Peptide Antibiotics

Discovery, Modes of Action, and Applications

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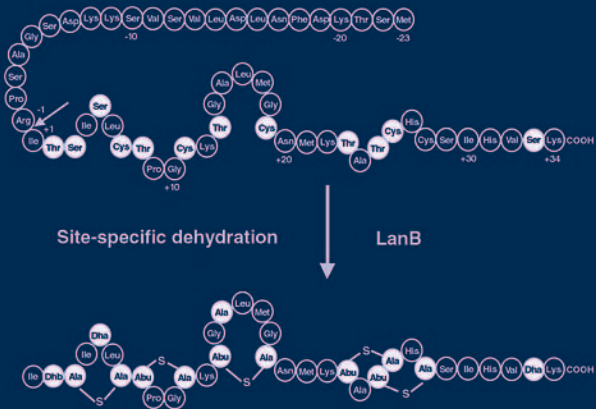
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Preface

The search for agents active against infective bacteria has been pursued from the depths of the oceans, to the highest mountains, in remote jungles and deserts, and in virtually every place on Earth where a novel organism or plant might be imagined to exist. It is of particular interest that the peptide antibiotics, one of today's most active areas of research, are found literally under our noses (in our mouths and on our skin), and are also synthesized by such commonly encountered organisms as frogs, insects, and the bacteria that abound in dairy products.

Contributors to this book describe the discovery of these novel compounds and the application of biotechnology to many aspects of their development. While the origins of the peptides covered in this book are diverse, common themes can be readily identified. Peptides originally found in frogs and insects are now produced by bacterial fermentation, and site-directed mutagenesis has been brought to bear to produce novel analogs of these agents. Recent discoveries in the biosynthesis of these unique compounds, their natural role in host defense systems, and the expanding commercial use of these compounds in food preservation are all described by experts in the field. The results of basic studies elucidating the membrane interactions and pore-forming ability of peptide antibiotics are presented and, finally, the progress toward harnessing the potential utility of these novel com-

pounds in the physician's armamentarium against multiple-drug resistant, disease-causing bacteria is also discussed.

This book will be of interest to researchers in the fields of anti-infective agents and peptides, and to students of chemistry, biology, and related disciplines. The current and potential applications of peptide antibiotics will be of interest to those in the fields of medical, veterinary, and agricultural research and to food scientists.

Finally, we would like to thank the chapter authors for their hard work and enthusiasm during the preparation of this book and to all at Marcel Dekker, Inc., who were involved in its production.

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Potential Applications of Peptides

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Introduction to the Peptide Antibiotics

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I. INTRODUCTION

The content of this volume is a testament to the importance of antimicrobial peptides in the lives of all organisms. Not only do microbial cells themselves produce these small peptides to influence the growth or activity of other microbes in their environments, but an immense body of evidence now points to the essential and diverse roles played by these peptides throughout nature in the innate immunity of plants and invertebrates, and in both innate and adaptive immunity of vertebrates—including humans. The review by Ganz and Lehrer (1) should be consulted for a brief treatment of the biology and applications of antimicrobial peptides from higher eukaryotes. Hancock and Diamond (2) have provided a recent overview of the roles of peptides in innate immunity and relate these to adaptive immunity. Hoffmann et al. (3) provide an in-depth review of innate immunity across species boundaries, and the commentary by Ganz (4) provides a perspective on vertebrate defensins. References in each of these publications should be consulted for further reading. The biological functions of antimicrobial peptides and their utility in practical applications are treated comprehensively in the chapters of this

volume, and include lists of references enabling the reader to pursue many interests in depth.

Antimicrobial peptides were discovered by investigators working in a variety of different systems. The principal classes of organisms capable of producing these peptides are covered in this volume and need not be reiterated here. One major group of organisms not treated here is the plant kingdom, but the focus of the present authors is on human health, including food preservation. In addition to coverage of naturally occurring peptides, a chapter on synthetic and hybrid peptides (Chapter 1) is included, in which the structural features of natural peptides that are responsible for their varied activities are addressed. Because this is an introductory chapter, what follows is an attempt to summarize generalities that apply to peptides from many biological systems while attempting to avoid redundancy with other chapters. It is presented from the standpoint of someone who has worked in the field of antibiotic permeability and resistance, and the molecular biology of bacterial membranes (5,6). The author has viewed the rapid growth of information about—and application of—antimicrobial peptides with increasing excitement, and the contributions to this volume succeed in conveying a sense of how quickly this group of structurally simple but functionally complex molecules has gained such wide attention.

II. THE VARIETY OF ANTIMICROBIAL PEPTIDE STRUCTURES

The majority of antimicrobial peptides have a net cationic charge, although this feature is not found universally, as some peptides with net neutral or anionic character recently have been described. The cationic peptides are generally classified into four groups based on their structure. One group is characterized by arrangement of the peptide chain into β-sheet structures (with internal disulfide bonding), the second by extended structures that can form amphipathic α-helices in the proper environment, the third by extended peptide chains (often with a predominant amino acid being present), and the fourth by a loop structure formed via a single internal disulfide bond (7). Many species have ex-

amples of all of these structural classes, as well as redundancy within each class (see, for example, Chapter 6 of this volume). Hancock and Diamond (2) have suggested four possible reasons for this structural diversity: (a) any given peptide will not have an antimicrobial spectrum that encompasses all pathogens that the producer species might encounter, so it is beneficial to have multiple peptides with overlapping activities; (b) peptides of different structures may work in synergy with each other to inhibit or kill pathogenic microorganisms; (c) nonantimicrobial functions that appear to vary from one peptide to another—such as the chemotactic and proinflammatory activities that have been identified in mammals—may complement one another; (d) in metazoans, different tissues tend to express a subset of the total repertoire that a particular species possesses, perhaps related either to the susceptibility of that tissue to pathogen invasion or to differential needs among tissues for nonantimicrobial functions of the peptides. In Chapter 6 of this volume, Bevins and Diamond provide a succinct review of these functions.

Antimicrobial peptides produced by gram-positive bacteria, which are treated in Chapters 3, 4, and 7 of this volume, are usually considered to constitute two classes. Class I (Chapter 3) is made up of small (approximately 20–35 amino acid residues), highly modified peptides that are termed lantibiotics because they contain intramolecular thioether rings formed by lanthionine and 3-methyllanthionine (structural diagrams in Chapter 3). Class II peptides are generally larger, approximately 30–70 amino acid residues, and, by contrast with Class I, are unmodified; Class II peptides from the lactic acid bacterial group are described in detail in Chapter 4. Higher molecular weight polypeptides called bacteriocins are also produced by both gram-positive and gram-negative bacteria, but because of their protein-sized molecular weights and apparently different mode of action, they are not usually included in the family of antimicrobial peptides.

Extensive programs of structural modification, and of synthetic invention of variants that differ from naturally occurring peptides by slight alterations or by more substantial changes, have been undertaken over the past two decades. Andreu and Rivas (this volume, Chapter 1) provide a well-referenced overview of these efforts, together with analysis of the changes in activities of the peptides brought

about by such modification. Some of the synthesis and alteration studies have been oriented to deeper understanding of the mechanism of antimicrobial action and of the structural determinants of peptide cytolytic effects on mammalian cells. Other studies have had a more direct bearing on the preparation of therapeutic compounds, and many have had a deliberate commercial purpose.

III. GENETIC ELEMENTS GOVERNING STRUCTURE AND FORMATION OF ANTIMICROBIAL PEPTIDES

Antimicrobial peptides in all systems thus far studied are formed from longer prepropeptide precursors encoded by open reading frames in the genome. The so-called pro-sequences must be proteolytically cleaved from the N terminus of the precursors to form the active peptide structure. In the majority of biological systems, the propeptide also has a pre- or signal sequence N-terminal to the pro-sequence; the signal sequence provides targeting to an intracellular membrane structure, and must also be posttranslationally cleaved to form the mature, biologically active peptide. In eukaryotes, the membrane structure is the endoplasmic reticulum system; in prokaryotes, it is the cytoplasmic membrane. Where systematic studies have been done, modifications to amino acid structure (see below) appear to be carried out prior to proteolytic cleavages.

In bacteria (this volume, Chapter 3), genes encoding the peptide precursors are commonly clustered together with those encoding (a) factors that control transcription of the precursor genes, (b) enzymes responsible for modification of the prepropeptide, and (c) proteases that subsequently process the prepeptide to the mature peptide. Additional functions such as export and producer self-protection are also encoded in these gene clusters. In some situations, the cluster may be located on a mobile element such as a transposon. Operons within the clusters are differentially regulated to ensure an appropriate balance between synthesis, modification, and export. The genetic location and organization, together with transcriptional regulation of the clusters encoding lantibiotics from three bacterial species (*Lactococcus lactis*, *Staphylococcus epidermidis*, *Bacillus subtilis*) are outlined in Chapter 3.

In mammals, antimicrobial peptides are encoded by typical exon-containing genes that occur as highly homologous gene families in localized chromosomal regions (this volume, Chapter 6). The α- and β-defensin gene families are substantially different from each other but maintain close sequence similarities within each family; these similarities include not only the regions encoding the propeptide and the mature peptide sequence, but also the signal sequence (2). Evolutionary relationships within families have been inferred from similarities in exon frequency and placement, in regulatory regions, and in tissue-specific expression. Transcriptional induction by specific inflammatory agents also has utility in grouping defensins (this volume, Chapter 6), as does the inducibility and constitutivity of expression. An extraordinary finding was made recently by Tang and co-workers (8); neutrophils from a species of monkey contain a member of a new class of defensins called θ (theta)-defensins. This defensin is composed of two truncated α-defensin molecules ligated head-to-tail by peptide splicing to form a cyclic structure. As pointed out by Ganz (4), the θ-defensins have properties that make them interesting candidates for modification and development as novel antibiotics.

The duplication of mammalian genes for antimicrobial peptides suggests a familiar evolutionary strategy: with one or more copies of the original gene intact, sequence divergence and exploration of the antimicrobial efficacy of new but related amino acid arrangements can occur without significant risk to the defensive armamentarium of the species.

IV. MODIFICATION AND PROCESSING

In the present context, the term *modification* will be used only to describe any chemical changes that are introduced into the amino acids making up the peptide structure following translation. The proteolytic events that convert the prepropeptide to its mature form will be discussed in the final paragraph of this section.

Eukaryotic cells appear to introduce relatively simple modifications to the primary amino acid sequence of their peptides, consisting largely of internal disulfide bond linkages. The *positioning* of the

cystine bonds (i.e., which specific pair of cysteines is involved in a given bond) determines the classification of mammalian defensins into the α and β groups (9). Arthropods (this volume, Chapter 5) have four classes of peptides carrying disulfide bridges: (a) a single bridge near the C terminus (e.g., thanatin); (b) two bridges (represented by the scorpion peptide androctonin); (c) three disulfide bridges (the insect defensins); and (d) four bridges (as in the *Drosophila* peptide drosomycin). Amphibians (this volume, Chapter 8) also have one class of peptides with a single disulfide bridge (e.g., brevinin I from the *Rana* genus, with strong sequence similarities to insect thanatin). Some linear amphibian peptides (lacking bridges) possess D-amino acids, apparently the product of posttranslational modification.

When we turn to the prokaryotes, we find much more extensive modifications among some classes of peptides. The lantibiotics (this volume, Chapter 3) contain not only intramolecular thioether bonds, but also an array of modified amino acids not otherwise found in proteins. Class II bacteriocin peptides (this volume, Chapter 4), on the other hand, do not contain modified amino acids but may have disulfide bridges (e.g., the pediocin group).

The peptide ligation event discovered by Tang et al. (8) thus far represents a unique posttranslational event and places this peptide in a class by itself (the θ-defensins). However, with new peptides being continually discovered and the details of their formation becoming available, more systems with unusual posttranslational events are likely to be found.

Most, but not all, antimicrobial peptides are synthesized with an N-terminal leader sequence that targets the prepropeptide structure to a membrane system, either the endoplasmic reticulum in the case of eukaryotic cells or the cytoplasmic membrane in the case of prokaryotes. This is cleaved proteolytically following the targeting event. There appears to be a variety of methods by which the pro-sequence is removed to create the active peptide; it may be coupled with transport in bacterial producers or with incorporation into neutrophil granules, as with bovine β-defensins. Alternatively, storage in the propeptide form, as with bovine cathelicidins, is followed by processing to the mature form when an induction signal is received.

V. REGULATION OF SYNTHESIS AND PROCESSING

Chapter 3 of this volume, by Pag and Sahl, reviews the genetic elements governing the formation of lantibiotics by gram-positive bacteria. Transcriptional regulation of these genes commonly appears to be governed by two-component systems consisting of proteins called sensor kinases and response regulators. Two-component signaling systems are prevalent throughout the bacterial world and function via environmental sensing by the sensor kinase, which then relays the signal to the response regulator by site-specific phosphorylation of the regulator. The latter effector then interacts with a regulatory sequence on the bacterial chromosome to modulate transcription. The signal for turn-on of lantibiotic structural gene transcription is not known in some cases, but for nisin gene transcription, the response regulator is nisin itself. Thus the two-component system appears to act as a quorum sensor, that is, as a mechanism for assessing the cell density of a growing population. When the low constitutive level of nisin production results in a sufficiently high extracellular concentration of the peptide following accumulation of enough cells to produce this threshold level, the two-component system serves to activate the operons responsible for encoding the primary structure of the pre-propeptide, together with the processing and modification functions. Formation of additional nisin ensures that production will continue to occur at a high level by continued stimulation of the transcriptional activation system.

Genes encoding the Class II bacteriocin peptides (this volume, Chapter 4), like those for the lantibiotics, are under two-component system transcriptional control. The signal molecule is not the bacteriocin peptide itself, but a bacteriocin-like molecule that is constitutively produced and serves as a quorum-sensing device mediated through the two-component signaling system. As with nisin, the pheromones for Class II bacteriocins also stimulate their own formation, leading to autoinduction when a threshold level of pheromone is reached.

