line 120A PTH analyzer, and Matrix Assisted

Laser Desorption Ionization , Time-of-Flight (MALDI-TOF) mass spectrometry was done on a Voyager Elite (PerSeptive Biosystems, TX) apparatus. Amino acid analysis was performed by AAA Analysis (Mercer Island, WA) on a Beckman 7300 Analyzer following twenty hours of digestion at 115° C in 6N HCl, 0.05% mercaptoethanol, and 0.02% phenol.

Carboxy-terminal sequencing was done as previously described(130, 131) using Carboxypeptidase Y and reagents contained in the Sequazyme kit (PerSeptive Biosystems, Framingham, MA) and mass spectrometry. Briefly, dilutions of the enzyme were incubated with the target protein, and after a short period of time the reaction was quenched and analyzed by mass spectrometry. The amino acids lost from the C-terminus were then determined by the mass lost from the target protein.

Tryptic fragmentation and analysis were done with material which had been purified by rapid HPLC in 1-propanol (as above) followed by a second rapid HPLC in .05% TFA/acetonitrile. The single peak obtained from multiple runs of this second HPLC was submitted to the Harvard Microchemistry Facility for analysis. Tryptic digests were separated by HPLC on a Zorbax C18 column. Edman degradation at Harvard was on an Applied Biosystems 477A Protein Sequencer with a 120A PTH analyzer, and fragment sequences were verified by MALDI-TOF mass spectrometry on a Finnegan TSQ Triple Quadrupole Mass Spectrometer with automatic calibration.

Results

Rapid HPLC Separates S. epidermidis PSM into Two Active Peaks

RP-HPLC in acetonitrile with .05% TFA was observed to have a strongly detrimental effect on the activity of PSM, so a variety of other solvent systems were

explored. Well over a hundred different combinations of solvent, buffer, elution time, and flow rates were tested, and almost every combination provided poor resolution of the active peptides. A system utilizing a C4 column and 1-propanol buffered with ammonium acetate was found to give two peaks of protein, and activity recovery was typically about 25% (Figure 8A) as long as the run was carried out quickly. Neither peak was found to contain phosphorous. SDS-PAGE of active peaks revealed only material which ran, unresolved, at the dye front (<6 kDa) and stained positively with silver stain and Coomassie Blue. Silver staining was not intensified with Alcian Blue, suggesting an absence of carbohydrate (Figure 9). Mass spectrometry revealed the presence of one major peptide in each peak and a smaller secondary peptide in the first peak (Figure8B). The small component of the first peak was named Phenol Soluble Modulin Alpha (PSMα), the larger component of that peak PSMβ, and the protein in the second peak was termed PSMγ. Amino acid analysis, shown in Table V, revealed a complete lack of proline, histidine, arginine, and tyrosine in either of the two peaks.

Anion Exchange Chromatography

A series of ion-exchange separations were performed using a variety of buffers and additives. Tris and piperazine buffers were employed at several pHs, and ethylene glycol, 1-butanol, and detergent were used in an effort to prevent aggregation. It was observed that tris buffer at with 0.1% detergent separated the peak of activity from the peak of protein (as measured by absorbance at 280nm) (Figure 10A). The recovery of activity was fairly high, ranging from 50-100% of a sample spiked with crude material, and mass spectrometry of the active fractions revealed the presence of both PSMα and β (Figure 10B).

HPLC was performed on the active fractions from anion exchange chromatography (Figure 11A). Although the active material was predominantly one peak by HPLC, mass spectrometry revealed that this single peak contained both PSMα and PSMβ (Figure 11B) (compare to HPLC alone, Figure 8B). Although some activity was still recovered, this series of steps was severely detrimental to the overall quantitative recovery. As the anion exchange chromatography step did not appear to contribute to purity, subsequent analyses were done with material purified by HPLC alone.

Initial Analysis of HPLC Purified Material

The two peaks from HPLC were submitted for Edman degradation (Figure 12). This analysis revealed that PSMβ had a high degree of homology to the *S.hemolyticus* antigonococcal proteins (addressed in Chapter 4) and the PSMγ was likely to be *S. epidermidis* delta toxin (Table VI). The structure of PSMα was not clarified, as it was in a lower concentration relative to PSMβ. Amino acid analysis of these two peaks was also consistent with these identified homologies, although the composition of the first peak was somewhat obfuscated by the presence of PSMα; most noteworthy in this analysis is the high degree of correlation with Peak 2 and *S. epidermidis* delta toxin.

Carboxy-terminal sequencing was performed on the first peak in order to elucidate more sequence information. HPLC-isolated material was subjected to incomplete carboxypeptidase digestion and analyzed by mass spectrometry (Figure 13). This analysis revealed that the last three amino acids were F G F; from this point the carboxypeptidase chewed back several amino acids without any observable intermediates. The structure of PSMα was obscured by PSMβ.

Tryptic Digestion and Sequencing of Peak 1

Material purified in 1-propanol by HPLC was re-chromatographed in acetonitrile/.05% TFA in an effort to separate the two components, and when this step failed to resolve them the combination was subjected to tryptic digestion, RP-HPLC, and mass spectrometry and sequencing of the fragments (Figure 14). Edman degradation of material which had been subjected to this second HPLC step resulted in heterogenous data, with two different amino acids giving strong signals in each cycle (data shown in Chapter four). As this work progressed, it was found that the two components of peak 1 could be separated by slow HPLC in 1-propanol (Figure 15), allowing for Edman degradation of the minor component in peak 1 (PSMα) and less ambiguous assembly of the tryptic fragments from this peak, which reflected sequence from both PSMα and β (Figure 16). Such lengthy HPLC severely compromised the activity of the material; overall recovery of activity was typically under one percent.

Discussion

The three proteins isolated here are remarkable for the amount they have in common despite possessing almost no sequence similarity. All lack histidine, proline, tyrosine, and arginine, and Peak 2 also lacked glutamine and glutamic acid. Compared to general amino acid usage in staphylococci, all the hydrophobic amino acids were found in a higher concentration in these proteins. PSMα and γ and the N-terminal half of PSMβ would be predicted to adopt a helical conformation according to the method of Burkhard Rost.(132-134) Perhaps the most compelling aspect of their similarity is their chromatographic behavior, which in all but the most extreme cases was nearly identical.

The tenacity with which these proteins aggregate is impressive. Anion exchange chromatography was the only method which appeared to pull the peak of activity away from the bulk of the protein. It is worth noting, however, that although the peaks of protein and activity are shifted relative to each other, 71% of the protein (as measured by integration of the absorbance at 280nm) is still under the peak of activity. Additionally, it was observed that the PSM proteins were all intensely hydrophobic, eluting very late by conventional RP-HPLC, and this may have caused post-detector retention in the plastic tubing of the FPLC. This would shift the activity from the measured protein peak.

The loss of biological activity which accompanies chromatographic separations can be particularly troublesome if the biological activity is a result of a cooperative interaction between two or more components in the crude mixture. It remains a matter of conjecture whether the association of these proteins is merely hydrophobic aggregation or the formation of a complex with biological function. The possibility of a covalent linkage has not been ruled out, although one would expect that the proteins would remain linked during mass spectrometry even with a linkage as labile as an ester.

The characterization of these proteins is a good study in the powers and limitations of the techniques available today. Perhaps the strongest asset available today is the access to computer-assisted database searching, but finding a match is a Pyrric victory: this method is most effective only when the unidentified protein has already been characterized. One can match an unknown protein to a database using properties as disparate as pI and MW, and with less than ten amino acids from Edman degradation an unambiguous identity can be established. It was precisely this approach which allowed for the identification of PSMγ as *S. epidermidis* delta toxin (Table VI).

PSMα and PSMβ are a bit more troublesome to characterize by chemical means. Part of this trouble stems from the difficulty in chromatographically separating them, as any of the chemical techniques suffer somewhat from impure samples. A notable exception to this is C-terminal sequencing of the largest protein in a mixture, as was the case for PSMβ: because the results are obtained via mass spectrometry it is possible to observe the degradation of the largest element as long as the fragments remain larger than the other components in the sample. In light of this advantage, it is unfortunate that this method has serious limitations in other respects: one typically retrieves only a small amount of sequence, and the mass of an amino acid doesn't distinguish isobaric residues (e.g. leucine and isoleucine). It is best employed as a method to verify what is already known. In the case of PSMβ, the last three residues could be unambiguously identified as F G F despite the fact that phenylalanine and oxidized methionine have the same mass; methionine was ruled out in these positions because amino acid analysis had indicated a low level of this residue and Edman degradation had placed what little there was at the beginning of the protein. The prior four amino acids, L G K I, were cleaved off without a measurable intermediate, but their theoretical mass of 397.5 corresponds with the observed mass loss of 397.7, serving to verify what was already known.

From the A_{214} peak areas of these HPLCs and the molecular weights of the PSMs the molar ratio of the components can be determined. The ratio is about 1α : 1.8β : 4.8γ, or 1 : 2 : 5. In light of this, the activity of PSMα is even more compelling, as it only composes about 13% of the total.

Mass spectrometry was used extensively in this study to verify purity and identity of proteins and peptides and to aid in their characterization. It is such a powerful technique that one can easily forget its limitations, probably the most important of which is its tendency to give artifactual, or non-representative results.

Although we observed reproducibility of masses to under 2% or so, peaks often appeared complexed with sodium or water. The size or intensity of the peak has more to do with the particular "ionizability" of a compound than the amount of it which is truly present, making this a somewhat dicey technique for judging purity. In the case of PSMα and PSMβ, it was known that both of these proteins were visible by MALDI-TOF mass spectrometry, but the relative amounts of these two could not be accurately assessed until they were separated, as in Figure 15. In this case, the peak intensity of the mass spectrum did accurately reflect the relative concentrations of the two proteins, although the two peaks did vary somewhat in their relative height depending upon what particular spot the ionizing laser was aimed at and how the machine was tuned.