For induction of peptide gene expression in eukaryotic species, there appears to be widespread utilization of a particular transcriptional effector family, the nuclear factor kappa B (NF-κB) family. This factor

often is employed in combination with other transcription factors. In Chapter 8 of this volume, on amphibian peptides, Zasloff cites recent work on a frog species in which NF-kB binding sites have been found in the 5´ region upstream from peptide structural genes. In mammalian systems, a careful study has been carried out with the bovine β-defensin TAP (tracheal airway protein) in cultured tracheal cells; this is described in Chapter 6 of this volume. Transcription of the TAP structural gene depends on NF-κB, and the appropriate binding sites are found in the region upstream of the gene. A transcriptional response is seen after treatment with bacterial surface polymer preparations or with specific interleukins.

In Chapter 5 of this volume, on insect cationic antimicrobial peptides, Hetru et al. describe the regulatory cascade that controls formation of the antifungal peptides produced in *Drosophila*, an organism that produces both antifungal and antibacterial peptides. This cascade depends in part on components of an intricate embryonic patterning sequence that include proteolysis, transmembrane signaling, nuclear localization, and transcriptional activation. Analysis using appropriate mutants affecting the patterning sequence, and comparison of these with mutants in which an independent immune function is compromised, show that formation of antifungal and antibacterial peptides occurs by different pathways, and that these pathways are responsible in vivo for resistance to the respective pathogen classes.

VI. SELF-PROTECTION EXHIBITED BY PRODUCING CELLS

Protection against damage to eukaryotic cell types that produce antimicrobial peptides appears to be inherent in the structure of eukaryotic membranes, which have significantly different lipid composition by comparison with their functional counterparts in bacteria. Cytoplasmic membranes of bacteria have anionic groups exposed to the exterior, while eukaryotic cells have most anionic groups sequestered to the cytoplasmic side of the membrane. The cationic character of peptides appears to be involved in their activity by sequestration of posi-

tively charged amino acid residues to the anionic face of the susceptible microbial membrane.

Protection is also afforded in mammalian systems by the structure of the unprocessed pro-form of the peptide, in which the anionic pro-sequence serves to neutralize the cationic groups of the mature peptide until the pro-sequence is proteolytically removed. There are additional mechanisms for protection—for example, the storage of peptides in neutrophil granules either in their mature form, as with indolicin, or in their inactive pro-form (e.g., bactenecin), which is processed to the active peptide following fusion with protease-containing granules that are also contained within the neutrophil. It is evident that the neutrophil must retain tight control of degranulation in response to microbial challenge in order to avoid damage to itself (refs. 1, 2, and this volume, Chapter 6).

Since bacteria that produce antimicrobial peptides have cytoplasmic membranes that would be susceptible to their own peptides, they must rely on mechanisms for protection other than those used by multicellular species (this volume, Chapters 3 and 4). The gram-positive lantibiotic and Class II bacteriocin producers have immunity systems that appear to be highly specific for the peptide produced by each individual species; that is, a lantibiotic produced by species 1 will be fully active against the lantibiotic producer species 2, even though species 2 has an immunity protein for its own peptide type. The converse would also be true. This immunity pattern resembles that of the classical heat-labile bacteriocins. The immunity systems examined in detail have either one or two components that function by quite different mechanisms. The first of these is an immunity protein capable of binding to the cytoplasmic membrane of the producer cell by a lipid modification or by transmembrane domains; its mechanism of action is not yet clear. Presumably it functions by interfering with the ability of the peptide to disrupt normal membrane structure and function. The second immunity component is an outwardly oriented ABC transporter system that removes peptide from the cytoplasmic membrane following binding from the external milieu, and does so at a rate sufficient to prevent accumulation of the peptide within the membrane. In some systems, a given ABC transport system will be utilized both for protection and for postsynthesis export of the

active peptide; in other systems, different transporters are assigned to the two tasks.

VII.   FOCUSING PEPTIDE CONCENTRATIONS TO LOCALIZED REGIONS

In epithelial layers of some amphibians, as discussed in Chapter 8 of this volume, there are specialized structures that appear to be sites for localized synthesis and secretion of antimicrobial peptides. In the skin of frogs, these structures are termed granular glands and respond to both external and systemic stimuli. In particular, injury to the skin will cause discharge of these glands located close to the site of insult. Structures similar to the granular glands occur in the digestive tracts of amphibians.

With their complex innate immune systems and wide variety of tissue types, mammals have ample opportunity to utilize specialized cells to place peptides at the most effective sites to resist pathogens. In Chapter 6 of this volume, Bevins and Diamond treat this topic by bodily location, i.e., those mechanisms in the myeloid blood cells and the different epithelial layers (intestine, skin and other squamous cell layers, respiratory tract). Epithelial cells deliver their peptides extra-cellularly to the adjacent luminal space, while phagocytic cells engulf pathogens and fuse granules containing antimicrobial substances to the phagolysosome. In the latter circumstance, extremely high con-centrations of peptides may be attained within the closed confines of the phagolysosome.

VIII.   MODE OF ACTION OF ANTIMICROBIAL PEPTIDES

A precise description of the mechanisms by which peptides act to de-stroy pathogens is still not available, although the general outlines are reasonably clear. This subject is treated—at least briefly—by all con-

tributors to this volume. That biological membranes are involved is likely, although whether disruption of their function is the decisive occurrence is not known. The fact that antimicrobial peptides are commonly cationic, and that those with α-helical structures frequently can assume amphipathic structures in the presence of membrane bilayers, is consistent with this. However, the existence of α-helical antimicrobial peptides that are not amphipathic, together with a large number of β-sheet peptides with strong antimicrobial effects, suggests that this is not essential. Epand and Vogel (9) have presented an overview of the diversity of peptides and their mechanisms of action as part of a single-topic journal issue devoted largely to the biophysical events inherent in peptide–membrane interactions. In a systematic study of several structural classes of peptides with artificial and biological membranes, Wu et al. (10) found no correlation between the minimal inhibitory concentration for bacterial killing and membrane depolarization. This suggests that models for antimicrobial effects that focus on membrane breakdown as the primary bactericidal event may not be appropriate. Loss of membrane potential by bacteria is ordinarily reversible, for example following exposure to protonophores or other ionophores.

However, as discussed by Pag and Sahl in Chapter 3 of this volume, there are significant data, based on work with the lantibiotic nisin, indicating that pores are formed with lifetimes that would allow loss of cellular solutes. The recent results that show a specific affinity of both nisin and epidermin for the membrane-bound bacterial cell wall precursor lipid II (ref. 11, discussed in this volume, Chapter 3) are most striking, and suggest that the peptide utilizes lipid II as a cell membrane receptor prior to pore formation. These results also point to cell wall synthesis inhibition as an additional target for peptide action, an activity that is emphasized by the early finding that autolysis of gram-positive bacteria is stimulated by lantibiotics (this volume, Chapter 3). There will need to be systematic reconciliation of intact cell viability studies with results obtained through isolated membranes, particularly artificial membranes. It is also likely that there will be a variety of mechanisms by which different peptides have their effects.

IX.   IMPLICATIONS OF ANTIMICROBIAL PEPTIDES
FOR MEDICINE AND PUBLIC HEALTH

As discussed by Bevins and Diamond in Chapter 6 of this volume, to develop effective peptide antibiotic-based therapies, it is crucial that we gain much greater knowledge of how underexpression (or overexpression) of individual antimicrobial peptides modulates innate immunity. To do this, we will need to learn substantially more about the actions of these molecules in both acute and chronic inflammatory processes. A distant possibility might be the development of drugs that act as inducers for individual peptides or sets of peptides. In a review of approaches to coping with bacterial antibiotic resistance as a general problem, Tan et al. (12) acknowledge the potential for creation of new antimicrobial peptides and their use as novel antibiotics. However, they do caution that bacterial resistance to some peptides can arise in patients with chronic infections (e.g., cystic fibrosis).

During the latter half of the twentieth century, the discovery and commercial availability of antibiotics directed against microbial pathogens led to their widespread adoption and, not uncommonly, their overuse. For these compounds to be effective, the human or animal being treated must be able to mount an appropriate immune response to clear the pathogen. Antimicrobial peptides form a large part of the innate arm of the immune response in vertebrate and invertebrate species. In humans, these peptides, together with antimicrobial proteins, complement pathways, and small bactericidal molecules such as hydrogen peroxide, form the first line of defense against pathogens. Investigation of therapies that involve supplementation with peptides or targeted stimulation of release of these molecules should be pursued; indeed, as discussed by Zasloff in Chapter 8 of this volume, topical peptide preparations are likely to be employed for treatment of localized infections. There may be a distinct benefit to utilizing peptides from both mammalian and nonmammalian sources for these endeavors in view of our relative lack of knowledge about how these peptides affect the inflammatory response and adaptive immunity. Clearly, workers in the field are carefully weighing the possibility of

negatively impacting an endogenous immune response by improper manipulation.

The genomics revolution may enhance our ability to assess the functional levels of antimicrobial peptides, particularly where chronic susceptibility to infection is seen. Analyses of the relevant genes could identify alterations pinpointing loss of ability to produce specific peptides, which might then be supplied exogenously. Other nucleotide sequence polymorphisms found in transcriptional regulatory regions might indicate significant up- or downregulation of individual peptides. Similarly, array-based analyses of transcript abundance can be made in readily available tissues such as blood. The transcripts measured should be not only those for peptide precursors, but also transcripts encoding peptide modification and processing functions, export, and granule formation. High-throughput measurements, coupled with sensitive antibody-based detection of peptides from fluids overlying epithelial tissues, have the potential to provide a comprehensive assessment of the contribution that peptides can make to innate defenses in individual patients. Such measurements will depend on sustained research on the genetics of peptide formation, together with continued development of analytic techniques.

Vector-borne disease has been a traditional scourge of the developing world, and is now being recognized as a significant threat to human and animal health in economically advanced societies. Arthropod-borne pathogens can spread rapidly by epizootic routes, as shown recently for West Nile virus in New York State and elsewhere in the northeastern United States. Also, the rapid movement of people and goods across international boundaries has brought diseases (and their vectors) formerly thought to be confined to tropical areas to more temperate regions. One approach, discussed by Hoffmann et al. (3), may be to intervene in the innate immune system of mosquitoes, with a view toward reduction of parasite survival in the vector. Significant advances in public health thus may become possible by a thorough understanding not only of human innate immunity, but also of comparable systems in our distantly related cousins, the invertebrates.

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Chemistry and Applications of Synthetic Antimicrobial Peptides

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I. INTRODUCTION

Synthetic methods of peptide chemistry have played a significant role in the spectacular development of antimicrobial peptide research over the last two decades. Interest in antibiotic peptides coincided with—and benefited from—a period when synthetic peptide chemistry had achieved an unprecedented level of proficiency. In his 1984 Nobel lecture (1), Bruce Merrifield referred to the synthetic work on cecropins currently being done at his laboratory as an example of the achievements in solid phase synthetic methodology. Although many substantial discoveries in antimicrobial peptide research have been possible only through molecular biology methods, as the chapters in this volume illustrate, it is no less true that many of those findings could be confirmed and developed further only by ready access to synthetic versions and analogs of the original antimicrobial sequences. In this chapter we will briefly consider the basic features of synthetic chemistry pertinent to antimicrobial peptides and then review some of its applications to areas such as structure confirmation, validation of putative

antimicrobial sequences, design of analogs to study structure–activity relationships and mechanisms of action, or de novo design.

II. SYNTHETIC METHODS

As noted above, advances in peptide synthesis methodology in the 1970s and 1980s were instrumental in the rapid development of antimicrobial peptide research. The relative structural simplicity of most antimicrobial peptides described up to then made them rather suitable targets for stepwise solid phase synthesis, a methodology invented by Merrifield (2) that revolutionized peptide chemistry and was continuously refined during that period.

The basic principles of stepwise solid phase synthesis are outlined in Figure 1. Extensive reviews (3–9), as well as some very recent textbooks [10-12], deal in more detail than this chapter will allow with the key chemical aspects of the methodology, namely, *protection* and *activation*. Protection strategies are meant to provide the necessary chemo- and regioselectivity for building a particular peptide sequence in a well-defined, straightforward way. Activation refers to the coupling chemistry required to ensure quantitative formation of every peptide bond in the sequence, a strict requirement in any stepwise synthetic process.

The two protection schemes that have come to enjoy general acceptance over the last several years are usually referred to by the names of their respective *N*-protecting groups: *tert*–butyloxycarbonyl (Boc) and *N*-(9-fluorenylmethoxycarbonyl) (Fmoc) (Figs. 2 and 3, respectively). In Boc chemistry (4), the original Merrifield proposal, the necessary chemoselectivity between the temporary (*N*-terminal) protecting group, on the one hand, and the permanent (side chain) protecting groups and the anchoring to the solid support, on the other hand, relies on graduated differences in acid stability. Temporary protection at the *N* terminus is provided by the Boc urethane group, which is labile to trifluoroacetic acid in dichloromethane. Benzyl-type (ester, amide) anchorings and side chain protections (ester, ether, urethane) remain essentially unaltered under these conditions, thus providing permanent protection throughout the buildup of the sequence. Once the synthesis
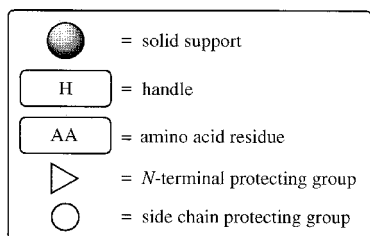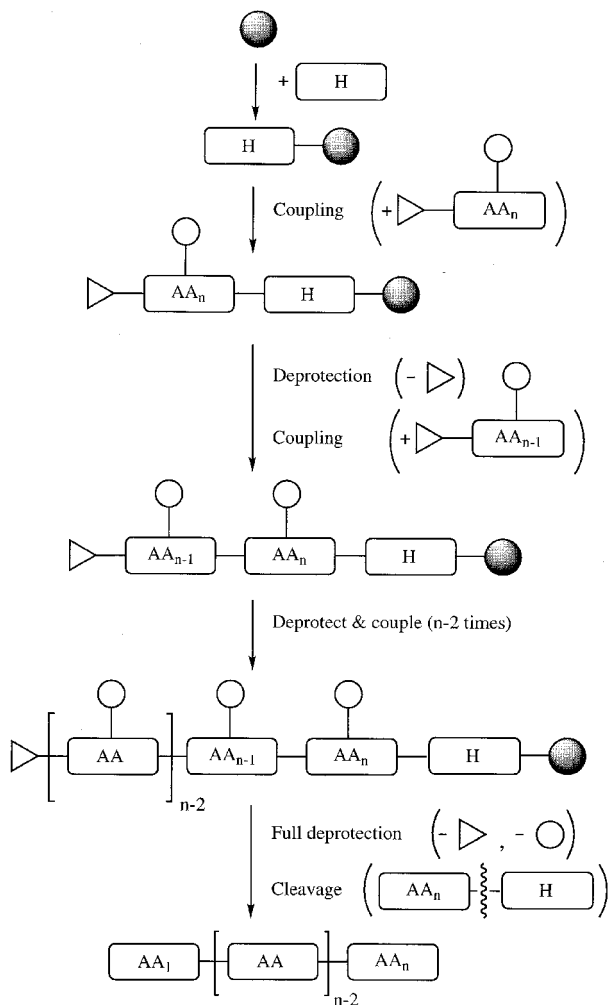
Figure 1 General scheme of solid phase peptide synthesis. The C-terminus of the growing peptide chain is attached to the resin support through a bifunctional handle. Stepwise chain elongation takes place by repetitive steps of deprotection and coupling until the desired sequence is assembled. The protected peptide-resin is then fully deprotected and cleaved from the solid support to give the free peptide.
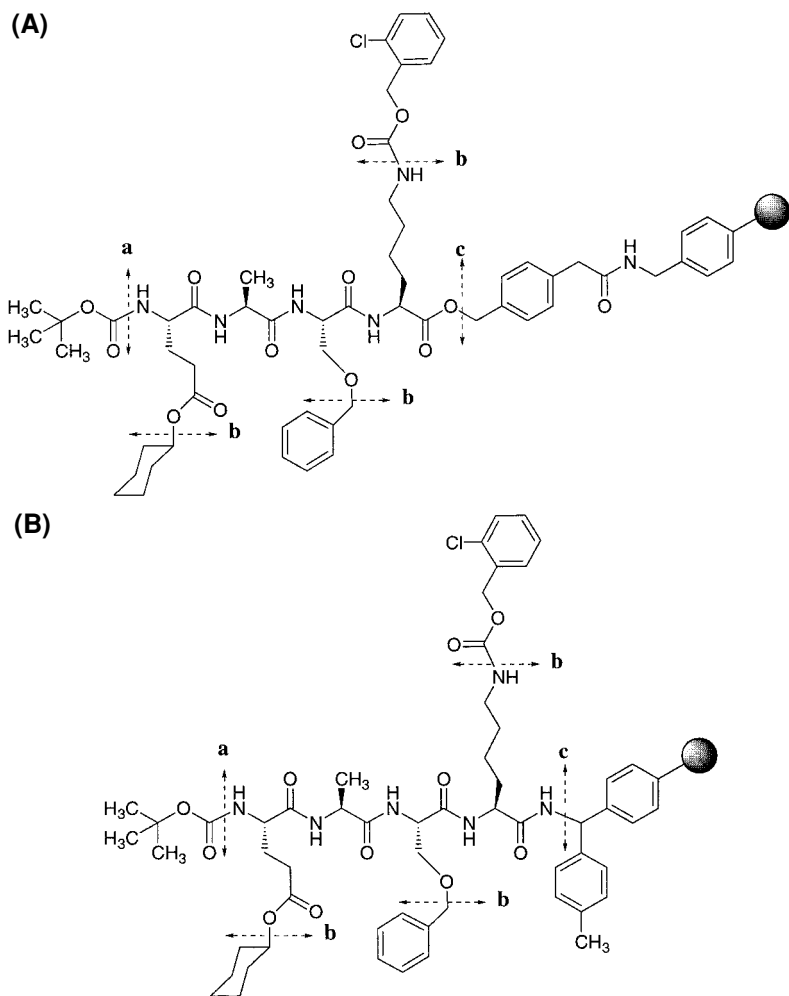
Figure 2 The solid phase synthesis of a Glu-Ala-Ser-Lys sequence illustrates the main features of the Boc/benzyl protection scheme. The Boc group serves as temporary protection of the N^α group, removable by trifluoroacetic acid in dichloromethane (a). The side chains of the three trifunctional residues bear permanent protecting groups: cyclohexyl for Glu, benzyl for Ser, and 2–chlorobenzyloxycarbonyl for Lys. These groups are stable to the repetitive N^α deprotection cycles and must be removed by strong acidolysis [e.g., anhydrous hydrogen fluoride (HF)] at the end of the synthesis (b). Anchoring to the polymer support takes place through (A) p-acetamido benzyl ester or (B) 4′-methylbenzhydryl amide linkages that are cleaved by HF (c) to give C-terminal carboxyl or carboxamide funtions, respectively.

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(A)

(B)
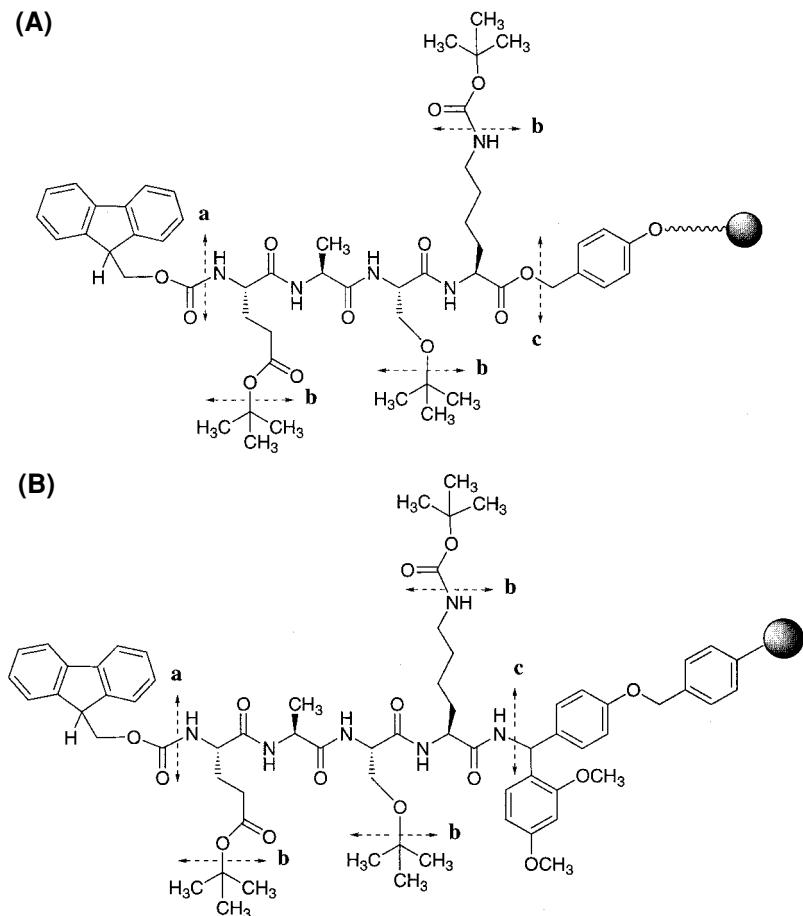
Figure 3 Synthesis of the Glu-Ala-Ser-Lys sequence is representative of the Fmoc/*t*-butyl protection scheme. Temporary N$^\alpha$ protection is provided by the Fmoc group, removable by piperidine (A). Permanent protection of the side chains of Glu, Ser and Lys is achieved respectively by *t*-butyl ester, ether, and urethane groups, all removable with TFA (b) at the end of the synthesis. Anchoring to the polymer support takes place through (A) *p*-alkoxy benzyl ester or (B) methoxy-substituted benzhydryl amide linkages that are cleaved by TFA (c) to give C-terminal carboxyl or carboxamide funtions, respectively.

is completed, the peptide is cleaved from the support and simultaneously deprotected at the side chains under strong acidolysis conditions, e.g., anhydrous hydrogen fluoride. The robustness, reliability, ease of automation, and low costs of Boc chemistry made it for many years the

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main choice in many laboratories. In the early years of antimicrobial peptide research, most synthetic versions of newly described structures and their analogs were prepared by this type of chemistry (Table 1).

The alternative Fmoc protection scheme has gained increased popularity in many laboratories in recent years (45). This strategy (Fig. 3) provides a higher degree of chemoselectivity (also refered to as orthogonality) than Boc chemistry, the *N*-terminal protecting group being removable under basic conditions (piperidine,1,8-diazabicyclo[5.4.0]undec-7-ene [DBU]) that leave acid-labile side chain protections unaltered. Anchoring to the resin is also done through acid-labile handles that can be fine-tuned to allow, for instance, the preparation of protected peptide segments (46,47), or peptides carrying glycosylated (Table 1, entries 21 and 22; Fig. 4), phosphorylated, or sulfated residues (48). This higher

Table 1 Representative Syntheses of Antimicrobial Peptides

Entry no.	Peptide	Synthetic chemistry	Disulfides	Glycan units	Reference
1	Cecropins	Boc	—	—	13–15
2	Melittin	Boc	—	—	16, 17
3	PGLa	Boc	—	—	18
4	Magainins	Boc	—	—	19, 20
5	Dermaseptins	Fmoc	—	—	21, 22
6	Indolicidin	Fmoc	—	—	23, 24
7	FALL-39	Boc	—	—	25
8	PR-39	Boc	—	—	26
9	Histatin 5	Fmoc	—	—	27
10	Bactenecin	Boc	1	—	28
11	Mesentericin Y 105[37]	Fmoc	1	—	29
12	Protegrins	Fmoc	2	—	30, 31
13	Tachyplesin	Fmoc	2	—	32
14	Androctonin	Fmoc	2	—	33
15	Defensins	Boc, Fmoc	3	—	34–36
16	MBP-1	Fmoc	3	—	37
17	Circulins	Boc	3	—	38, 39
18	Cyclic protegrin	Boc	1	—	40
19	Cyclic tachyplesin	Boc	2	—	41
20	Cyclic defensin (cNP-1)	Boc	3	—	42
21	Drosocin	Fmoc	—	2	43
22	Pyrrhocoricin	Fmoc	—	2	44
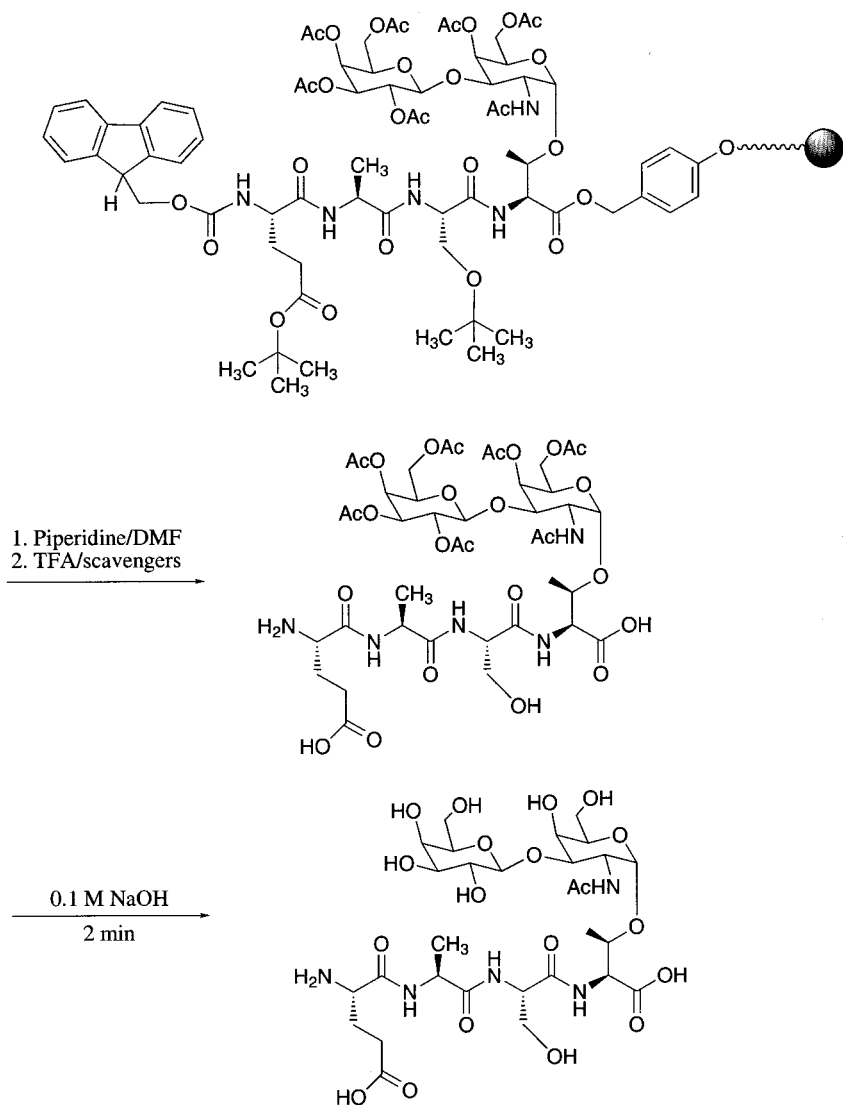
Figure 4 Solid phase Fmoc glycopeptide synthesis is illustrated by a Glu-Ala-Ser-Thr sequence glycosylated at the Thr with Gal-1→3β-GalNac. The synthesis relies on a Thr derivative with two protected glycan units that is fully compatible with conventional Fmoc synthetic chemistry. To obtain the glycopeptide, the regular deprotection steps are followed by an additional treatment with base that removes the acetyl groups from the glycan units. (Adapted from Refs. 43 and 44.)