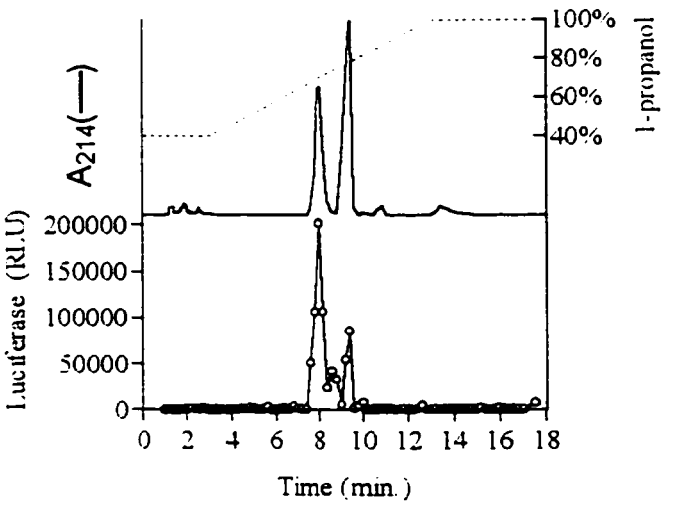
Edman degradation has evolved into probably the most effective method for obtaining sequence information from an unknown protein, and this technique was central to the analysis of these PSMs. In combination with enzymatic digestion and HPLC, it is possible to get complete sequences of even very large proteins. The data suffers from a certain amount of ambiguity, especially if the sample is not rigorously pure, and doublets or missing residues are fairly common. N-terminal blockage is also fairly common, (approximately 60% of prokaryotic proteins are blocked) leading to the possibility that no sequence at all will be obtained. Some blockage, such as formylation, is easily removed by acid treatment (often the acidification employed in Edman degradation is enough), while others require more radical treatments: tryptic digestion, enzyme-mediated cleavage of pyroglutamate or N-acetyl methionine. PSMα proved to be fairly difficult to sequence, and either no sequence would be obtained or the first three amino acids appeared at a very low level. This was probably fortuitous, as it allowed for the sequencing of PSMβ in the presence of PSMα. Treatment with enzymes to remove N-actyl methionine and pyroglutamic acid from highly purified

PSMα did not clarify the Edman degradation, so the chemical methods employed here left the first few amino acids of PSMα open to some question. It is likely that PSMα is formylated, as re-chromatographing Peak 1 material in acetonitrile with .05% TFA did not separate PSMα and PSMβ but did unblock PSMα: the N-terminal sequence obtained from this material was a mixture of the two sequences (addressed in chapter Four). The first few amino acids are typically somewhat troublesome for Edman degradation, as these cycles are often plagued by minor contaminants in the sample, and the sequence generally gets less ambiguous after the first three or four cycles.

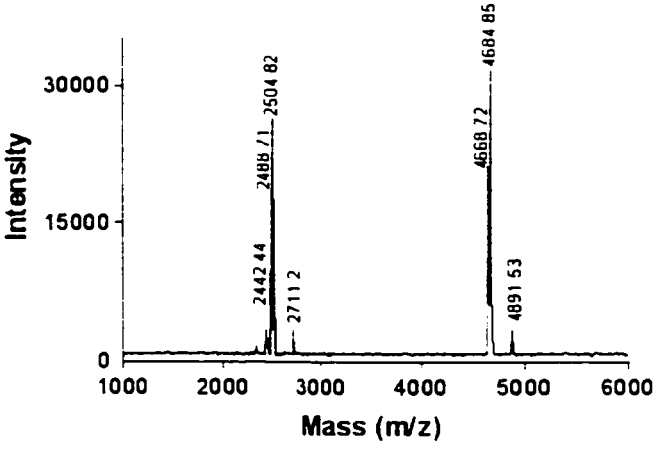
The assignment of the tryptic fragments as shown in Figure 16 followed fairly naturally from the initial N-terminal sequencing of the proteins, as enough sequence was obtained from the N terminus to provide overlap for almost all the fragments. The sequence of the fragments was more certain than the original sequencing because they were a good deal more pure and were verified by mass spectrometry. In positions where the data conflict between fragment sequence and initial N-terminal sequence, therefore, the fragment assignment was used.

In summary, we found that the active material from *S. epidermidis* UW-3 supernatants contains an aggregate of three polypeptides, PSMα, β, and γ. The polypeptides are all intensely hydrophobic and lack arginine, histidine, proline and tyrosine. Enough sequence was obtained by chemical means to provide the primary amino acid sequence of PSMα and β with about 90% certainty, and PSMγ could at this point be predicted to be at least 90% identical to *S. epidermidis* delta toxin. At this point, though, we had pretty much reached the limit of what direct chemical analysis could tell us, and to completely and unambiguously define the PSMs had to resort to isolating their genes.

A.



B.



C.

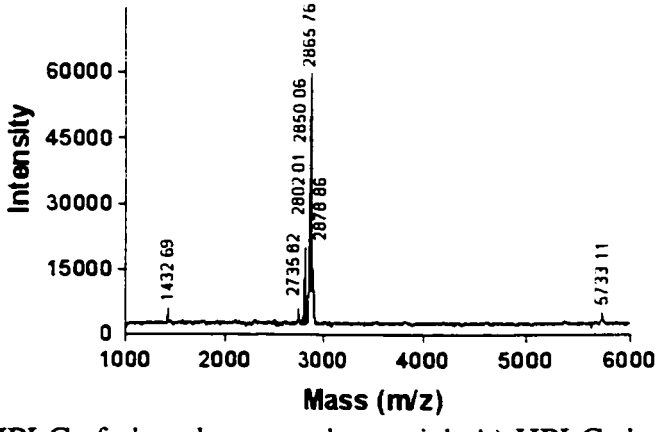


Figure 8. Rapid HPLC of phenol extracted material. **A**) HPLC absorbance at 214nm and activity in RLU. **B**) and **C**), mass spectra of Peak 1 and Peak 2, respectively. Peak 2 was calibrated with insulin, and these peaks were removed for clarity.

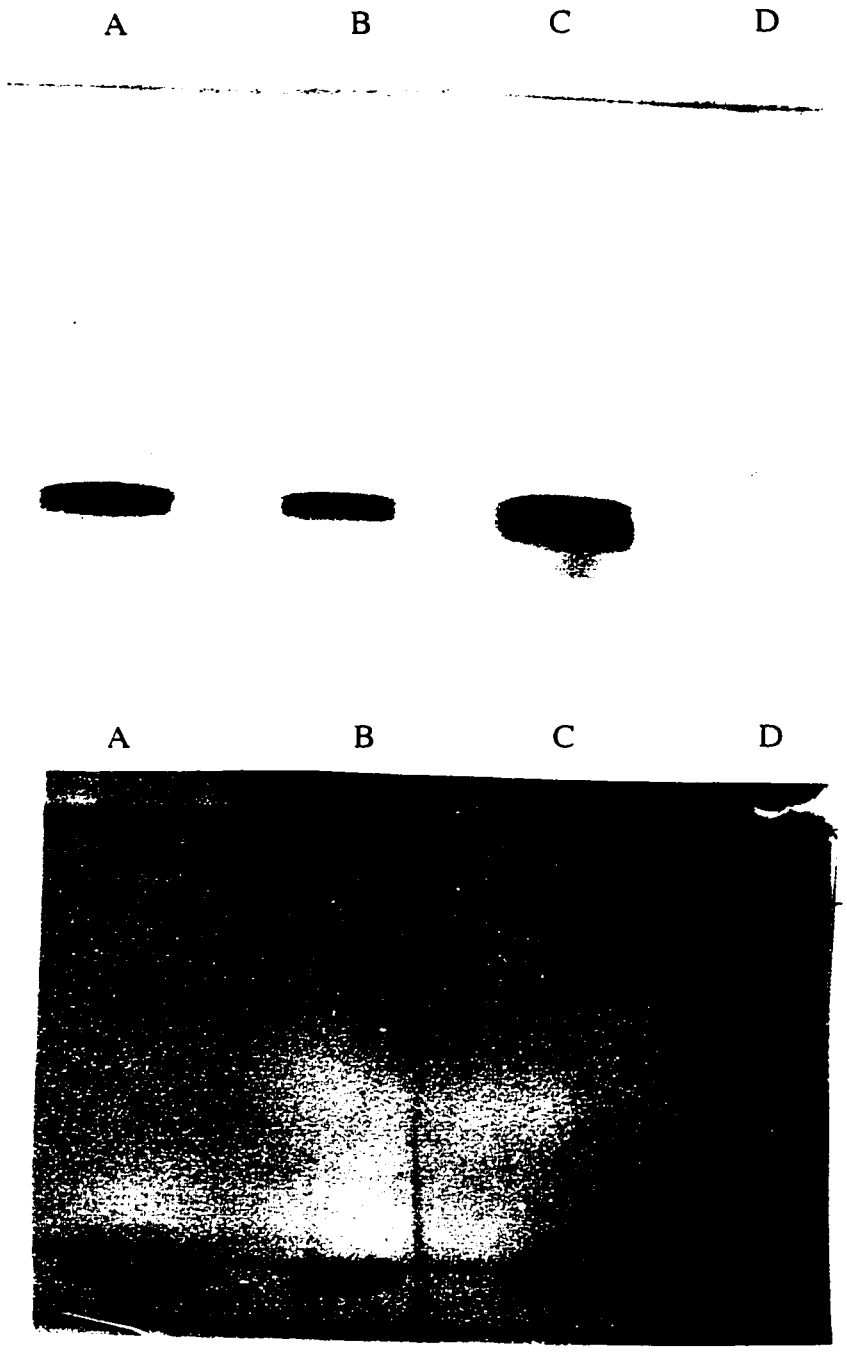


Figure 9. SDS-PAGE of PSM following rapid HPLC. Gels were 12.5% acrylamide and contained identical samples. Lane A is Peak 1, lane B is from between Peak 1 and Two (containing some of each), lane C is Peak 2, and lane D is 1ug of lipoteichoic acid. Molecular weight markers (not shown) did not run down to the sample bands. Gels were stained with either Coomassie Blue and Silver Stains (top) or Alcian Blue and silver stains (below). Note the intense staining of lipoteichoic acid with Alcian Blue/silver and the lack of such staining with PSM.

Table V. Amino Acid Composition of Peaks One and Two

Amino Acid	Total in *S. Aureus*	Peak 1	Peak 2
Alanine	9.54%	7.56%	5.63%
Arginine	4.99	0	0
Asp/Asn	12.2	10.2	18.3
Glu/Gln	15.9	13.8	.543
Glycine	10.3	4.88	2.40
Histidine	1.52	0	0
Isoleucine	6.22	11.2	17.4
Leucine	5.90	7.16	4.54
Lysine	11.0	13.2	21.0
Methionine	2.68	3.71	5.14
Phenylalanine	2.76	6.52	6.14
Proline	2.93	0	0
Serine	3.81	6.15	3.43
Threonine	3.73	5.92	7.94
Tyrosine	1.41	0	0
Valine	5.06	9.83	7.63

Amino acid analysis of Peak 1 and Peak 2 material after rapid HPLC of crude PSM. *S. aureus* amino acid composition is shown for comparison adapted from D. L. Watson.(135)

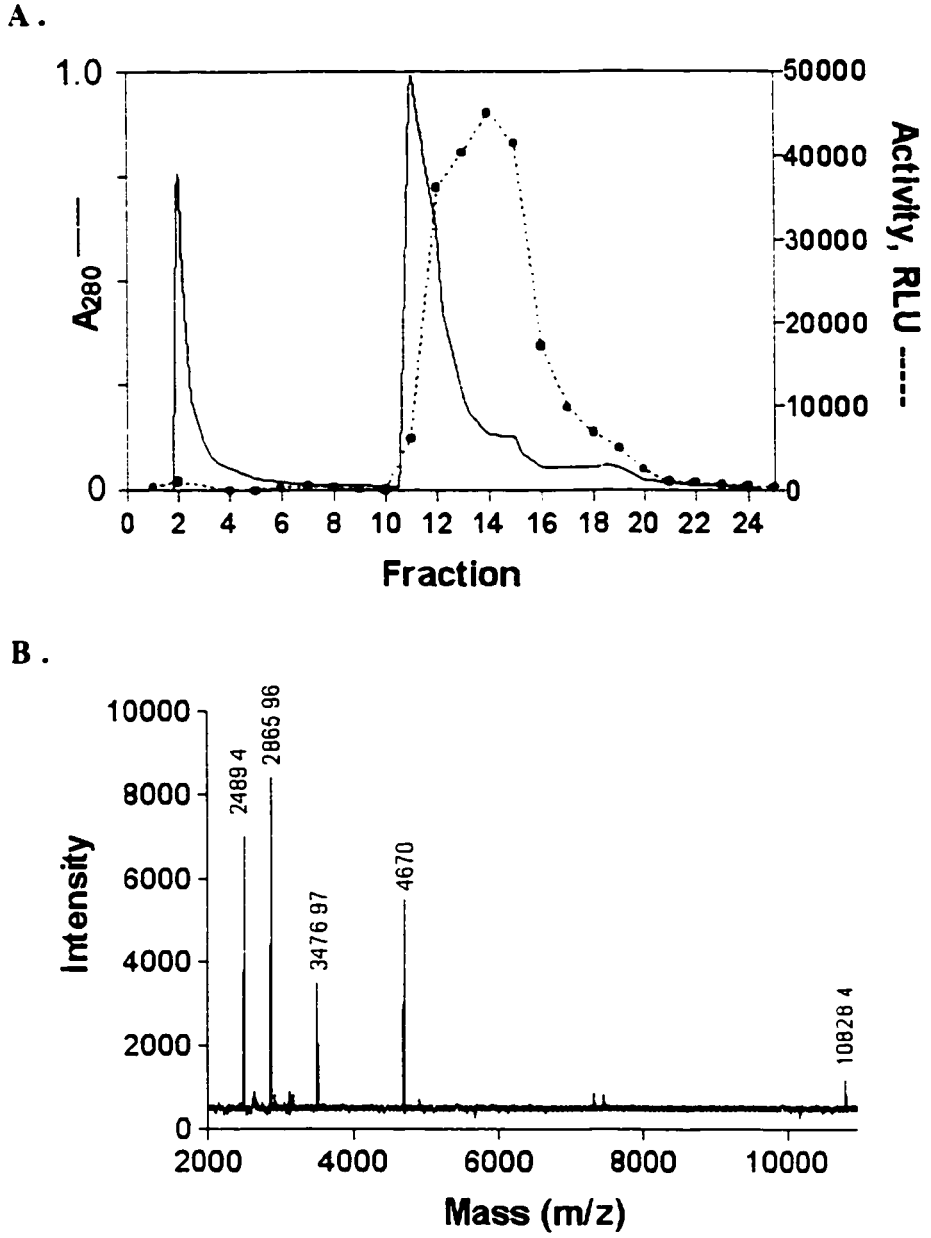


Figure 10. **A**) Anion exchange (Mono Q) chromatography of phenol-extracted material. **B**) Mass spectrum of active fractions (13, 14, and 15) from column in **A**.

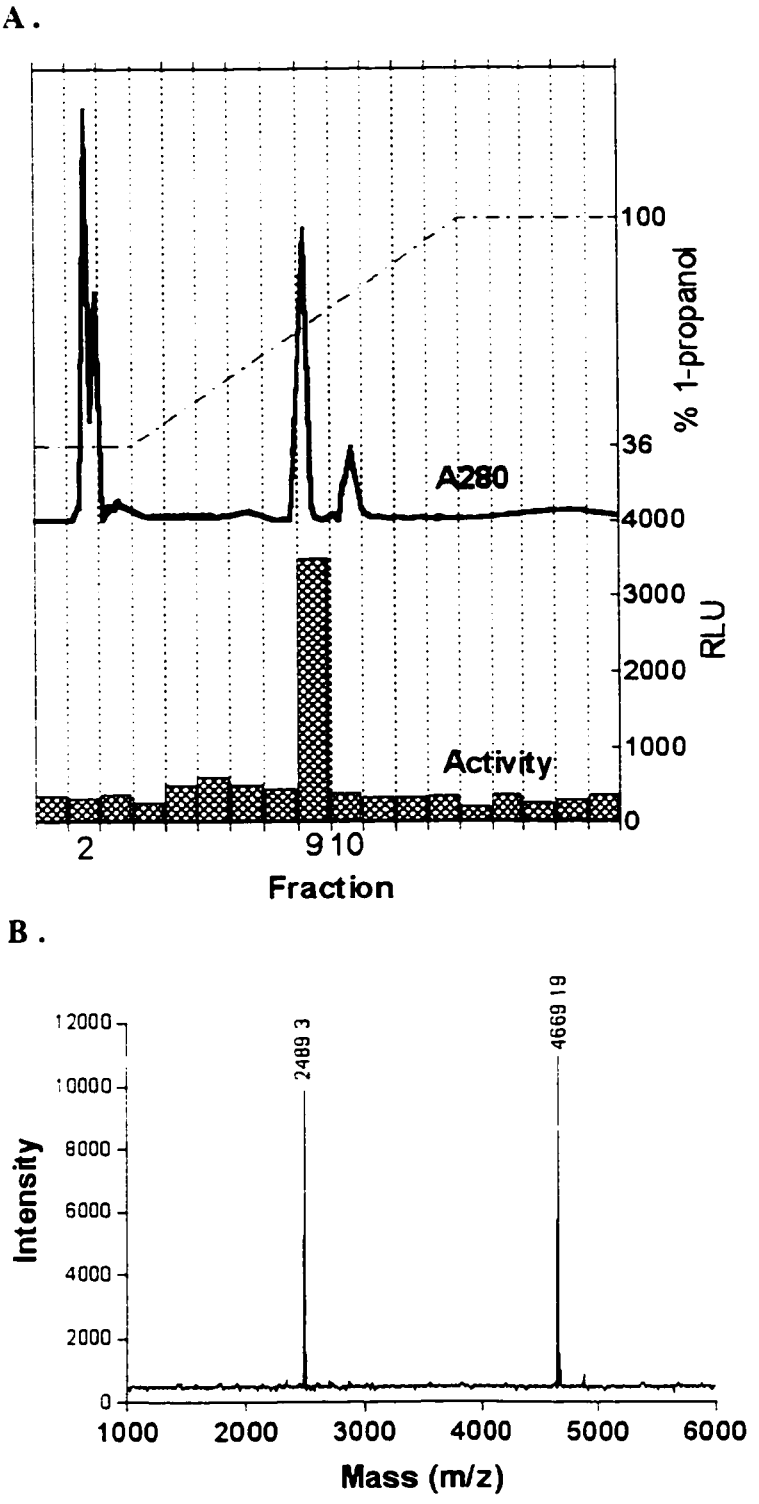


Figure 11. **A**) HPLC of active fractions from anion exchange chromatography (Figure 10). Activity of HPLC fractions is displayed below the absorbance at 214nm. **B**) Mass spectrum of fraction nine from HPLC; compare to Figure 8B.

Peak 1: M A/S K L I E [A] I V N/D T V K A A D Q W T K G

[T] X I

Peak 2: M A [A] D I F/I S/K T I G D [L] V K W I

Figure 12. Initial Edman Degradation of peaks one and two. Weak amino acids are shown in brackets, ambiguous ones are shown with a slash.

Table VI. Peak Two and Delta Toxin

Peak 2 Sequence: M A A D I[F]S T I G D L V K W I

Delta Toxin: M A A D I I S T I G D L V K W I

Amino Acid Analysis:

		Delta Toxin	
Amino Acid	**Peak 2**	**Actual**	**Found**
Alanine	2	2	2
Arginine	0	0	0
Asx	4	4	4
Glx	0 (slt.)	0	0
Glycine	1	2	1
Histidine	0	0	0
Isoleucine	4	4	5
Leucine	1	1	1
Lysine	4	4	4
Methionine	1	1	1
Phenylalanine	1	1	1
Proline	0	0	0
Serine	1	1	1
Threonine	2	2	2
Tyrosine	0	0	0
Valine	2	2	2

Comparison of chemical data on Peak 2 with that published for *S. epidermidis* delta toxin. Sequence data is at the top; a weak phenylalanine signal was seen in Edman degradation of Peak 2, as shown. Background signal at this point was ambiguous (I/L). The bottom table shows amino acid analysis, normalized as molar equivalents. The delta toxin columns have the molar equivalents based on amino acid analysis (found) and cloning (actual).

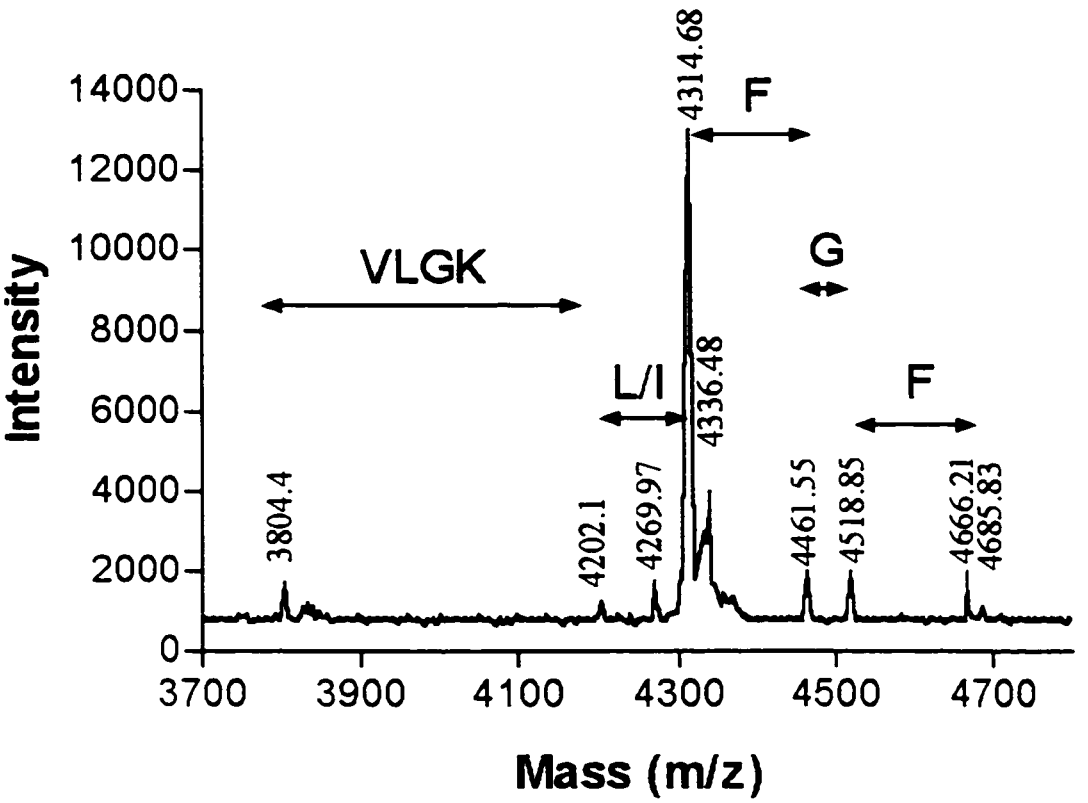
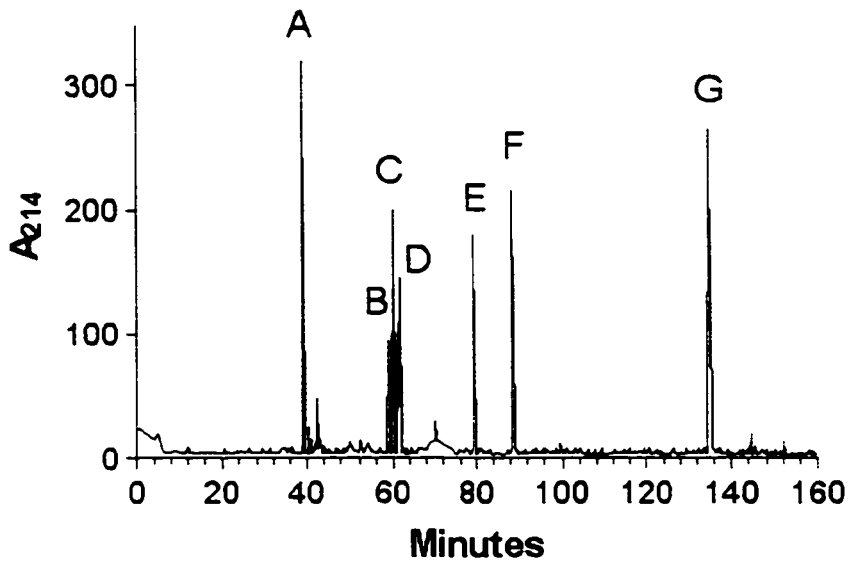