(A)
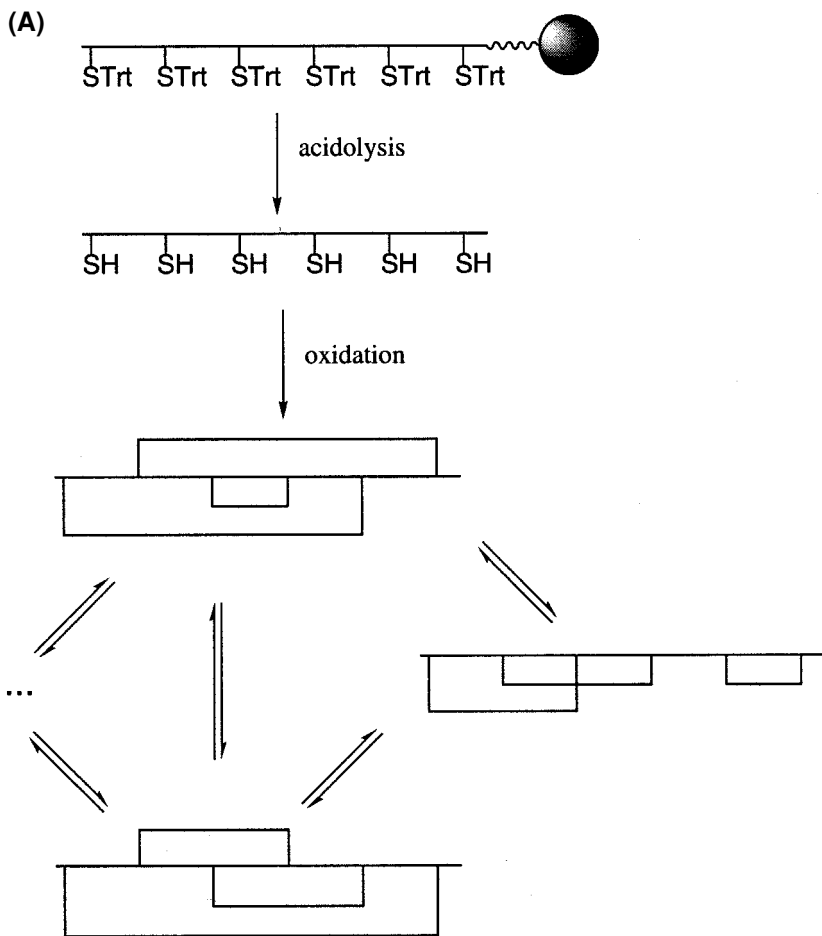
Figure 5   Two synthetic approaches to a triple disulfide peptide (e.g., defensin). In strategy A, the same protecting group is used for all six Cys residues. The hexathiol obtained after acidolysis is folded at high dilution under oxidizing conditions that favor thiol-disulfide exchange (e.g., glutathion in reduced and oxidized forms). Success requires the thermodynamically predominant species to correspond to the native folding. In strategy B, selective protection of Cys residues allows the desired cystine pairings to be formed one at a time by using an appropriate sequence of deprotection and oxidation conditions. (Adapted from Refs. 35 and 37 (A) and 36 (B), respectively. For further details, see Refs. 49–53.)
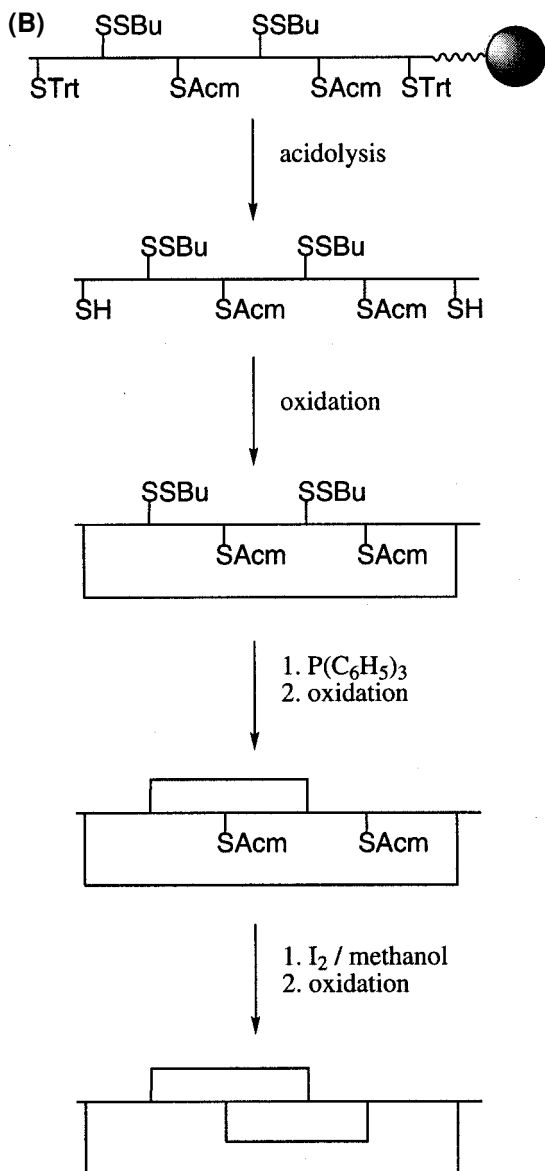
Figure 5 Continued

flexibility in the synthetic design, as well as the avoidance of hazardous hydrogen fluoride as cleavage-deprotection reagent, have helped to make Fmoc chemistry a very attractive option. This trend is also reflected in the growing number of antimicrobial peptides prepared by this approach in recent years (Table 1).

The earliest accounts (up to ca. 1990) of synthetic antimicrobial peptides dealt primarily with linear structures, in agreement with the majority of sequences described thus far. However, starting with the defensins, a growing number of active structures folded around one or several intramolecular disulfide bridges were discovered and a demand for the synthesis of such structures ensued. Although disulfide-linked peptides still pose a substantial challenge to peptide chemists, synthetic approaches developed in recent years (reviewed in refs. 49–53) have also been applied with relative success to antimicrobial peptide targets with single or multiple internal disulfides (Table 1). The most favored approach for multiple disulfide peptides involves oxidation of the corresponding polythiol precursor under conditions that allow equilibration among different cysteine pairings (Fig. 5A). If the native form of the target peptide is thermodynamically favored, this is the most simple and promising approach. Alternatively, disulfide bonds may be formed sequentially, by judicious choice of cysteine protecting groups and deprotection/oxidation conditions (Fig. 5B). This strategy is much more chemically demanding but can be advantageous whenever the target peptide does not fold spontaneously into the correct disulfide pattern.

In recent years, the development of methods (*chemical* or *native ligation*) that make possible the assembly of large, complex peptide molecules in solution from unprotected segments (54–56) has opened new prospects for the production of proteins by chemical synthesis, with all its associated possibilities, such as specific labeling, use of noncoded amino acids, avoidance of expression problems, etc. (reviewed in ref. 57). Specific chemistries have been developed for these purposes, largely relying on classical Boc protection schemes, which provide the most straightforward routes to the relevant precursors. This novel, complex, but extremely powerful synthetic chemistry has been successfully applied by the group of Tam to prepare unusual cyclic antimicrobial peptides such as the circulins (Fig. 6), as well as circularized (head-to-tail) versions of natural peptides (entries 18–21, Table 1).
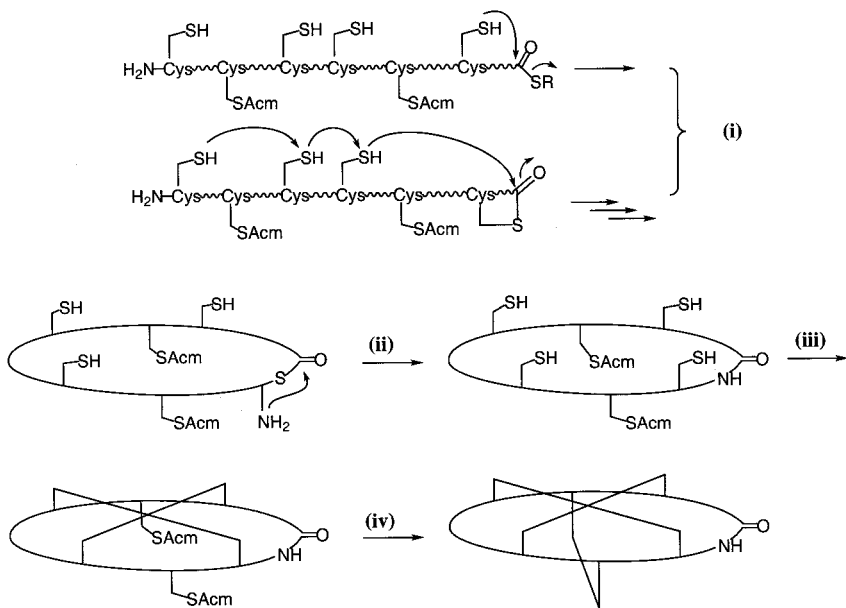
Figure 6 Synthesis of a complex cyclic peptide with a cystine knot pattern (e.g., circulin) requires a precursor with an N-terminal Cys residue and a C-terminal thioester group. This precursor undergoes intramolecular transthioesterifications by the free thiol groups of the various Cys residues (i), leading to thiolactones of different ring sizes (thia-zip cyclization). Eventually, the largest thiolactone rearranges irreversibly to a head-to-tail cyclic peptide by intramolecular aminolysis (ii). DMSO-promoted oxidation (iii) followed by deprotection-oxidation of the remaining Cys pair (iv) leads to the target tetracyclic peptide. The reaction conditions in step iii can be adjusted to favor formation of one of the three possible cystine pairings. (Adapted from Refs. 38 and 39.)

III. APPLICATIONS OF SYNTHETIC ANTIMICROBIAL PEPTIDES

The synthetic methodologies briefly outlined in the preceding section have been applied to almost every known type of antimicrobial peptide, with a view to confirming their structure, unraveling their mechanisms of action, enhancing their activity or bioavailability, or simply

obtaining practical amounts of material for basic or clinical microbiological studies. The literature covering such endeavors has grown so spectacularly over the last two decades that it is virtually impossible to review in detail. Hence, rather than an exhaustive (and possibly rather tedious) enumeration, a selection is provided in Table 2, with representative examples arranged according to the main directions of application.

Confirmation of structure, a classical goal of the synthesis of natural products, was the earliest area where synthetic versions of antimicrobial peptides were useful. For instance, synthesis served to complete the structural elucidation of cecropins A and B (13,14) by verifying the presence of a *C*-terminal carboxamide in the native forms. In other instances, particularly in the cathelicidin family (58–61), the synthetic peptides have been useful in confirming putative sequences deduced from cDNA or genomic data and in providing more conclusive evidence of their expression and function. A somewhat related approach has been the study of the posttranslational processing of cecropins A and B (62) and dermaseptin b [63], both carried out directly on synthetic preparations of the full precursors.

The role of intramolecular disulfide bridges in antimicrobial peptides has also been explored by means of synthetic peptides. Like other posttranslational modifications, disulfide bridges do not have a unique function, and in some instances they display opposite effects, depending on the peptide and activity assayed. Total or partial loss of activity upon disulfide reduction or Cys replacement is the somewhat anticipated finding in a number of cases, such as CAP37 (64) or NK-lysin (125). In contrast, bactenecin requires intact disulfide bonds for full activity against gram-negative bacteria such as *Escherichia*, *Pseudomonas*, or *Salmonella* (65), but retains activity toward mutants of the first two bacteria defective in the outer membrane, and even appears to expand its spectrum to gram-positive microorganisms such as *Staphylococcus epidermidis* or *Enterococcus faecalis* when in reduced form or with Ser for Cys replacements (for an earlier, partly conflicting result, see ref. 124). In the protegrins, the ability to alter membrane permeability is lost upon reduction of the two disulfides (66,67), but a substantial level of antimicrobial activity is still observed for either the fully reduced or the S-protected (tetra-acetamidomethyl) forms (66). A

Table 2 Representative Applications of Synthetic Antimicrobial Peptides and Their Analogs

Application	Peptides	Reference
A. Confirmation of bioactive structure		
Isolated from natural sources		
	Cecropins	13, 14
	Magainins	19
Inferred from gene sequence		
	LL-37	58
	CRAMP	59
	BMAP-34	60
	SMAP-29	61
B. Functionality of post-translational modifications		
Processing of precursor		
	Preprocecropins	62
	Dermaseptin b	63
	FALL-39/LL-37	25, 58
Role of disulfide bridges		
	CAP37	64
	Bactenecin	65
	Protegrins	29, 66, 67
	Tachyplesins	68, 69
Glycosylation		
	Lebocin	70
	Pyrrhocoricin	43
	Myrmecin	71
	Drosocin	44
	Diptericin	72
Amidation		
	Magainin	73, 74
	Protegrin	66, 67
Phosphorylation		
	Enkelytin	75
Native D-amino acids		
	Bombinin H	76
C. Structural optimization		
Amphipathicity, helical content,		
role of specific residues, size, etc.		
	Cecropins	77
	Magainins	20, 74, 78
	Melittin	79, 80
	Indolicidin	81–83
	Protegrin	67, 84
	Histatin	85

Table 2 Continued

		Thanatin	86
		Seminalplasmin fragment	87
	Chimeric peptides		
		Cecropin A/D	15
		Cecropin-melittin	88–95
		Cecropin-magainin	94–96
		BPI fragments	97
	Enantiomers: active		
		Cecropins and hybrids	89, 92, 98
		Magainin	89, 99
		Protegrin	67
		Thanatin	86
		HPF IV C-terminal peptide	100
		Lactoferricin fragments	101
	Enantiomers: inactive		
		Drosocin	43
		Apidaecin	102
	Topoisomers (retro and retroenantio)		
		Cecropin and hybrids	98, 103, 104
		Melittin and hybrids	105
	Diastereomers		
		Pardaxin	106, 107
		Melittin	118
		Magainin	109, 110
	Stabilization or disruption of helices		
		Cecropin and hybrids	111-113
		CAP18(106–125)	114
		Histatin	115
		Pardaxin	116
		Seminalplasmin fragment	117
D.	Miscellaneous modifications		
	Halogenated amino acids		
		Hagfish intestinal peptides	118
		Seminalplasmin fragment	119
	Acylation		
		Histatin	115
		Cathepsin G sequence	120
		Lactoferricin B fragment	121
	Protected peptide		
		Pardaxin	122
	Carrier- or polymer-bound peptide		
		BPI fragment	96
		Magainin	123

similar segregation of functions has been observed for the tachyplesins (68,69), in which the cyclic form is capable of translocating across lipid bilayers, while the reduced or protected forms cause disruption of the bilayer in a detergent-like fashion and both forms possess antimicrobial activity.