Figure 13. C-terminal sequencing of Peak 1 material. Shown is the MALDI-TOF mass spectrum of material partially digested with carboxypeptidase. Arrows and letters indicate mass losses which correspond to specific amino acids. Only the last three (F G F) were unambiguously determined from this experiment; the other amino acids were determined by other means, but the mass losses which correspond to these residues are consistent with the designations shown.



A: A A Q D Q D W T K

B: I V E I V K

C: L A E A I A N T V K

D: L A E A I A . . . (indeterminate)

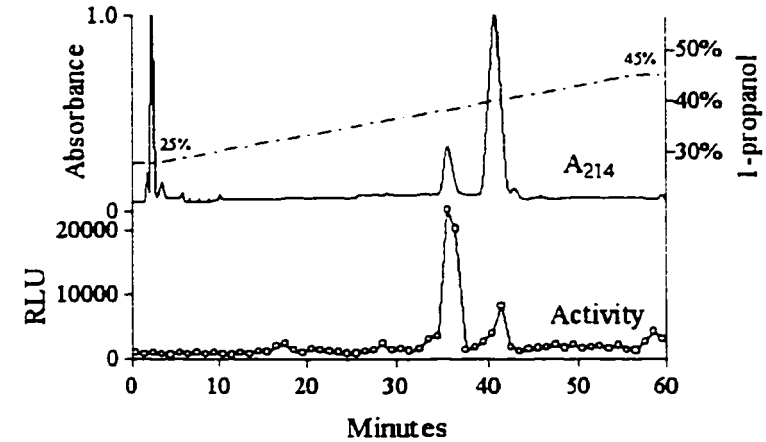
E: G L I D Q F T Q K

F: I F G

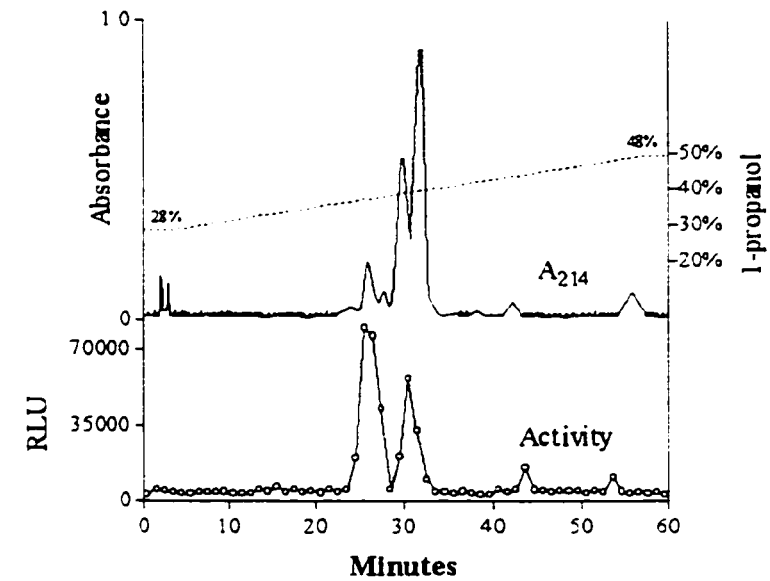
G: L G T S I V D I V E S G V S V L G K

Figure 14. HPLC of the tryptic fragments from Peak 1 material (above) and the sequence of these fragments (below) via Edman degradation. Fragment D was assumed to be a breakdown product of fragment C. All sequences were verified by mass spectrometry; mass spectrometry of fragment F indicated the presence of another phenylalanine.

A.



B.



C.

M [Q] [Q/K] V I A K I V X I V X

Figure 15. Isolation and Edman degradation of PSMα. **A**) Second HPLC of Peak 1 material. **B**) Slow HPLC of crude PSM. The absorbance is shown on the top and the activity in the THP-1 LTR_{LUC} assay is shown below for both Figures. **C**) Edman degradation of material from the first peak in **A**, above (PSMα).

PSMα

```
M Q Q/K V I A K I V X I V X [N][L]

                I V E I V K
                          G L I D Q F T Q K
-------------------------------------------
M Q  K  V I A K I V E I V K G L I D Q F T Q K
```

Theoretical Mass: 2530.11
Actual Mass: 2488-2504

PSMβ

```
M A/S K L I E A I V N/D T V K A A Q D Q[D]W T K L G[T]X I

        L A E A I A  N  T V K
                        A A Q D Q D W T K
                                        L G T S I V D I V E S G V S V L G K
                                                                          I F G
                                                                            F G F
-------------------------------------------------------------------------------
M  S  K L A E A I A  N  T V K A A Q D Q D W T K L G T S I V D I V E S G V S V L G K I F G F
```

Theoretical Mass: 4639.34
Actual Mass: 4668-4686

Figure 16. Assembly of tryptic fragments from Peak One.

Chapter Four

Characterization of the Genes of the Phenol Soluble Modulins

Introduction

Chemical methods provided a fairly good amino acid sequence of the proteins involved in PSM, but the data obtained in this manner left open the possibility that some of the amino acids were incorrect. In order to unambiguously determine the total sequences, an approach was desired which would define the genes which encode the PSMs. This would have the added benefit of determining whether these proteins are produced as larger precursors. The sequencing of nucleic acids has advantages over the sequencing of proteins, and the wide array of techniques available today for isolating genes greatly increases the possibility of finding a successful approach.

While Edman degradation of proteins typically returns less than twenty amino acids of sequence, sequencing DNA routinely gives over six hundred bases (corresponding to 200 amino acids). Much of the nucleic acid sequence is likely to be from non-coding regions, but even this information can be useful in providing clues to the regulation or initial transcription of the protein. Although DNA sequences typically degenerate about 600 bases from the primer, the error rate is well under 1%. The chief stumbling block in moving from known protein sequence to the gene sequence is the degeneracy of the amino acid coding -- that is, each amino acid typically has several possible coding triplets of DNA. The coding bias of an organism can aid in the creation of primers, but several mismatches are to be expected (see Table VII).

Many techniques for isolating genes rely on the creation of a genetic library and screening this library to pull out the clone of interest. This has the advantages of maintaining a high degree of sequence fidelity and providing a library which can be screened for many different genes. The chief disadvantage to this method is that toxic genes are often excluded from the library, as they tend to kill the *E. coli* host cells. Although much effort has been put into preventing the bacteria from expressing the products of the library elements they carry, these repressor systems are typically leaky and may not operate at all in cases where the library insert contains bacterial promoters.

In response to these concerns with library-based systems, other approaches based on the Polymerase Chain Reaction (PCR) have been developed. PCR has the advantages of being quick and not requiring host bacteria, but its chief disadvantages are that it can introduce errors into products and that it is highly susceptible to artifacts arising from trace contaminants. Also, cloning strategies relying on PCR provide, initially, sequence adjacent to the gene of interest, requiring additional steps to find the sequence of the gene. With TA-rich species (such as *Staphylococci*), one typically needs a large amount of amino acid sequence to design a primer with a reasonable melting point for PCR (about 15 amino acids, usually). Inverse PCR is a PCR cloning technique wherein the genetic DNA is cut into pieces and allowed to ligate into circles.(136) Primers directed out of known sequence can then be used to amplify adjacent regions. Of course, two primers are required for this approach. Anchor PCR(137, 138) only requires one gene-specific primer; the restricted genomic DNA is ligated to an adapter and adjacent sequence is amplified using one gene-specific and one adapter-specific primer.

In practice, these methods can often be used together. PCR can be used to verify that a library contains the desired insert and to provide for unambiguous

sequences with which to probe the library. Anchor PCR can give long stretches of adjacent sequence from which inverse PCR primers can be designed.

Materials and Methods

Reagents

All chemicals were obtained from Sigma and all restriction enzymes from New England Biolabs (Beverly, MA) unless otherwise indicated. Tryptic Soy Broth (T-Soy) was from BBL (Cockeysville, MD). TAQ was supplied by Boehringer-Mannheim (Mannheim, Germany), radiolabelled P32-ATP was from New England Nuclear (Boston, MA), desalting columns were manufactured by Princeton Separations (Centri-Sep, Aldelphia, NJ), spin columns for removing DNA from agarose gels were from Amicon (Micropure .22 with nebulizer, Beverly, MA), nylon membrane for Southern Blots was from Amersham (Hybond N, Buckinghamshire, England), and reinforced nitrocellulose membranes for colony lifts was obtained from MSI (NitroPlus, Westboro, MA). Plasmids were isolated using a Quiagen kit (Santa Clarita, CA), and sequencing was done by fluorescent dye termination on an ABI Prism Model 377 at the University of Washington Biochemistry Facility using ABI dRhodamine or BigDye kits. Oligonucleotides were synthesized by Gibco/BRL.