Glycosylation is a common feature in a group of proline-rich antibacterial peptides from insects (reviewed in ref. 126; see also ref. 127). Peptides with one (GalNAc), two (Gal→GalNAc), or three (Glc→Gal(GalNAc), glycan units linked α-glycosydically to a Thr hydroxyl group have been described. The solid phase synthesis of glycopeptides (see Fig. 4) of medium-large size with complex glycosyl substituents is not yet a strictly routine procedure in most laboratories, partly due to the fact that the corresponding glycosyl amino acid moieties are not readily available in appropriately protected form. This probably explains why only a few of these structures and some partial sequences (72) have been synthetically reproduced in glycosylated form (Table 1, entries 21 and 22). This synthetic work, along with the synthesis of nonglycosylated versions of other peptides in this family (70,71), allows one to conclude that integrity of the oligosaccharide chain is generally necessary for optimal antimicrobial activity. The only reported exception is pyrrhocoricin (44), which has been synthesized in nonglycosylated form and shown to be more potent than the natural glycopeptide. Another recent interesting finding is that the glycosydic bond between the Thr residue and the sugar moiety in α-anomeric configuration is not a strict requirement for activity: the sugar units can be attached to the peptide chain through oxime linkages without significant change in antimicrobial activity (128,129). The nonglycosylated D-enantiomer of diptericin has been synthesized (43) and shown to be significantly less potent than the deglycosylated natural form, suggesting that membrane permeabilization may not be the main mechanism of action for this group of peptides.

The role of amidation, a common posttranslational modification of antimicrobial peptides (127), has also been explored in peptides such as magainin (73,74) and protegrin (66) by means of synthetic analogs. In both cases, an increase in antimicrobial potency is observed for the carboxamide versus the carboxyl form. Assuming that these peptides adopt helical conformations in their bioactive states, the

enhanced activity could be associated with an increased α-helix macrodipole, as well as the additional possibilities for hydrogen bonding and thus helix formation provided by the carboxamide. Similar results have been observed for other peptide families, e.g., the dermaseptins (130). For other peptides, however, the carboxyl form is as potent as the amide or even more potent. Thus, increased activity of protegrin acid over the naturally occurring amide has been described against *Chlamydia tracomatis* (67) (though for many other susceptible organisms the opposite effect has been reported [ref. 66]). For clavanin A, the acid form is significantly more potent than the amide (131).

Synthetic chemistry has also contributed to elucidate the role of other, less frequent posttranslational modifications such as phosphorylation or the presence of D-amino acids in native structures. The first case is well illustrated by enkelytin, an antimicrobial peptide derived from proenkephalin that is phosphorylated at two Ser residues (132). Both phosphorylated and nonphosphorylated forms of enkelytin have been synthesized (74), and a marked drop in potency has been found for the latter version. Furthermore, the synthetic phosphopeptide has been found to adopt several conformations (associated to cis-trans proline isomerization), only a fraction of which reproduce the activity of the native form.

The occurrence of D-amino acids in animal peptides is a relatively recent finding. It was first observed in the dermorphins and deltorphins, opioid peptides isolated from the skin of the *Phyllomedusa* amphibian species and later on in neurohormonal peptides from crustaceans and molluscs (133,134). Chemical synthesis proved to be very valuable in the confirmation of this relatively elusive modification, which has also been identified in bombinin H (76), one of the antimicrobial peptides from the frog, *Bombina variegata*.

Section C of Table 2 deals with a rather abundant and heterogeneous group of synthetic analogs of natural antimicrobial peptides designed to improve the effectiveness of the native forms by one or more criteria, e.g., broader spectrum, higher activity, lower cytotoxicity. Most of these analogs can also provide valuable insights into the mechanism of action of the parent peptides. Their design is usually guided by physicochemical considerations such as helical content, amphipathicity, charge distribution, etc., which can be estimated a

priori by criteria such as helical wheel plots (135), hydrophobic moment profiles (136), or secondary structure prediction algorithms. The process is usually interactive, with experimental validation of the design provided by conformational [circular dichroism (CD), nuclear magnetic resonance (NMR)], biophysical (membrane), and microbiological studies. A detailed discussion of the principles that underlie this design process is beyond the scope of this chapter. In addition to the examples from Table 2 discussed in the following paragraphs, the reader is directed to several reviews in a recent dedicated issue (137–141) that provide a thorough treatment of the subject.

A particularly fruitful idea in the design of improved analogs of natural antimicrobial peptides has been the hybridization between peptide sequences with complementary structures and/or activities. The sequence hybridization concept, which had already been useful in the design of analogs of peptide hormones (142), was first applied to antibacterial peptides by Fink et al. (15), who designed a series of cecropin A/D hybrids with improved activity over either parent molecule. The next step came also from collaborative work between the Merrifield and Boman laboratories, leading to cecropin A–melittin hybrids (88,89,98). These chimeric peptides had the *N*-terminal region of cecropin A (hydrophilic) followed by the corresponding region of melittin (hydrophobic), so that the overall hydrophilic-hydrophobic pattern resembled that of cecropin A. The most potent hybrids were cecropin A(1–13)melittin(1–13) [CA(1–13)M(1–13)] and cecropin A(1–8)melittin(1–18) [CA(1–8)-M(1–18)]. These 26-residue peptides were two-thirds the size of cecropin A, had a better antibiotic spectrum and greater potency than the two parent molecules, and lacked the undesirable cytolytic effect of melittin. A reverse hybridization pattern, with a hydrophobic *N* terminus followed by a hydrophilic region, did not lead to productive analogs (143). Over the last decade, CA(1–8)M(1–18) has become one of the most widely studied nonnatural peptide antibiotics, including work in our laboratories on its permeabilization (144,145), macrophage triggering (147), and leishmanicidal (148) properties. Another group has taken CA(1–8)M(1–18) (renamed CEME) as the lead compound to develop a group of analogs that shed light on the mechanism of action of this type of peptides (93). We have also shown that further size reduction of the CA(1–8)M(1–18) structure

down to 15-residue (90) or even 12-residue (91) analogs preserves antibacterial activity. Several of these short hybrids have been used in biophysical studies on membrane activity (146), conformation, and self-association (149,150). Their efficacy against several fungal plant pathogens (151) and in an in vivo ocular infection model (152) has also been demonstrated. Parallel approaches with cecropin–magainin hybrids (94–96) or with bactericidal permeability/increasing protein (BPI) fragments (97) have been described, with similar results.

A substantial contribution to the understanding of the mechanism of action of many antibacterial peptides came from the intuition that the interaction between the peptide and the bacterial membrane might not be stereospecific (i.e., involve chiral receptors or enzymes) and thus that a synthetic D-enantiomer of the peptide would be as active [98] as its natural counterpart. This supposition was first confirmed in 1990 for cecropin, melittin, and their hybrids (89,92), as well as for magainin (89,99): the D-enantiomers were equipotent with the natural forms against a representative panel of microorganisms and, additionally, resistant to proteolysis. These findings were later substantiated for other antimicrobial structures such as protegrin (67), lactoferricin fragments (100), and the human platelet factor IV *C*-terminal peptides (101). In the latter case, an in vivo model showed that the D-enantiomers act synergistically with low levels of β-lactams in protecting mice against infection. For D-thanatin (86), a mixed activity profile has been described: the D-enantiomer is active against fungi and gram-positive, but not gram-negative, bacteria. In this way, the lack of activity of the D-enantiomer becomes substantial evidence that the peptide exerts its activity through mechanisms other than membrane permeabilization, as is the case with drosocin (43), apidaecin (102), or PR-39 (153).

A further step in understanding the relationship between the topology and antimicrobial activity of linear cationic peptides came from the study of their retro (inverted sequence) and retroenantio (inverted sequence and all-D amino acids) versions, also known as topoisomers (98,103). For both topoisomers of CA(1–7)M(2–9), activity against *Escherichia coli*, *Bacillus megaterium*, *Micrococcus luteus*, and *Streptococcus pyogenes* was undistinguishable from the parent compound, while for *Pseudomonas aeruginosa* and *Staphylococcus aureus* a certain decrease in activity was found. From these and further studies (104), including those involving melittin topoisomers (105), it

has been possible to conclude that sequence composition or helix dipole (CO→NH vs. NH→CO direction of peptide bonds), individually but not together, and not chirality, are important requirements for activity.

Single point mutations with the corresponding D-amino acid at selected positions of a largely helical peptide produce diastereomers with considerable disruption of the integrity of the helix. Despite such loss in helicity, diastereomer analogs of pardaxin (a fish cytotoxic peptide) (106,107) or melittin (108) retain the potential to cause substantial destabilization of the membrane by a carpet-like mechanism (see Chapter 5). These somewhat controversial results question the generally accepted concept of an amphipathic helix as a requirement for activity and offer new hints for the design of membrane-active sequences, including some where antimicrobial and cytolytic activities can be segregated.

An even more pronounced destabilization of a local helical conformation can be achieved by double replacement of adjacent amino acids by their D-enantiomers. This method was first used to determine which regions of neuropeptide Y were interacting with membrane phospholipids (154) and then was applied to the magainins (109,110). The results again indicate that the amphipathic helix is important only for action on neutral lipid bilayers and is less crucial for permeabilization of negatively charged (i.e., bacteria-like) membranes. These studies on peptides with single or double replacements offer new insights into the relevance of the helicoid form for peptide binding to and permeabilization of lipid membranes. Their implications for antimicrobial peptide design will no doubt soon become evident.

Alternative approaches used to probe the role of helical regions in antimicrobial activity have involved substitution by Pro, causing helix disruption (111,114), or replacement of Pro (helix stabilization) (112) at strategic positions. On the other hand, replacement of the single Pro residue in the amphibian peptide buforin disrupts the hinge at the middle of the sequence and causes loss of activity (155,156), as previously found for cecropin A (112). Attempts have also been made to induce/stabilize a local helical conformation by introducing mobility restrictions such as a side chain lactam (113) or a disulfide between the N and C termini (117), or by optimizing electrostatic interactions between side chains (116).

Section D of Table 2 groups assorted examples of how synthetic methods have dealt with several other issues in antimicrobial

peptide research. These include the potential role of halogenated residues (118,119), the influence of N-terminal blocking with acetyl or fatty acid units (115,120,121), and the activity of peptide sequences in unusual forms of presentation such as side chain–protected (122), bound to carrier proteins (96), or bound to insoluble polymer supports (122). The majority of these applications remain to be explored in further detail.

IV.   NEW APPROACHES TO SYNTHETIC ANTIMICROBIAL PEPTIDES: DE NOVO DESIGN AND COMBINATORIAL PEPTIDE LIBRARIES

As the preceding section has shown, the activity of antimicrobial peptides, particularly those belonging in the linear, helical group of the original classification (157), can be reasonably well understood on the basis of a few structural parameters such as positive charge distribution, helical content, hydrophobic moment, amphipathicity, etc. (137–141). This realization underlies the next logical step, which is the development of purely artificial antimicrobial peptides designed according to those physicochemical principles and with minimal or no reference to natural structures. This goal was set in the early days of antimicrobial peptide research, mainly on the basis of the work of Kaiser and coworkers in de novo design of peptides that bound to biological interfaces (158–161). The secondary structural considerations guiding those approaches were first applied to melittin (16), extended by De Grado and co-workers to model ion channels (162), and generalized to a number of other structures (163,164).

  In the literature on antimicrobial peptides, the boundary between purely de novo design and what we have called structural optimization (Table 2) of natural structures is often blurred. A necessarily concise selection of examples should include, among the former category, the early (precombinatorial) work by Houghten's group (165). In their design, the amphipathic α-helix was modeled by a simple combination of only Leu and Lys residues arrayed in appropriate periodicity. The resulting peptides displayed quite potent activities but had substantial cytolytic effects. This fairly simple approach has subsequently been pursued in other laboratories, with comparable results (166–168). Alternative approaches have relied more on homology considerations,

defining sequence templates from which α-helical (169,170) or even β-strand (171) active structures can be derived.

In recent years, the development of combinatorial chemistry (172,173) has had a significant impact on the process of drug discovery. Not surprisingly, one of the areas where the combinatorial search for active structures produced incipient rewards was precisely the antimicrobial peptide field (174–176). A number of active sequences, ranging from 4 to 18 residues, have been since reported by the Houghten group (177) and by other laboratories (178,179). The subject has been exhaustively covered in a recent review (180).

This section would be incomplete without noting the excellent antimicrobial data very recently reported by the De Grado (181) and Gellman (182) groups for de novo-designed β-peptides. These oligomers with unnatural backbone fold into predictable helical conformations (183–185) and thus an amphipathic helical pattern can be built by appropriate choice of β-amino acid residues. The protease-resistant β-peptide backbone makes this new class of peptides very promising antibiotic candidates (186).

In view of the growing number of active antimicrobial sequences unraveled by either rational design or combinatorial search, one would expect that some of them might eventually be developed into therapeutically useful drugs. At this time, all ongoing trials of antimicrobial peptides that we are aware of involve only molecules closely related to natural structures (see the next section). The reasons for this may have to do with the cost of synthesis (see the next section). In any event, one may conclude that the two methodologies outlined above have the potential to identify a wealth of candidate antimicrobial molecules for further development.