S.epidermidis UW-3 DNA

Bacteria were grown overnight at 37° C with shaking in two liters of T-Soy broth. DNA was obtained from these bacteria using a slight variation of established procedure.(139) Bacteria were harvested by centrifugation, resuspended in 95ml of TE (tris-EDTA)(140) with 5mg of lysostaphin (Sigma), and incubated with gentle shaking

at 37° C for two hours. The rest of the procedure was performed as for Gram-negative bacteria: briefly, the polysaccharides and proteins were removed by precipitation with CTAB (hexadecyltrimethyl ammonium bromide) and extraction with chloroform / iso-amyl alcohol (24:1). The supernatant was extracted with phenol / chloroform / isoamyl alcohol (25:24:1) and again with chloroform / isoamyl alcohol. DNA was precipitated with the addition of .6 volumes of isopropanol and spooled onto a bent pasteur pipette. This DNA was rinsed twice with 70% ethanol, air-dried, and dissolved in TE with gentle mixing overnight at 4° C for a final yield of about 3mg of genomic DNA.

Polymerase Chain Reaction

What will in this paper be called "standard" PCR was done on a water-cooled Eppendorf MicroCycler according to established procedures (T. Maniatis *Molecular Cloning* 2nd ed. p. 14.2-14.4). $MgCl_2$ was at 1.5 mM, dNTPs were each at 200uM, 5 pMol each primer, and 2.5 units of TAQ were mixed with the TAQ polymerase buffer (Boehringer-Mannheim) in a final reaction volume of 100ul; this was overlaid with mineral oil to prevent evaporation. The cycles ran from a denaturation at 94° C for fifteen seconds, to annealing at 55° C for fifteen seconds, and extension at 72° C for ninety seconds (the number of cycles for each case is given in the results). These cycles were preceeded by a four minute incubation at 94° C and followed by a nine minute, thirty second extension at 72° C after the last cycle.

Hybridization

Hybridization techniques, including Southern Blot and colony lifts, employed established methods.(141) For Southern Blots of genomic DNA, 15ug of genomic DNA was digested with each restriction endonuclease and run on a 1.2% agarose gel.

This was stained with ethidium bromide, photographed, and transferred to nylon membrane by capillary transfer and crosslinked by UV irradiation. Blots were probed with 24 pmol of radiolabelled oligonucleotides in 6X SSC, 5X Denhardts, 1% SDS (with 100ug/ml sheared, denatured salmon sperm DNA and 100ug/ml Torula RNA) after at least overnight prehybridization in the same buffer). Prehybridization and hybridization were carried out at 55° C with gentle rocking. Blots were rinsed with 6X SSC at 50° C with repeated buffer changes until they reached near-background level of radioactivity with a handheld meter. They were then exposed to film with an intensifying screen at -70° C for visualization.

Primers and Adapters

The initial primers used were based on amino acid sequence data and are shown in Figure 17. From experiments with these came the second-generation primers listed in Figure 18. *Acc I* adapters were taken up in TE at a concentration of 100 pmol/ul; 25ul of each longer oligomer was combined with 50ul of the shorter, the combination was held at 70° C for 15 minutes and then allowed to cool to room temperature at one degree per minute in a PCR machine (MJ Research PTC-100, Watertown, MA). Adapters were stored at -20° C until use.

Anchor PCR with Exonuclease III Digestion (PSMα)

50 ug of genomic DNA was digested with *Acc I* and ligated to 1.25 nM of *Acc* I - specific adapters. This material was gel purified and the 1.5 kB region was extracted using spin columns, precipitated, and taken up in 50ul TE. 2ul of this target was subjected to single-strand PCR using only the gene-specific (forward) primer and the following temperature cycles, thirty times: 1 minute at 94° C followed by ninety

seconds at 55° C and two minutes at 72° C. This was preceeded by a four minute incubation at 94° C and followed by nine minutes, thirty seconds at 72° C. To 50ul of this PCR mixture was added 5ul of buffer containing 400mM Tris, pH 8, and 35mM $MgCl_2$ followed by 500 units (5ul) of Exonuclease III. This was mixed and incubated for fifteen minutes at 37° C, followed by twenty minutes at 70°C. Small molecular weight contaminants were removed on a Centri-Sep column and 4ul of this was used as a target in standard PCR (35 cycles) using gene-specific and adapter-specific primers. 1ul of this PCR was used in another PCR (35 cycles) with the same primers.

Anchor PCR for PSMβ

The 4kB region of *Eco RV* cut genomic DNA was isolated by agarose gel electrophoresis and spin-column purification. 100ng of this was ligated to 60 ng of *Sma I* cut pBS SK+ (Stratagene, LaJolla, CA), gel-purified plasmid. 2ul of this was used after overnight incubation and heat inactivation as a target in (anchor) PCR using one primer specific for the beginning of the PSMβ (Figure 17) and one specific for the vector (T7 primer). A four-minute presoak at 94° C was followed by thirty cycles of one minute at 94° C, ninety seconds at 50° C, and two minutes at 72° C, and this was followed by a nine minute, thirty second incubation at 72° C. The 350bp product obtained in this manner was cloned using Invitrogen's (Carlsbad, CA) TA kit and sequenced.

Colony Lifts for PSMβ

The 5kB region of *Eco RI* cut genomic DNA was purified as for anchor PCR (above), and 320 ng of this was ligated into 250ng *Eco RI* cut pBS plasmid. This was used to transform Supercompetent XL-1 Blue *E. coli* (Stratagene) and plated onto four

standard petri dishes. Colonies (about 150 per plate) were adsorbed onto nitrocellulose membranes and lysed using standard procedures. The DNA was crosslinked by UV irradiation and probed as detailed above using a mixture of gene-specific (anchor PCR probes in Figure 17) and intergenic (Figure 18) oligonucleotides, radiolabelled with P-32. Three positive colonies per plate were picked and grown out; the positive insert verified by Southern blotting, and two inserts were sequenced (both strands) in their entirety. This was accomplished by digesting the two plasmids with restriction endonucleases and subcloning the smaller constructs as well as the generation of several new primers (not shown).

Anchor PCR for PSMγ

200ng *Acc I* cut, adapter-linked genomic DNA (from anchor PCR for PSMα) was cut with *Eag I*, gel purified, and ligated into 500ng of *Not I* pBS (2:1 insert:vector molar ratio). The ligase was heat killed, and 5ul of this ligated mixture was used as a target in (anchor) PCR using a Gamma-specific primer (Figure 17) and vector -specific primer (M13 Reverse) in a standard, 35 cycle reaction. No bands were seen on an ethidium-stained agarose gel following this, so 1ul of the PCR reaction was used as a template in a second PCR with the same primers and reaction conditions. The 550bp product obtained from this was sequenced directly.

Inverse PCR for PSMγ, downstream sequence for PSMα

10ug of genomic DNA was digested with *Sau 3AI* and the enzyme was then heat-inactivated. 1ug of this digest was combined with 500U of ligase (NEB) in a total volume of 1ml and incubated overnight at 16° C. The ligase was heat inactivated, and 10ul of the circularized DNA was used as a template in a standard PCR, 35 cycles,

using the inverse PCR primers shown in Table II. One microliter of this PCR was then used as a target in a second, identical, PCR, and the product was sequenced directly using the same primers.

Results

The complete nucleic acid and corresponding amino acid sequences are shown in Figures nineteen, twenty, and twenty-one for all three PSMs. The amino acid sequence obtained by chemical means (Chapter Three) is listed for comparison in italics where it differs from the deduced sequence. Anchor PCR was found to be a very useful technique for the isolation of all three genes, but only PSMβ could be retrieved from a library. This may be because PSMα and PSMγ are toxic to the *E. coli* used as host cells for the library: this is supported by the observation that PCR products containing the genes for PSMα and PSMγ could not be cloned. All operations for PSMα and PSMγ had to be done strictly *in vitro*: all PCR products containing these genes were sequenced directly, without cloning.

PSMα

Sequence immediately downstream of PSMα was obtained by a variation of anchor PCR.(142) Southern blotting of genomic DNA (probe sequence in Figure 17) digested with a panel of restriction endonucleases revealed that *Acc I* liberated a fragment of about 1.5 kb containing PSMα. Genomic DNA was digested with Acc I, ligated to Acc I - specific adapters, and cycled as for PCR with only the gene-specific primer, creating single-stranded products. The remaining double-stranded DNA was digested with Exonuclease III to leave only single-stranded target DNA which was

freed of low molecular weight contaminants with a desalting column. PCR was performed on this single-stranded target using gene-specific and adapter-specific primers, and two bands resulted: one was about 1.5kb and the other was about 250kb. A small amount of this PCR reaction was used as a target in a PCR containing the same primers and only the lower size band was amplified. The identity of this band was verified by sequencing, as it was noted that the sequence contained the last two amino acids that PSMα was predicted to have, directly adjacent to the gene-specific probe. It was also noted that the adapter had ligated to the DNA fragment aberrently, as the *Acc I* cut site was not present. Enough sequence data was obtained, however, for generating Inverse PCR primers (Figure 18). Inverse PCR using *Sau 3AI* digested, cyclized DNA provided only downstream sequence due to the presence of a *Sau 3AI* site close to the end of the gene; this was used to generate the Alpha 3' primer (downstream of the inverse PCR primers). A new anchor PCR template was created using Acc I cut DNA blunted with Klenow and ligated into the *Sma I* (blunt) site of the Bluescript plasmid. This was used directly as template DNA in a PCR reaction containing an antisense primer specific for the region after PSMα ("Inverse PCR, Back", Figure 18) and a plasmid-specific primer (M13 Reverse). Sequencing this product gave the final gene sequence for PSMα as well as preceding sequence, from which the Alpha 5' primer was made. This sequence was verified by several PCRs using the Alpha 5' and Alpha 3' primers specific for the upstream and downstream regions of the genomic DNA.

PSMβ

It was noted by Southern Blot that *Eco RV* and *Eco RI* liberated 4kb and 5kb fragments, respectively, containing PSMβ (probes shown in Figure 17). The 4kb Eco RV fragment was ligated into the Sma I site of the Bluescript vector for generating a

library and for anchor PCR. This *Eco RV* fragment was also digested with *Sau 3AI* and religated for use as an inverse PCR template. Both the anchor PCR and inverse PCR revealed that PSMβ appeared in two copies, and it was also shown that Eco RV cut within the downstream gene. A similar approach was therefore used with the Eco RI fragment except that the intergenic region was used as a primer for anchor PCR and as a probe for a size-selected library. Two clones were selected for sequencing as they represented the two patterns seen upon enzymatic digestion and Southern blotting. These clones contained identical constructs (although the inserts were reversed), and anchor PCR retrieved the same sequence. The gene appeared about 1kb away from the end of the construct, and anchor PCR was only able to retrieve this shorter end despite all efforts to increase the length of the PCR products obtained in the other direction. All 5kb of each of the clones was sequenced and no further copies of the genes were found.

PSMγ

Downstream sequence for PSMγ was obtained by anchor PCR from an Acc I fragment of genomic DNA. As was the case with PSMα, Acc I liberated a 1.5kB fragment which was shown by Southern Blot to contain PSMγ sequence. Size-selected Acc I fragments were ligated to adapters. These adapters were then cut with Eag I and ligated into the (compatible) Not I site of Bluescript. Anchor PCR using a gene-specific primer and a plasmid-specific primer provided enough downstream sequence to create two primers for inverse PCR. Genomic DNA was digested with Sau 3AI and allowed to ligate into circles, and this was used as a template for inverse PCR. The sequence thus obtained was verified by sequencing the products of four separate PCR reactions using primers specific for regions upstream and downstream of PSMγ.

Discussion

The isolation of the genes for these three PSMs provided the first wholly unambiguous determination of their primary structure. In hindsight, it is remarkable how good the chemical determination (Edman degradation) was; only a couple of amino acids were different, and the correct amino acid generally was present as a background signal. Figure 17 illustrates how disparate the initial primers were from the actual sequence -- it is somewhat surprising that these primers worked at all. As the troublesome areas of the Edman degradation were avoided, the degeneracy of the amino acid coding is mostly to blame for these inaccuracies.

PSMα is the most novel of the three PSMs, and a standard BLAST search demonstrates no homologies. Closer inspection, however, reveals that there is a fairly high degree of similarity between this protein and *S. epidermidis* delta toxin (Figure 22). Lowering the stringency of the BLAST search demonstrated distant homology to many proteins related to PSMα, and of those found only delta toxin was a small peptide. *S. epidermidis* delta toxin was not retrieved with this search despite its similarity to *S. aureus* delta toxin; it is likely that the gaps required for alignment weighed heavily against its statistical similarity to PSMα. The theoretical mass of PSMα is 2460.0, slightly less than the 2489-2505 (M+1) actually determined (this is despite including one molecule of water in the theoretical mass). Formylation adds 28.0 to the mass (total of 2488), and oxidation of the methionine adds 16.0 (total of 2504). Thus, it seems reasonable to conclude that PSMα is formylated and that the chromatographic processes involved in its isolation often resulted in the oxidation of the methionine. The mass spectrum of crude PSM prior to chromatography (Figure 7) shows all three PSMs with masses 16 lighter than those found after chromatography, so it is likely that methionine

oxidation is a side product of HPLC. As mentioned in Chapter Three, PSMα is predicted to be α helical in structure along its entire length. Extending this analysis to a helical wheel (Figure 23), it can be seen that the helix is amphipathic in nature, with hydrophobic residues largely on one side and hydrophilic residues on the other. Such a structure is typical of pore-forming peptides and may provide a clue as to how PSMα functions.

PSMβ was found to have a high degree of similarity to antibacterial and hemolytic peptides previously isolated from other staphylococci (Figure 24). It is interesting that both the SLUSH proteins and the antigonococcal proteins were found as complexes or aggregates of three proteins. PSMβ has a theoretical mass (with one molecule of water) of 4639.3, less than the 4669-4686 (M+1) observed by mass spectrometry. As was the case for PSMα, formylation and methionine oxidation bring the theoretical values (4667.3, 4685.3) very close to those observed. PSMβ is predicted to be about half α helical.

PSMγ, with a sequence identical to that of *S. epidermidis* delta toxin, is the major component of PSM. The theoretical mass of 2820.8 is, again, short of the observed mass of 2864.8 (M + 1 = 2865.8) by an amount corresponding to a formylated protein with an oxidized methionine (total 2864.8). Delta toxin is known to be encoded within the *agr* locus of staphylococci, and the entire sequence of the *agr* locus for *S. epidermidis* has recently been reported.(143) The sequence obtained from *S. epidermidis* UW-3 was more than 99% identical to the previously published sequence, and none of the bases within the coding region were different.

All the PSMs had stop codons after their last amino acid and the initial ATGs were preceeded by reasonable Shine-Delgarno sequences (Table VIII). It had been previously suggested that the antigonococcal proteins of *S. haemolyticus* represented

cleaved-off signal sequences for larger proteins.(144) but the presence of stop codons makes this unlikely for PSMs. These genes were not found to be neighboring on the *S. epidermidis* chromosome despite sequencing several hundred (or, in the case of PSMβ, thousand) bases on either side of the genes.

In an early effort to separate PSMα and PSMβ, material from Peak 1 (Chapter 3) was subjected to a second rapid HPLC in acetonitrile with .05% TFA. This chromatographic step still resulted in only a single peak, but when this was subjected to Edman degradation mixed results were obtained (Figure 25). This probably resulted from the unblocking of both PSMs, and although the results were puzzling at the time they are a lot easier to understand now given the complete sequence of both proteins.

It is noteworthy that neither PSMα or PSMβ were contained within the *agr* virulence locus. This locus is the site of several virulence-associated genes in *S. aureus*, including α-toxin, β-toxin, δ-toxin, serine protease, DNAse, fibrinolysin, enterotoxin B and toxic shock syndrome toxin-1, all of which share regulatory systems.(143) In *S. epidermidis*, these proteins are largely absent, with the exception of the *S.epidermidis* delta toxin. The finding of proteins which may be associated with virulence outside of this locus suggests that the *agr* locus may not play as significant a role in *S. epidermidis* as it does in *S. aureus*. Additionally, it may be an indication that Staphylococci have "virulence genes" with more disparate locations than had previously been thought.

Table VI. Codon Bias Utilized for *S. epidermidis*

Amino Acid	Codon Used	% Exact Match	% First Two
Alanine	GCA	45	100
Arginine	AGA	44	51
Aspartic A.	GAT	76	100
Asparagine	AAT	66	100
Cysteine	TGT	62	100
Glutamic A.	GAA	83	100
Glutamine	CAA	87	100
Glycine	GGT	54	100
Histidine	CAT	76	100
Isoleucine	ATT	54	100
Leucine	TTA	53	68
Lysine	AAA	81	100
Methionine	ATG	100	100
Phenylalanine	TTT	70	100
Proline	CCA	47	100
Serine	TCA	28	60
Threonine	ACA	52	100
Tyrosine	TAT	80	100
Valine	GTT	39	100

The table above lists the codon bias of *Staphylococcus aureus*; this information was used in generating degenerate oligonucleotides. As can be seen from this data, there is a good deal of heterogenity in codon preference. *Data based on Genbank entries and information posted on the internet by J. Michael Cherry.*

PSMα

```
 V   I   A   K   I   V   E   I   V   K   G   L   I   D   Q   F
GTT ATT GCA AAA ATT GTT GAA ATT GTT AAA GGT TTA ATT GAT CAA TTT
--A --C --T --- --- --- --- --- --- --- --C --- --- --- --- --C
```

Tm (theoretical): 54.4° C Tm(adjusted): 48.2° C

PSMβ

Inverse PCR Primers

```
 I   V   N   T   V   K   A   A   Q     D   Q   D   W   T   K   L   G   T
AAT GTT AAT ACT GTT AAA GCT GCT       GAT CAA GAT TGG ACT AAA TTA GGT ACT
--- -CA --- --A --A --- --A --A       --- --- --- --- --- --- --- --A ---
```

Tm(theoretical): 43.7° C Tm(adjusted): 36.2° C

(primer was reverse complimented)

Tm (theoretical): 48.6° C Tm (adjusted): 47.3° C

Anchor PCR Primers

```
 E   A   I   A   N   T   V   K   A . . . E   S   G   V   S   V   L   G   K
GAA GCA ATT GCA AAT ACA GTA AAA G       GAA TCA GGT GTT TCA GTT TTA GGT A
--- --T --- --- --- --- --T --- -       --- AGT --C --- AGC --- --- --- -
```

Tm (theoretical) 46.0° C Tm (adjusted): 43.5° C

Tm (theoretical): 47.7° C Tm(adjusted): 38.9° C

(primer was reverse complimented)

PSMγ

```
 A   D   I   S   T   I   G   D   L   V   K   W   I   I   D   T   V   N   K
GCA GAT ATT TCA ACA ATT GGT GAT TTA GTT AAA TGG ATT ATT GAT ACA GTT AAT AAA
--- --- --C --T --- --C --- --- --- --A --- --- --- --C --- --- --- - - ---
        ATT
```

Tm (theoretical) :58.1° C Tm (adjusted): 48.1° C

Figure 17. Primers used in the initial screening of PSMs. The primers are shown with the amino acid sequence from which they were deduced. Melting temperatures were calculated using standard technigues; adjusted melting temperatures reflect the mismatches observed after the genes were isolated and sequenced.

Alpha Inverse PCR

Back

GAAATGTATTTAGAGATGGTTGTTGAATATTCAAAAGCTAAC

Forward

GAAAGGAGGTCATGAACATGAGCATCGTATC

Alpha 5'

CGAATAATACTATTAATATATTTTAAATGAGCAAGAGTGTCAATGG

Alpha 3' CATGGTTGTAAAACATATAAAAATGCTAATGAGTGTGACAATAATTGATGA

Beta Intergenic

TCTTAGTTTTTTAAAATATAAATTTAAATAATTAATTAGGGAGAGATA

Gamma Inverse PCR

Back

TGCTTCTCACTTGCTTAGTTTATATTAGTAAATTATTAAGTTGGGATGGCTCAACAACT
C

Forward

TTTGCTAGTAACTGTAGTTTCCTTGGACTCAGTGTTACGTATTATTCTTAGCTACCTTA
A

Gamma 5'

GATATTTTACCATATTTAGTTTTACAGTTGAGTACTAAATATTGCTAT

Gamma 3'

CCACATCTTTATAAATAGCATAGTTAAAGCCGTGAGC

Acc I Adapters

Eag I
V

ATCTCGAGTCGGCCGTACGAGCT

50/50 mix of these two

CGCTCGAGTCGGCCGTACGAGCT

GAGCTCAGCCGGCATGCTCGA

Figure 18. Second generation primers. These primers were obtained from sequencing of inserts or clones, as described in the text. The Alpha and Gamma primers were used to amplify their respective genes by PCR or inverse PCR, as indicated, and the Beta oligomer (sense strand given) was used to probe colony lifts and in anchor PCR. The Acc I adapters were mixed, heated, and allowed to anneal as detailed in Materials and Methods. All primers are shown 5' to 3' except the shorter adapter primer, which is reversed to show how it annealed.

```
TCACTTAAGA CATTGATATT TTAATTTAGG GACTATGCAC ATATTTAATT
CAAATTCCAG TTACACTATA GATAACAAAT TAGTAAGGGA GGTCATTTAA

ATG GCA GAT GTA ATC GCT AAA ATT GTT GAA ATT GTT AAA GGC
 M   A   D   V   I   A   K   I   V   E   I   V   K   G
     Q   K

TTA ATT GAT CAA TTT ACT CAA AAA TAA
 L   I   D   Q   F   T   Q   K   .

TATTTAAAAG TTACGTATTT TATAAGTTAG CTTTTGAATA TTCAACAACC
ATCTCTAAAT ACATTTCCCT TTTGAAAGGA GGTCATGAAC ATGAGCATCG
```