V. SYNTHETIC ANTIMICROBIAL PEPTIDES AS DRUG CANDIDATES

It is becoming increasingly obvious that antimicrobial peptides are one of not too many potential lines of defense against the now widespread occurrence of resistance to conventional antibiotics. Many of the desirable properties that would be sought in a new class of antibiotic compounds are actually realized in these peptides: broad spectrum, fast kinetics, scarce—if any—induction of resistant strains,

demonstrable in vivo activity, and synergy with other antimicrobial agents. These advantages notwithstanding, progress in the therapeutic application of these peptides is slower than was originally expected. After the nonapproval by the Food and Drug Administration of Locilex® (Magainin Pharmaceuticals, Plymouth Meeting, PA), a topical cream for diabetic foot ulcers based on the magainin analog Pexiganan®, only two clinical trials of antimicrobial peptides are currently underway. One is for MBI-226, a peptide developed by Micrologix Biotech (Vancouver, Canada) for treating catheters to prevent bacterial biofilm formation. The other is IB-367, a protegrin analog (Intrabiotics, Inc., Mountain View, CA) targeted for oral mucositis in cancer patients. Several reasons may explain the relative paucity of clinical applications for antimicrobial peptides: (a) The cost of the peptides on a molar basis is about tenfold that of classical antibiotics; hence production by recombinant techniques is a likely prospect—not yet fully realized—for peptides composed exclusively of natural amino acids. (b) For peptides acting on membranes, an increase in affinity is always attained at the risk of a concurrent loss of specificity; hence some kind of trade-off is often required. (c) Most peptides are extremely susceptible to proteolytic degradation, which must be minimized by modification or replacement of particular amino acids, by encapsulation, or by use of all-D enantiomers. In the latter case, issues such as metabolic clearance pathways and potential toxicity remain to be explored. (d) In addition to proteolytic degradation, low tissue penetrability has so far limited clinical applications to topical use or bloodstream infections; the possibility of coupling active antimicrobial sequences to penetration enhancers or other translocating peptides (187) is a potential solution that deserves consideration.

ACKNOWLEDGMENTS

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3
Lanthionine-Containing Bacterial Peptides

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I. INTRODUCTION

Lantibiotics constitute a group of bacteriocin-like antimicrobial peptides exclusively produced by, and mainly active against, gram-positive bacteria. The unique structural feature of lantibiotics is the presence of the amino acids lanthionine and β-methyllanthionine, which form characteristic intrachain ring structures (1). In contrast to classical peptide antibiotics such as gramicidin S or valinomycin, which result from the activity of multienzyme complexes (2–4), lantibiotics arise from ribosomally synthesized precursor peptides by posttranslational modifications. On the basis of their structures, lantibiotics are currently divided into two major groups: the elongated amphiphilic type A lantibiotics with pore-forming activity and the globular, enzyme-inhibitory type B lantibiotics (5).

In this chapter we will summarize the biosynthesis and genetics of lantibiotics, focusing on nisin, epidermin, and mersacidin. These peptides are the best-studied examples and have either already found extensive applications or have excellent potential for future use in biomedical or agricultural industries.

II. PARTICULAR STRUCTURAL FEATURES OF LANTIBIOTICS

Lantibiotics are ribosomally synthesized and posttranslationally modified antibiotic peptides that contain intramolecular rings formed by the thioether amino acids lanthionine (Lan) and 3-methyllanthionine (MeLan). Additionally, a variety of other modified nonprotein amino acids, including 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb), S-aminovinyl-D-cysteine and S-aminovinyl-D-methylcysteine, lysinoalanine, hydroxyaspartic acid, D-alanine, 2-oxobutyrate and hydroxypyruvate, have been identified in various lantibiotics. The key reaction that leads to the majority of these residues is the dehydration of hydroxy amino acids, generating the dehydro residues to which suitably positioned sulfhydryl or amino groups may be added (5–7).

The presence of the modified residues, in particular the dehydroamino acids, poses special challenges for the structural elucidation of lantibiotics. Dehydroamino acids, located at the N terminus of lantibiotics, or when becoming N-terminally exposed during Edman degradation, are unstable and spontaneously deaminate, blocking further sequencing reactions (5). Meyer et al. (8) demonstrated that treatment of the peptides with thiol compounds removes the N-terminal and internal blocking groups, thus allowing direct sequencing of dehydroamino acids and thioethers. The bridging pattern can be determined by two-dimensional nuclear magnetic resonance (2D NMR) techniques; in the case of natural variants of peptides with known structures, it may be partially deduced from the gene sequence.

Jung and Sahl (5) defined two major subgroups, the type A and the type B lantibiotics, based on structural and functional aspects. The type A lantibiotics, e.g. nisin and epidermin (Fig. 1), are elongated amphiphilic peptides that kill susceptible cells by forming pores in the cytoplasmic membrane. Their molecular masses range from 2164 to 3764 Da, and the peptides either have no net charge or carry up to seven net positive charges. In contrast, the smaller type B lantibiotics (molecular mass 1835–2042 Da) such as mersacidin and actagardine (Fig. 1) possess a compact globular structure having either no net charge or a net negative charge. These peptides inhibit various enzyme

(A)

(B)

(C)   **(D)**
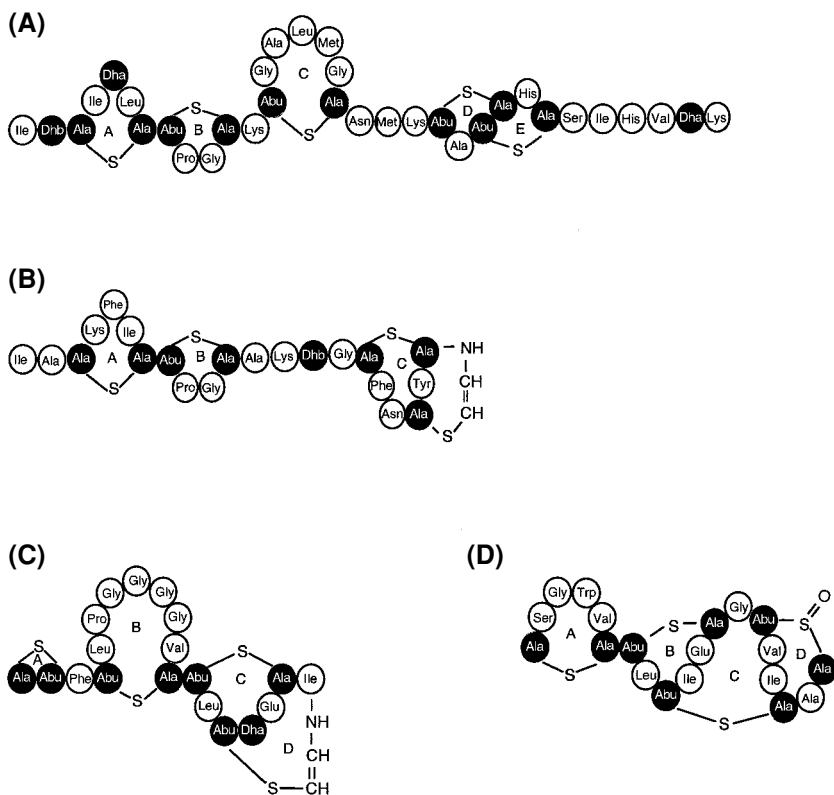
Figure 1 The primary structure of the type A lantibiotics nisin (A) and epidermin (B), and of the type B lantibiotics mersacidin (C) and actagardine (D). Amino acid residues involved in posttranslational modifications are highlighted by shading (see text for details).

functions by binding to membrane lipids. In recent years, a number of new lantibiotics with intermediate features have been identified, making classification more difficult.

Nisin, the most prominent lantibiotic, is produced by numerous *Lactococcus lactis* subsp. *lactis* strains. It was first described in 1928 (9) as a Lancefield group N streptococci inhibitory substance. As early as 1952 (10) it was shown to contain the amino acid Lan, but it was not until 1971 that Gross and Morell (1) were able to report the

complete primary structure. In addition to two Dha resides and one Dhb residue, the 3353-Da peptide contains a single Lan and four MeLan residues, resulting in five intramolecular ring structures. With the exception of the small rings, nisin is relatively unstructured in aqueous solution and has no preferred overall conformation. However, in lipophilic solvents such as trifluorethanol or dimethyl sulfoxide (DMSO), which mimic a membrane environment, nisin adopts an amphiphilic, elongated, corkscrew-like structure presenting hydrophilic and hydrophobic residues on opposite sides of the molecule (11–15). The amphiphilic properties of nisin are reported to be the basis for its biological activity.

The lantibiotic epidermin is produced by *Staphylococcus epidermidis* (16). The tetracyclic peptide has a mass of 2164 Da. It contains two Lan residues and a single MeLan residue. Its N-terminal region, including thioether rings A and B, is closely related to that of nisin, whereas the C-terminal region of this peptide differs and contains an *S*-aminovinyl-D-cysteine as the terminal ring structure residue; Fig. 1). Like nisin, gallidermin, a natural variant of epidermin ([Leu[6]]epidermin), adopts an extended, screw-shaped, amphiphilic structure in an appropriate solvent.

With a molecular mass of 1825 Da, mersacidin, a typical type B lantibiotic, is the smallest lantibiotic known so far. The hydrophobic peptide is produced by a *Bacillus* strain (*Bacillus* HIL Y-85,54728) and contains four rings formed by three MeLan and one C-terminal S-aminovinyl-D-methylcysteine residue (17). In contrast to nisin and epidermin, mersacidin has a defined globular conformation due to overlapping bridging structures (18).

III. BIOSYNTHESIS OF NISIN, EPIDERMIN AND MERSACIDIN

A. Biosynthetic Gene Clusters

The lantibiotic prepeptides, encoded by the *lanA* genes, consist of an N-terminal segment designated as the leader peptide (19) and a C-terminal propeptide part in which the Ser, Thr, and Cys residues are posttranslationally modified to Lan and MeLan. The structural genes

(*lanA*) and the genes necessary for modification (*lanB*, *lanC*, *lanM*, *lanD*) and proteolytic processing (lanP), as well as for accessory functions such as transport (*lanT*, *lanH*), producer self-protection (*lanI*, *lanFEG*), and regulation (*lanR*, *lanK*, *lanQ*), are organized in biosynthetic gene clusters (Fig. 2). The gene clusters usually consist of several transcription units and are localized on the bacterial chromosome or on mobile elements such as plasmids or transposons. Regarding the enzymes that take part in modifications, export, and processing, two different classes of lantibiotics can be distinguished. As shown in Table 1, class I lantibiotics are modified by two enzymes, LanB and LanC, processed by a serine protease, LanP, and exported by an ATP binding cassette (ABC) transporter, LanT. In contrast, class II lantibiotics possess a single modification enzyme, LanM, and an ABC transporter, LanT(P), with an additional N-terminal protease domain. The differences in the biosynthetic machinery are reflected in some typical features of the prepeptides. While the leader peptides of class I lantibiotics show a conserved FN/DLD motif and a cleavage site with Pro in position –2, the class II lantibiotics possess a characteristic "double glycine" cleavage site and contain several conserved Glu residues (7).

The nisin A biosynthetic gene cluster is encoded on the 70-kb conjugative transposable element Tn*5301* from *L. lactis* NCFB894 (20,21) or Tn*5276* from *L. lactis* NIZO R5 (22). The genetic information for the production of its natural variant nisin Z ([Asn^{27}]nisin) is located on the transposon Tn*5278* from *L. lactis* subsp. *lactis* N8 (23). Additionally, all these transposons contain the genes necessary for utilization of sucrose (*sacA*, *sacB*, and *sacR*). After specific integration in the chromosome of the recipient strains, the transposons are flanked on both ends by 5′-TTTTTG-3′ direct repeats (21, 22). The nisin A gene cluster consists of three transcription units, *nisABTCIP*, *nisRK*, and *nisFEG* (24), while in the nisin Z gene cluster two operons, *nisZBTCPRK*, and *nisFEG*, are found (25). A single *nisA* or *nisZ* transcript, respectively, can be detected; however, this mRNA seems to result from internal processing rather than from transcription termination (25,26). The single transcript for the structural gene and the polycistronic transcript are detected in equal amounts, indicating that the terminator structure localized upstream of the structural gene
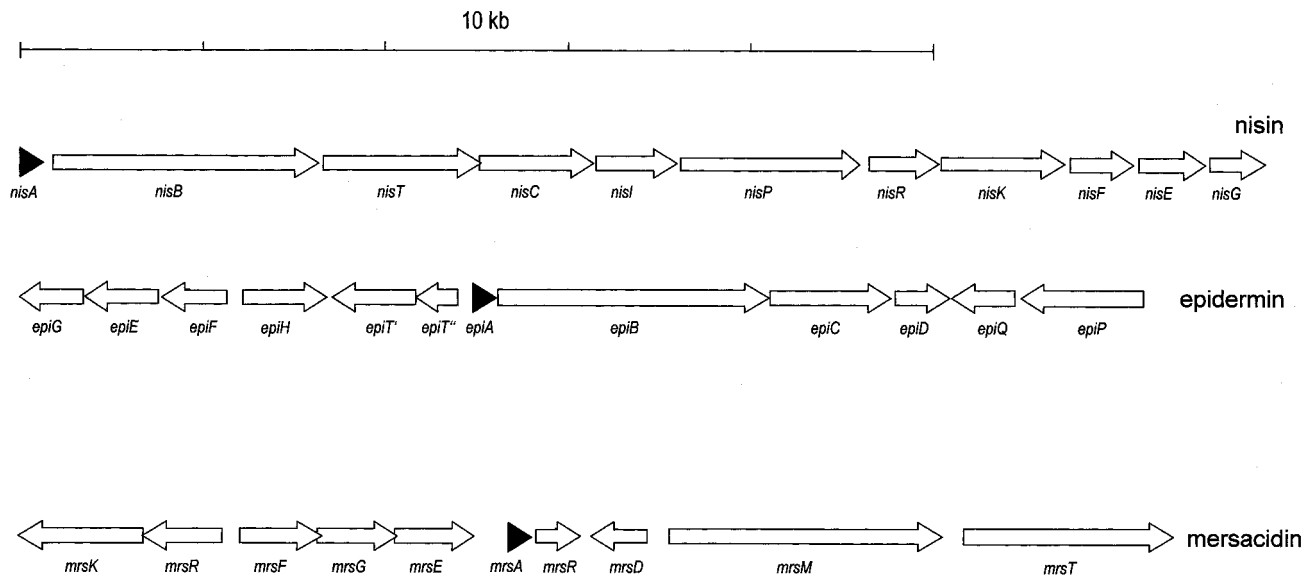
Figure 2 Biosynthetic gene clusters of lantibiotics. The structural genes *lanA* are shown in black; homologous genes are marked by the same suffix according to Refs. 19 and 41. The arrows indicate the direction of transcription.