Figure 19. The complete amino acid and genomic sequence for PSMα. Deduced amino acid sequence is in plain type, and amino acid identity from Edman degradation is shown in italics where it differed (Chapter Three). Nucleic acid sequence was obtained as described in the text and was verified by at least four separate PCRs, sequenced on both strands, with identical determinations. One hundred flanking bases are also shown.

```
TATTGTTGAA AATGGTGTTA GTATTATTTC TAAATTATTA GGTCAATAAT
TTAAAAATTA AAACTTTAAA ATTTAAATAA TTAATTAGGG AGAGATAAAC

ATG TCA AAA TTA GCA GAA GCT ATT GCA AAT ACA GTA AAA GCA
 M   S   K   L   A   E   A   I   A   N   T   V   K   A

GCA CAA GAT CAA GAT TGG ACT AAA TTA GGA ACT AGT ATC GTT
 A   Q   D   Q   D   W   T   K   L   G   T   S   I   V

GAC ATC GTA GAA AGT GGC GTT AGC GTA TTA GGT AAA ATC TTC
 D   I   V   E   S   G   V   S   V   L   G   K   I   F

GGA TTT TAA TTAAT
 G   F   .

CTTAGTTTTT TAAAATATAA ATTTAAATAA TTAATTAGGG AGAGATAAAC

ATG TCA AAA TTA GCA GAA GCT ATT GCA AAT ACA GTA AAA GCA
 M   S   K   L   A   E   A   I   A   N   T   V   K   A

GCA CAA GAC CAA GAT TGG ACT AAA TTA GGA ACT AGT ATC GTT
 A   Q   D   Q   D   W   T   K   L   G   T   S   I   V

GAT ATC GTA GAA AGT GGC GTT AGC GTA TTA GGT AAA ATC TTC
 D   I   V   E   S   G   V   S   V   L   G   K   I   F

GGT TTC TAA TACAC
 G   F   .

GATTAGGTAT TGGCCAGAGC GAATGCTCTG GTCTTTTTTT GTGCCATTTT
ATTTGTTACT ATTTTAGTAA AAAAAGATTG TTAATAAATA GGAGA
```

Figure 20. The complete amino acid and genomic sequence for both copies of PSMβ is shown. One hundred bases of flanking sequence and the intergenic region is also given. The (deduced) amino acid sequence of the two genes is the same, and there is a high degree of homology in the genetic sequence which precedes the two coding regions. Sequence which differs from the nearly identical upstream gene is underlined. Deduced amino acid sequence is identical to that obtained by Edman degradation.

```
GATGTGATTG AAAGATAGTT GAAAAATTTG CTTAATCTAG TCGAGTGAAT
GTTAAATTCA TTCGTATCCA TTACCTTAAT TCGAAAGGAG TGAAGTTATG

ATG GCA GCA GAT ATC ATT TCT ACA ATC GGT GAT TTA GTA AAA
 M   A   A   D   I   I   S   T   I   G   D   L   V   K
                     [F]

TGG ATT ATC GAT ACA GTT AAT AAA TTC AAA AAA TAA
 W   I   I   D   T   V   N   K   F   K   K   .

TTTTTGAATG AGTCTATTGT AACTTTTGTA ACTTTGTTTT CTTCGTATAA
TTAATACTAT TAGTGAGTTG TTGAGCCATC CCAACTTAAT AATTTACTAA
```

Figure 21. The complete genomic and amino acid sequence of PSMγ is shown as well as some flanking sequence. Sequence was determined as described in the text. Edman degradation obtained identical results for the first fifteen amino acids except for a weak phenylalanine signal at the position indicated: background signals at this point corresponded to L or I (Chapter Three). Underlined bases are different from those previously reported for this gene.

PSMα
M A D V **I** A K **I** V E I **V K** G L **I D** Q F T Q **K**
‡ ‡ ‡ + ‡ ‡ + + ‡ ‡ + ‡ ‡ + ‡
M A **A D** I **I** S T **I** G D L **V K** W I **I D** T V N **K** F K K
Delta Toxin

Figure 22. Alignment of PSMα and *S epidermidis* delta toxin. Identical residues are bold with a " ‡ ", homologous residues are indicated with a "+". and each protein has a single gap in order to facilitate alignment.

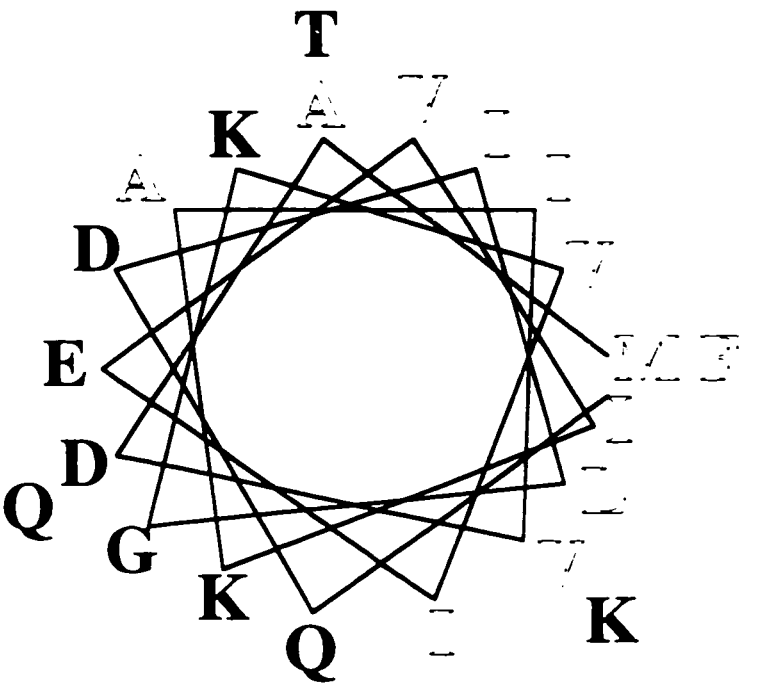


Figure 23. Helical wheel analysis of PSMα. Amino acids were spaced at 300° intervals around a circle. Solid residues are hydrophilic, open residues are hydrophobic. Glycine, as it has no side chain, is not considered to have either character.

```
PSMβ       MSKLAEAIANTVKAAQDQDWTKLGTSIVDIVESGVSVLGKIFGF
AGP-1      -Q-------AA-S-G--K--G-M-----G---N-IT--------
AGP-3      ----VQ--SDA-Q-Q-N---A-------G---N--GI---L---
AGP-2      -E-I-N-VKSAIE-G-N----------L---SN--TE-S-----
SLUSH-B    --GII---TKA-Q-GL-K--ATM----AEALAK-IDAISGL--
SLUSH-A    --GVID--TKA-Q-GL-K--ATMA---A-AIAK--DFIAGF-N
SLUSH-C    -DGIF---SKA-Q-GL-K--ATM----AEALAK--DFIIGL-H
```

Figure 24. Comparison of the sequence of PSMβ with other staphylococcal proteins. *S. haemolyticus* antigonococcal proteins and *S. lugdunensis* hemolytic proteins are shown. Dashes represent residues identical to PSMβ.

Table VIII. Shine-Delgarno Sequences

Alpha:	AGGACAT
Beta:	AGGGAGAG
Gamma:	AGGAGTG
S. aureus:	ATGAGAA
	AGGAGGG
	AGGAGGA
	CGGAGGG
	AGGAGTC
	CAGGAGGA
	AGGTGAT
	AGGAGAT
	AGAAGGA
	AGGATGA
	AGGAGGT
	GGGAGAT
	AGGAGTG
	AGGAGTG
	TGGAGTG
	TGGAGTG
	TTGAGGT
	AGGGGAT
	AGGAGTT
	AGGAGGG
	AGGAGGA
	AAGAGGG
	CGGAGGG
	TGGAGGAA
	TGGAGGT
	AGGGGGT
	TGGAGGA
	AAGAGGT
	AGGAGCA
	ACGGGGT
	ACGAGGT
	AGGAGAA

Comparison of Shine-Delgarno sequences from PSMs with those published for various genes from *S. aureus* (from *The Molecular Biology of Staphylococci*). All sequences were 4-8 bases upstream of the start codon.

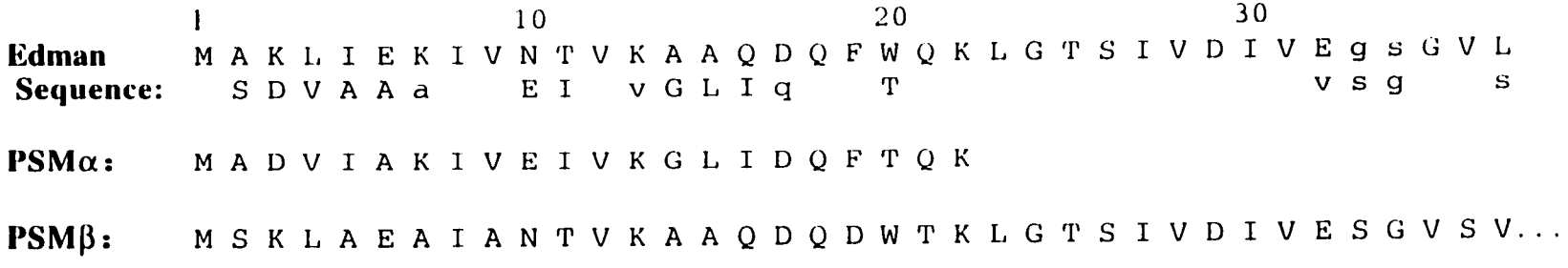


Figure 25. N-terminal sequence from Peak One following treatment with .05% TFA and acteonitrile. Peak One from several rapid HPLCs of Phenol Soluble Material in 1-propanol/ammonium acetate (as described in Materials and Methods) was rechromatographed in acetonitrile with .05% TFA. The resultant single peak was collected and subjected to Edman degradation, resulting in the heterogeneous data shown above. Weaker signals are denoted by small letters, and capital letters at equivalent positions were found in approximately equimolar amounts. The sequences of PSMα and PSMβ, as verified by genetic sequencing, are shown for comparison.

Chapter Five

Summary and Comparison to Delta Toxin

The activating factor from *S. epidermidis* has been shown to consist of three polypeptides: PSMα, β, and γ. PSMα is the most potent of the three, and while PSMγ showed some activity, PSMβ possessed only marginal stimulatory capacity at best. These analyses are somewhat confounded by the tendency for HPLC to destroy activity and the difficulty of getting PSMβ apart from the other two modulins, but it appears that α and γ are the most important of the three.

PSMα and γ show a high degree of similarity, with a 40% identity and a 60% homology, so it is likely that they operate in a similar manner. Much research has been done on *S. aureus* delta toxin, which differs from the *S. epidermidis* delta toxin (PSMγ) by only two residues (the *S. aureus* toxin has a glutamine instead of an alanine at position three and a threonine inserted prior to the final two lysines).(145) Given that all these polypeptides are so alike, it is reasonable to suppose that results obtained using the *S. aureus* molecule likely apply to the other two as well. Figures 26 and 27show PSMα and PSMγ modeled using the structure (based on Nuclear Magnetic Resonance) of the *S. aureus* delta toxin as a guide. The pores formed by these molecules are strikingly similar.

Delta toxin is thought to form amphipathic alpha helices which bundle into parallel groups of six polypeptides -- a quaternary structure known as the barrel stave model.(146) These insert into a variety of membranes, including phospholipid micelles (147) and erythrocytes (100), and the latter can be lysed by high concentrations (about

been observed in granulocytes. This influx was correlated with the release of Platelet Activating Factor and the generation of reactive oxygen species. (148)

Notably, the peak of activity in the LTR_{LLC} assay system occurred at concentrations much lower than those mentioned above. Significant TNFα production has been observed from monocytes with delta toxin concentrations as low as 35nM, (103) results which correlate with unpublished results from our laboratory using PSM. The pores form at an optimum concentration of 200nM in aqueous solution, inserting into phospholipid membranes as weakly cation selective channels of varying sizes. (146) Calcium infux in monocytes, the result of calcium ionophore or scavenger receptor signaling, induces phospholipases A2 and C, leading to the creation of diacylglycerol and the activation of protein kinase C.(149, 150) While it is an attractive hypothesis that pores formed by delta toxin allow the entry of calcium and initiate a similar signaling cascade, the observation that maximal signaling occurs at concentrations so much lower than pore formation casts some doubt on this theory.(148) In addition, PSM showed no activity on Jurkat cells (Klebanoff, unpublished observations), suggesting that a monocytie/macrophage receptor may be involved. Studies with *E. coli* hemolysin showed that although this toxin formed pores, derivatives of the hemolysin which were incapable of forming pores were still able to induce G-protein related responses in neutrophils, (151, 152) prompting speculation that the toxin is acting directly on these G-proteins via hydrophobic regions of the toxin.(153)

In data not presented in this thesis, PSM has been demonstrated to induce TNFα and IL-1 release from both THP-1 cells and monocytes. Following PSM exposure, the induction of an active NF-kB p65/p50 heterodimer has been shown in THP-1 cells by gel shift assays, but pathways upstream of NF-kB remain unclear.

These studies have raised more questions than they have answered. We know now that the protein component of PSM consists largely of an aggregate of three hydrophobic peptides, but we would like to know if this is simple hydrophobic aggregation or the formation of a higher-order complex. We know the sequence of the PSMs and the genes from which they are transcribed, but we know very little about their regulation and their distribution. And, finally, we have observed an inflammatory capacity from these polypeptides, but can only conjecture about their role in Gram positive sepsis. The elucidation of the structure of these PSMs is really much more of a beginning than an end. It is the first step in understanding what the greater importance of these molecules is and represents what I hope will be the birth of many research projects.

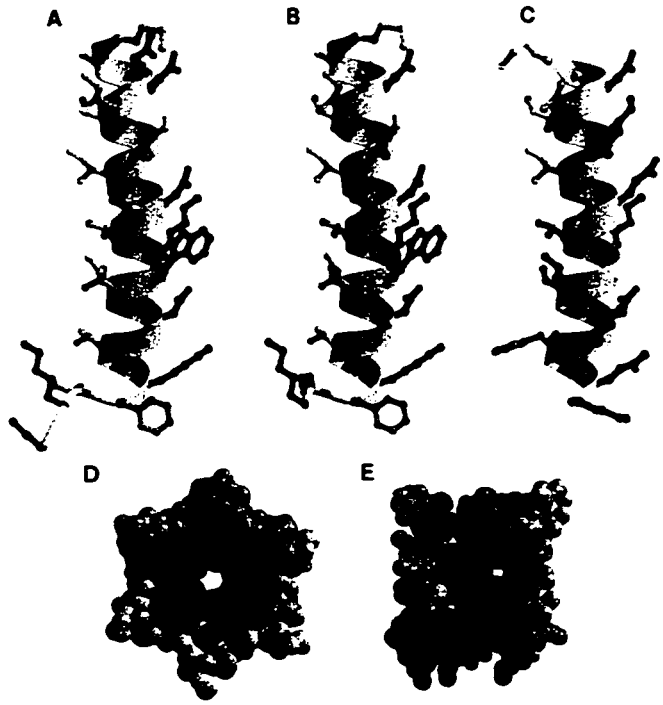


Figure 26. Computer modeling of PSMα and γ compared to the *S. aureus* delta toxin. **A**) *S. aureus* delta toxin. A single alpha helix is shown, the structure of which is based upon Nuclear Magetic Resonance data in the Protein Databank (154). **B**) PSMγ and **C**) PSMα were modeled by introducing the appropriate amino acid changes into the delta toxin helix. Amino acid identities are shown by color, and it can be seen that hydrophobic residues (G, A, V, I, light gray; L, dark gray; F, purple; W, dark purple; M, yellow) and hydrophilic residues (D, E, red; T, pink; K, blue; Q, green) are found largely on opposite sides of the helix. A hypothetical pore model shown viewed from the top in **D** and in cross-section in **E** was constructed by arraying the alpha helices of PSMα with sixfold symmetry, a structure loosely based on the models of I. D. Kerr. (155) This modeling was done by Elinor T. Adman (156) using the programs "O" (157), Molscript (158), and Raster 3D (159).

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PERSONAL

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EDUCATION

Undergraduate:

1986-90	Williams College, Williamstown, MA. B.A., Chemistry 1990.

Graduate:

1991-92	University of Illinois, Chicago , IL. Department of Chemistry. Graduate research assistantship under Dr. Michael Kahn synthesizing beta turn mimetics of GP120.
1992-94	University of Washington, Seattle, WA Department of Pathobiology. Graduate research assistantship under Dr. Michael Kahn synthesizing drugs to selectively kill Kinetoplastid parasites.
1994-98	University of Washington, Seattle, WA Departments of Pathobiology and Medicine. Graduate research assistanship under Dr. Seymour Klebanoff isolating, characterizing, and cloning inflammatory polypeptides. Ph.D. in Pathobiology 1998.

AWARDS AND HONORS

1989	Frederick C. Hagedron Award (Chemistry). Williams College.
1991-92	Teaching Assistantship, University of Illinois.
1992-98	Research Assistantship, University of Washington.
1996	Outstanding Student Scholarship, School of Public Health, University of Washington.

1996 STD/HIV Research Training Fellowship. School of Medicine, University of Washington.

PUBLICATIONS

Kiener, PA, Davis PM, Starling GC, Mehlin C, Klebanoff SJ, Ledbetter JA, Liles, WC. Differential induction of apoptosis by Fas-Fas ligand interactions in human monocytes and macrophages. J Exp Medicine. 1997; 185(8): 1511-6.

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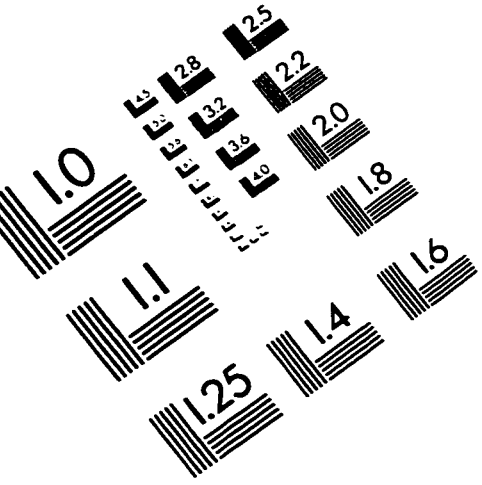
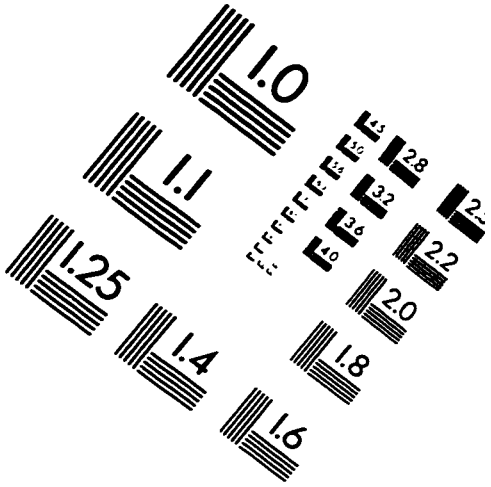
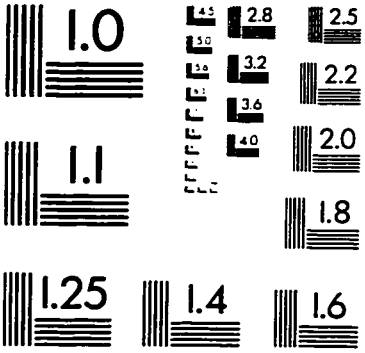
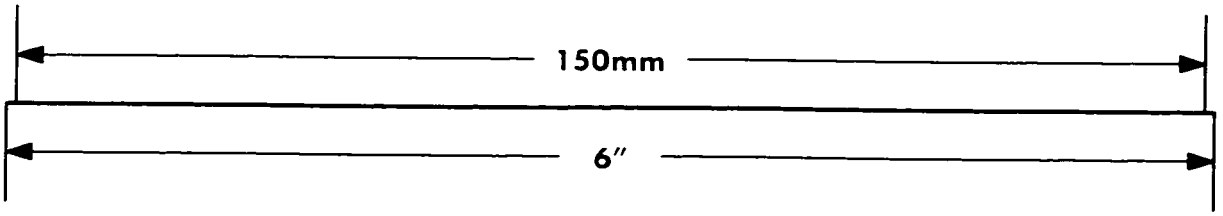
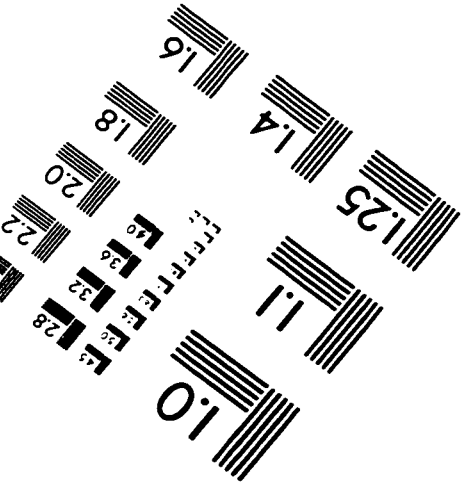
IMAGE EVALUATION TEST TARGET (QA-3)

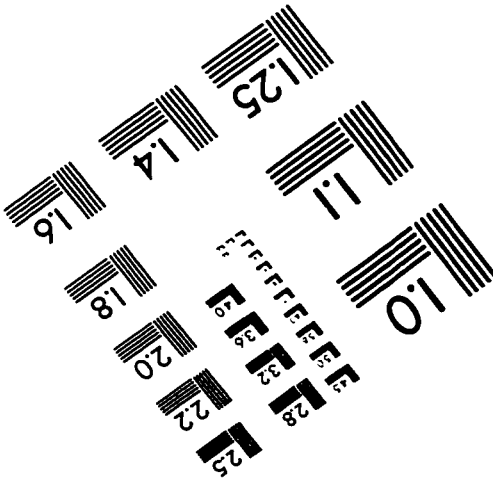












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