Table 1 Lantibiotics and Their Biosynthesis Machinery

Lantibiotic	Leader peptide		Modification enzymes		Processing and transport	
	FN/DLD-Type	GG-Type	LanB, LanC	LanM	Lan, LanT	LanT(P)
Class I						
Nisin	+		+		+	
Subtilin	+		+		+	
Pep5	+		+		+	
Epicidin 280	+		+		+	
Epilancin K7	+		n.k.		(+)	
Epidermin	+		+		+	
Lactocin S	−	−		+	+	
Class II						
Lacticin 481		+		+		+
Mutacin II[a]		+		+		+
Nukacin ISK-1[b]		+		+		+
Cytolysins		+		+		+
Lacticin 3147[c]		+		+		+
Staphylococcin 55[d]		+		+		+
Mersacidin[e]		+		+		+
Sublancin 168[f]		+		+		+

For literature see text and Ref. 7.

[a]Refs. 122, 123.

[b]T. Sashihara and K. Sonomoto (personal communication, 1998).

[c]Ref. 124.

[d]Ref. 125.

[e]Ref. 35.

[f]Ref. 126.

n.k.: not known; (+) Incomplete but conclusive sequence information available (Ref. 127).

functions as a signal for internal processing (25) rather than as a transcription terminator.

The biosynthesis of nisin is regulated by a two-component regulatory system, which consists of the membrane-bound sensor kinase NisK and the intracellular response regulator protein NisR (27). Kuipers et al. (28) have demonstrated that nisin itself represents the external signal that is recognized by the extracellular domain of NisK, resulting in the autophosphorylation of a conserved His residue in the intracellular domain of the kinase. The phosphate residue is then transferred to a conserved Asp residue in the N-terminal domain of NisR, which, after a conformational alteration, activates the transcription of the biosynthetic gene cluster. Interestingly, in the nisin A gene cluster, the operons *nisABTCIP* and *nisFEG* are induced by nisin, while *nisRK* is expressed constitutively (24); in contrast, in the nisin Z gene cluster, *nisRK* form a transcription unit with *nisZBTCP*, which is induced by nisin (25).

The epidermin biosynthetic gene cluster is located on the 54-kb plasmid pTü32 of *S. epidermidis* Tü298/DSM 3095. The respective genes are organized in at least five operons: *epiABCD*, *epiPQ*, *epiT*, *epiH*, and *epiFEG* (29–32). Upstream of *epiC* and *epiD*, additional promotor structures have been identified from which an *epiCD* as well as a single *epiD* mRNA could be transcribed (29). Downstream of the structural gene *epiA*, a terminator structure is located that allows partial readthrough, and therefore ensures an appropriate substrate to enzyme ratio by reducing the amount of *epiB*, *epiC*, and *epiD* mRNA (30). The production of epidermin is regulated by the transcriptional activator EpiQ, which interacts with the promoters upstream of *epiA*, *epiFEG*, *epiT*, and *epiH* (31–33).

The gene cluster for production of mersacidin is located on the chromosome of *Bacillus subtilis* HIL Y-85,54728 (34). The entire gene cluster comprises *mrsKRFGEAR1DMT* and is about 12.3 kb in size (35). The expression of mersacidin is regulated by a two-component regulatory system, *MrsRK*, as well as by an additional regulatory protein, MrsR1; in contrast to nisin, the external signal molecule for the sensor kinase, MrsK, has not yet been idenfied.

B. Modification Enzymes

In the first step of the posttranslational modification of the propeptide (Fig. 3), the hydroxyl amino acids Ser and Thr are selectively dehydrated, forming the α,β-unsaturated amino acids Dha and Dhb, respectively (36). In the second step, the sulfhydryl group from a Cys residue is stereospecifically added to the reactive double bond of a dehydroamino acid to form the acid stable thioether. While the leader peptides known so far do not contain Cys residues, Ser and Thr are frequently present; however, hydroxyl amino acids in this part of the prepeptide are not posttranslationally modified (36).

In the vicinity of the first identified structural genes (*nisA*, *epiA*, *spaA*) two genes, *lanB* and *lanC*, were identified, which did not show any sequence similarities to proteins in data bases. Since both genes were found to be essential for the biosynthesis of lantibiotics, it was tempting to assume that they could catalyze the key reaction in lantibiotic biosynthesis, i.e., dehydration and thioether formation (29,37). Subsequent inactivation experiments with the Pep5 biosynthetic gene cluster suggest that LanC is necessary for the formation of thioether bridges, possibly acting as a chaperone, whereas LanB is responsible for the dehydration of the hydroxyl amino acids (38).

The LanB enzymes consist of about 1000 amino acids and seem to be associated with the cytoplasmic membrane (39–41). Although the overall sequence similarity among LanB proteins is low (26–29%), seven conserved sequence motifs were identified and could be of functional significance (42). The LanC enzymes are approximately 400 amino acids in size and contain rather regularly alternating hydrophilic and hydrophobic segments (39). The hydrophobic segments show seven conserved sequence motifs, all of which contain a number of conserved Gly residues of potential structural relevance, whereas conserved His, Cys, and Trp residues are involved in catalysis, disulfide bond formation, or metal ion binding (42). In the case of nisin and subtilin, there is experimental evidence that LanB, LanC, and LanT may associate transiently to form an oligomeric biosynthetic complex attached to the cytoplasmic membrane (43,44); this was concluded from immunoprecipitation and two-hybrid system experiments demonstrat-
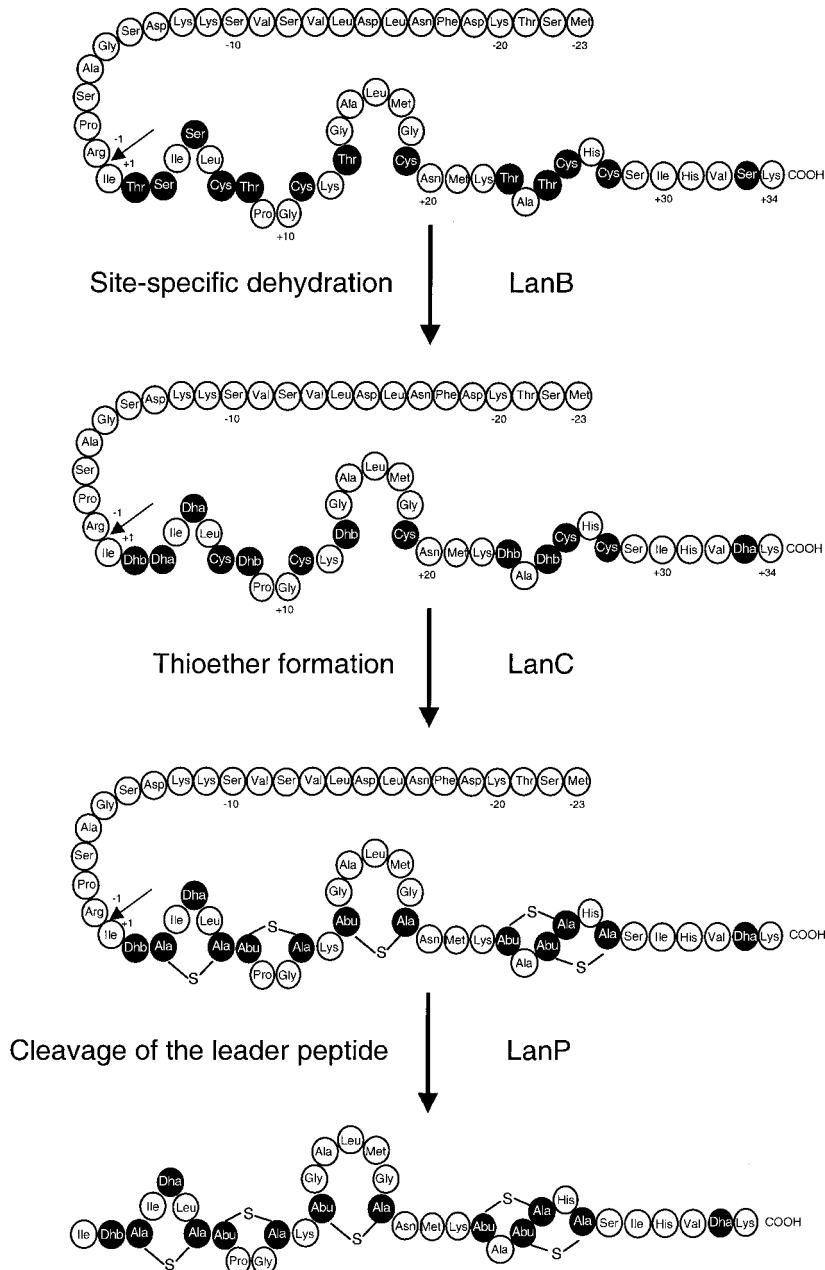
Figure 3 Schematic representation of the maturation of nisin (see text). The protease cleavage site (between residues −1 and +1) is indicated by the arrow. Amino acid residues involved in posttranslational modification are highlighted by shading.

ing that LanB, LanC, and LanT interact with themselves and with the lantibiotic precursor peptide.

In the gene clusters of class II lantibiotics such as mersacidin (Table 1), a single modification enzyme, LanM, of about 950 amino acids is encoded, which seems to substitute for the function of LanB and/or LanC. In the C-terminal domain, LanM proteins show sequence similarities to the LanC enzymes; the seven conserved motifs found in LanC are also found in LanM proteins. In contrast, the N-terminal domain of LanM displays no similarity to the LanB enzymes, which makes it unlikely that *lanM* genes originate from a gene fusion of *lanB* and *lanC* (42).

In the biosynthesis of epidermin and mersacidin a further modification enzyme, LanD, is involved. In the biosynthesis of epidermin the flavin mononucleotide (FMN)-containing oxidoreductase EpiD catalyzes the oxidative decarboxylation of the C-terminal Cys residue to a *S*-aminovinyl-D-cysteine (45,46). Two reducing equivalents from the C-terminal Cys residue of EpiA are removed, a double bond is formed, and the coenzyme FMN is reduced to $FMNH_2$. The decarboxylation occurs spontaneously or is catalyzed by EpiD, resulting in a (Z)-enethiol intermediate that subsequently reacts with the dehydroalanine to form S-aminovinyl-D-cysteine (47).

C. Accessory Enzymes

After modification of the prepeptide, the leader peptide segment is removed proteolytically. This reaction is catalyzed in type I lantibiotics by a protease, LanP, whereas in class II lantibiotics it is performed by a chimeric ABC transporter, LanT(P) as summarized in Table 1. The subtilisin-like serine protease LanP cleaves off the leader peptide before or after export from the cell at a conserved processing site consisting of a hydrophobic amino acid (Ala or Leu) in position –4, a negatively charged or polar amino acid (Glu or Ser) in position –3, a Pro in position –2 (with the exception of epicidin 280), and a positively charged or polar amino acid (Arg or Gln) in position –1 (5). The *nisP* gene codes for a protein of 682 amino acids containing an N-terminal prepro-sequence of about 220 amino acids, which directs NisP to the *sec* system and acts as an intramolecular chaperone. At the C terminus, a membrane anchor sequence with the consensus cell

wall attachment motif LPXTG is present; after export, the mature enzyme is probably bound to the peptidoglycan (27). EpiP consists of 461 amino acids and shows a high degree of homology to NisP (42% of amino acids are identical) (42); EpiP is also synthesized as a pre-pro-protein, but the C-terminal anchor sequence is lacking, so the mature enzyme is found in the culture supernatant (48). In contrast, PepP and LasP apparently function intracellularly, since they lack a signal sequence and a pro-sequence (38).

Class II lantibiotics are processed concomitantly with export by an ABC transporter LanT(P) that has an intrinsic leader peptidase function. The leader peptides of class II lantibiotics possess a conserved cleavage site with Gly in position –2 and Gly, Ser, or Ala in position –1, the so-called double glycine cleavage site, which is also found in various nonlantibiotic bacteriocin precursors. The transporter-associated proteases, i.e., the N-terminal proteolytic domains of the ABC transporters, probably belong to the family of cysteine proteases with a conserved Cys residue as part of the active site. The C-terminal transporter domain of the LanT(P) proteins shares all structural features with the regular LanT protein (49).

The leader peptides of lantibiotics differ significantly in length (23–59 amino acids) and may serve a number of functions. The presence of the leader peptide keeps the lantibiotic inactive and thus protects the producer cell; for example, the fully modified but unprocessed nisin prepeptide is inactive, and processing takes place after export from the cell (50). Furthermore, the leader peptide may interact with the unmodified propeptide and stabilize a conformation that is recognized by the modifying enzymes (5,51). Finally, the conserved FN/DLD motif in the leader peptide of class I lantibiotics might constitute a signal for an interaction with the modifying enzymes and/or transport system (50,52).

The gene clusters of class I lantibiotics contain a *lanT* gene encoding a transport protein of about 500 to 600 amino acids involved in the export of the lantibiotic or of the modified precursor from the producer cell. LanT shares homology with the ABC superfamily of transport proteins, which are characterized by an intracellular C-terminal domain with two conserved ATP binding motifs (Walker motifs) and by a membrane-spanning N-terminal domain consisting of six trans-

membrane helices; the active transporter in the membrane is apparently homodimeric (53). While NisT is essential for export (54), EpiT is inactive due to a frameshift mutation and two deletions in the gene; apparently, its function can be replaced by related transport systems (30). In addition, in the gene clusters of epidermin and of its natural variant, gallidermin, another gene, *lanH*, has been identified, encoding an accessory protein to EpiT or GdmT, respectively (32). Transformation of the epidermin-producing wild-type strain *S. epidermidis* Tü3298 harboring the defective *epiT* with a functional *gdmT* from the gallidermin gene cluster resulted in a twofold increase in production, which probably resulted from improved secretion of epidermin.

D. Producer Self-Protection

Since the production of lantibiotics is potentially lethal for the producer strain, the strain is protected against its own product by dedicated immunity peptides LanI and/or specialized ABC transporter systems LanFEG. In the nisin gene cluster an immunity peptide (NisI) of 245 amino acids is encoded (55), which contains an N-terminal *sec*-dependent signal sequence; following export it is anchored to the outside of the cytoplasmic membrane by a lipid-modified cysteine residue (56). The subtilin immunity peptide (SpaI) consists of 165 amino acids and is also a lipoprotein; however, it neither shows sequence similarity to NisI (57) nor confers cross-immunity to nisin, demonstrating the specificity of the immunity mechanism. The Pep5 immunity peptide (PepI) differs considerably from NisI and SpaI, since it is a small peptide of 69 amino acids. Although it lacks an N-terminal lipoprotein signal sequence, PepI seems to be associated with the extracellular side of the cytoplasmic membrane (58). The molecular mechanism by which the LanI peptides reduce producer strain sensitivity to the respective lantibiotic remains to be elucidated.

A second self-protection mechanism is based on the ABC transporter *LanFEG*, which codes for the gene clusters of nisin, subtilin, epidermin, and mersacidin (31,35,57,59). While LanT are typical group A exporters, which are encoded by a single gene, *LanFEG* belongs to type B ABC transporters, consisting of three separately encoded proteins. LanE, and LanG both form the membrane-spanning

subunits, whereas LanF contains the ATP-binding site; the active transporter is presumably composed of one LanG, one LanE and two LanF molecules. Recently, it has been demonstrated that such immunity ABC transporters function by exporting the lantibiotic out of the cytoplasmic membrane, thus keeping its concentration below a critical level and preventing its killing action (60).

IV. ENGINEERING OF NOVEL ANALOGUES— TECHNICAL PREREQUISITES

Lantibiotics are gene-encoded and, therefore, can be modified by site-directed mutagenesis. Although they are potent antibiotics, only the prototype lantibiotic nisin has found substantial application so far. Currently, nisin is approved as a biopreservative in approximately 50 countries as a food additive in processed cheese, various pasteurized dairy and liquid egg products, canned vegetables, natural cheeses, and salad dressings (61). Epidermin and its variant, gallidermin, are promising agents for the topical treatment of acne, since both peptides are very active against *Propionibacterium acnes* (5). Moreover, mersacidin and actagardine are effective against methicillin-resistant *Staphylococcus aureus* strains (MRSA) (17,62), as well as against enterococci expressing the VanA vancomycin-resistance phenotype (63). Recently, mersacidin has been shown to interact with the peptidoglycan precursur lipid II at a target site that is not attacked by any antibiotic currently in use, thus providing excellent potential for the development of novel antimicrobial compounds (64). Therefore, the optimization of lantibiotics with respect to antibiotic activity, stability, solubility, or protease insensitivity is of great importance.

A. Expression Systems

In order to express mutated structural genes, the producer strain must contain the enzymatic apparatus that is necessary for correct modification and processing of the engineered precursor. Several expression systems have been constructed for nisin (55,65–67), epidermin/gallidermin (68), subtilin (69), Pep5 (70), and mutacin (71).

In the most convenient system, the mutations are introduced into a plasmid-encoded structural gene of a closely related lantibiotic. For example, the expression of the first nisin Z mutant peptides was achieved in a host strain producing nisin A, which can be separated from nisin Z or engineered variants by reversed-phase high-pressure liquid chromatography (HPLC) (67). However, this method precludes the detection of colonies expressing mutant peptides by the agar overlay method. On the other hand, in the case of nisin, the simultaneous production of nisin A is advantageous, since nisin A induces the biosynthesis of the modification enzymes, while the mutant peptide may have a low induction capacity. Furthermore, the nisin yield can be increased threefold by placing *nisZ* under the control of the strong lactococcal promoter *lacA* (67).

Alternatively, mutated genes may be expressed from a plasmid using the wild-type producer strain after inactivation of the structural gene. This strategy has been employed for nisin by inactivating the chromosomally encoded *nisA* gene through a 4–bp deletion, causing a frameshift mutation. The truncated gene (*nisA* was then introduced into the biosynthetic gene cluster by double crossover (55). In another system, *nisA* was inactivated by integration of the insertion sequence IS*905* (65). Complementation to production is achieved by plasmid-encoded mutant *nisA* in both systems.

An expression system for epidermin and gallidermin structural genes was developed using ethyl methanosulfonate mutagenesis. The resulting *epiA* mutant (EMS6) of the epidermin producer strain *S. epidermidis* Tü3298, showing an Epi⁻ phenotype, was used as the expression host. The *Escherichia coli–Staphylococcus* shuttle vector pCU1 (29) or the staphylococcal vector pT181mcs was then employed to express natural and mutant *gdmA* and *epiA* genes (68).

The expression of the structural gene from a separate multicopy plasmid is not possible for Pep5 and subtilin; probably the copy number of the structural genes is higher than that in the wild-type strains, resulting in lethal overexpression of the lantibiotic. Therefore, in the case of Pep5, an expression vector (pGB9) was constructed that carries all information necessary for Pep5 production with the exception of *pepA* and pepI. A fragment covering *pepA* and *pepI* was employed for site-directed mutagenesis and inserted into pGB9; the resulting

plasmid was then transformed into a Pep5 minus variant of the wild-type producer strain (70). The subtilin biosynthetic gene cluster was transformed into *B. subitilis* 168 by competence transformation with chromosomal DNA from the natural producer of subtilin, *B. subtilis* ATCC 6633 (72). The chromosomal *spaA* gene was replaced by an erythromycin resistance gene, which was then replaced by a mutant copy of the subtilin gene by a double crossover, using a flanking chloramphenicol resistance gene as a selective marker (69).

A gene replacement system for variant nisin expression was also constructed. The nisin structural gene was inactivated by deletion of a 300-bp sequence including most of the proposed promoter region of *nisA*, which resulted in a nonproducing and sensitive strain. Site-specific mutations are introduced into the nisA gene using a fragment that contains the missing promoter regions. This fragment is then integrated into the chromosome by double crossover and after reconstitution of the *nisA* promoter, recombinant strains can be selected by their immunity level to nisin (66). For production of engineered mutacin, a similar host-vector expression system was developed. The structural gene was deleted from the wild-type strain *Streptococcus mutans* T8, resulting in a mutacin-negative phenotype. For genetic manipulation, a plasmid containing the polymerase chain reaction (PCR) product of *mutA* served as the target and was used to introduce the mutated *mutA* gene into the chromosome by a reciprocal double cross-over event (71).

B. Site-Directed Mutagenesis

The protein engineering of lantibiotics is a potent research tool for studying structure–function relationships, as well as for optimization of physical and chemical parameters. The role of the ring structures was investigated with several lantibiotics (67,68,71,73), demonstrating that the thioether bridges serve as stabilizers of conformations essential for activity and, at least in the case of Pep5, for protecting the lantibiotic against proteases of the producing strain. In epidermin, most of the mutations affecting the ring structures resulted in complete loss of activity (68).

The introduction or replacement of dehydroamino acids had dif-

ferent effects on the properties of the respective mutant peptides. In nisin Z, the replacement of the Dha residue in position 5 for Dhb reduced the antimicrobial activity by 2- to 10-fold (67) but improved the stability of the peptide (74). T2S nisin Z, M17Q/G18T nisin Z, and L6V gallidermin display higher antibacterial activity against at least some indicator strains, while S3T nisin Z shows dramatically decreased antimicrobial activity (67,68,75). The exchange of Dha5 for Ala abolishes any sporicidal activity of nisin but has no effect on the pore-forming activity (66,76). In contrast, an exchange of Dhb residues in the central part of Pep5 leads to a significant decrease in pore-forming activity, indicating a role in the stabilization of the active conformation (73). The importance of the flexible hinge region in the central part of type A lantibiotics for antimicrobial activity has been demonstrated by introduction of a novel lanthionine ring (73). Although the resulting peptide, A19C Pep5, became insensitive to destruction by chymotrypsin, it lost activity to a great extent. Various derivatives of nisin, gallidermin, and mutacin II mutated in the flexible region gave similar results (68,71,75).

The optimization of lantibiotics is of great interest with regard to applications. A disadvantage of nisin is its low solubility in water at neutral pH, which was significantly increased by introduction of a Lys residue (N27K and H31K nisin Z); the variant peptides maintained antimicrobial activity and spectrum (74). Dha residues are intrinsically unstable, since the double bond is susceptible to addition of a water molecule, finally resulting in degradation and cleavage of the peptide. Although subtilin and nisin are structurally homologous, subtilin is comparatively unstable at room temperature. The loss of activity is correlated with the degradation of the Dha residue in position 5, which is catalyzed by the neighboring Glu4 residue. The mutant E4I subtilin displayed a dramatically (57-fold) increased stability and a threefold higher sporicidal activity (69). Dha5Dhb nisin Z is more stable than the wild-type nisin but less active (74). Stabilization against proteolytic cleavage was achieved by constructing Dhb14P gallidermin and A12L gallidermin (68) as well as A19C Pep5 (73); however, the mutant peptides show reduced antibacterial activity.

C.   Limitations

The above examples demonstrate that the chemical and physical properties of a peptide can be optimized through protein engineering; on the other hand, it became obvious that the biosynthesis machinery does not tolerate certain amino acid substitutions. Mutagenesis of amino acids that are directly involved in thioether formation frequently results in a large decrease or in loss of production, as in the case of S19A epidermin, (C22 epidermin, S23A nisin A, and F23A Pep5 (68,70,75). Mutations of Val32 in nisin completely disturbed the dehydration of Ser33, probably inhibiting the dehydrating enzymes (77).

The rational optimization of the antimicrobial activity is still difficult. The effects of the substitutions on the antibacterial action of the modified peptide can hardly be predicted and, so far, no peptide has been constructed with improved activity against all indicator strains tested. Restraints result primarily from limited knowledge of the mode of action, especially the interaction of the type A lantibiotics with their target, the bacterial membrane (see below). The cytoplasmic membrane is highly dynamic, and its composition varies with different species and growth conditions (e.g., growth phase, temperature, pH) (78,79). Moreover, additional effects such as induction of autolysis (80), formation of slime capsules, or sporicidal activity modulate the antimicrobial activity of the respective peptide.

Another restriction on the development of highly active lantibiotics may come from the fact that such peptides could be toxic for the producing strain. However, this problem may possibly be circumvented by the construction of hyperimmune strains, as shown for Pep5 (81).

## V.   ACTIVITY OPTIMIZATION OF LANTIBIOTICS: WHAT DO WE REALLY KNOW ABOUT MOLECULAR TARGETS?

Lantibiotics are primarily active against gram-positive bacterial strains, among which the range of bacteria susceptible to the action of

different peptides varies considerably. Nisin exhibits a comparatively broad antimicrobial spectrum, while others, such as salivaricin A, inhibit only a limited number of species or even strains. Gram-negative bacteria are affected only when the outer membrane is disrupted by ion chelators such as ethylenediaminetetraacetic acid (EDTA) or citrate (82,83).

Nisin and other type A lantibiotics exert their antibacterial action predominantly by forming pores in the cytoplasmic membrane (Fig. 4). In aqueous solution the type A lantibiotics display high flexibility, which is restricted only by the rigid conformation of the thioether bridges (6). The cationic peptides initially bind to the membrane by ionic interaction with the anionic phospholipids (79,84,85) and adopt an amphiphilic, helical conformation, with the hydrophilic side chains interacting with the headgroups of the phospholipids and the N-terminal hydrophobic residues inserting into the lipophilic core of the membrane (75,86–88). Studies with nisin demonstrated that the C-terminal region (79,89) as well as the overall negative surface charge of the membrane (85,90–92) are important for binding and pore formation. In the presence of a trans-negative membrane potential of at least 50–100 mV (82,93,94) or, as shown for nisin, after activation by a transmembrane pH gradient (95), several peptide molecules aggregate and the C-terminal parts are translocated across the membrane, forming a pore (89). The water-filled, wedge-like pores possess diameters of up to 2 nm and lifetimes ranging from a few milliseconds up to 30 s (82,93,96,97). Sensitive cells are killed by the nonselective efflux of small metabolites such as amino acids or ATP (97,98), disrupting energy transduction (98,99) and leading to cessation of all cellular biosynthesis of protein, DNA, RNA, and polysaccharides (100). Although this model is able to explain many of the data obtained with model membrane systems, some points remain enigmatic, e.g., the molecular interactions taking place at or within the cytoplasmic membrane and the considerable differences in susceptibility of closely related strains. However, recent results provide new insight into the molecular activities of nisin and epidermin and demonstrate that the complete picture of the activity of these peptides is rather complex.

Numerous experiments with artificial vesicles and bilayers showed that type A lantibiotics, at least when applied in micromolar

A. Nisin and epidermin
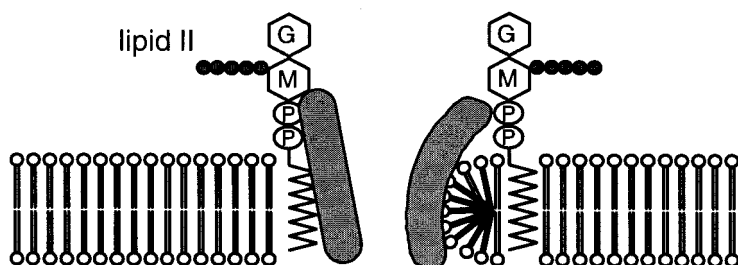
B. Mersacidin
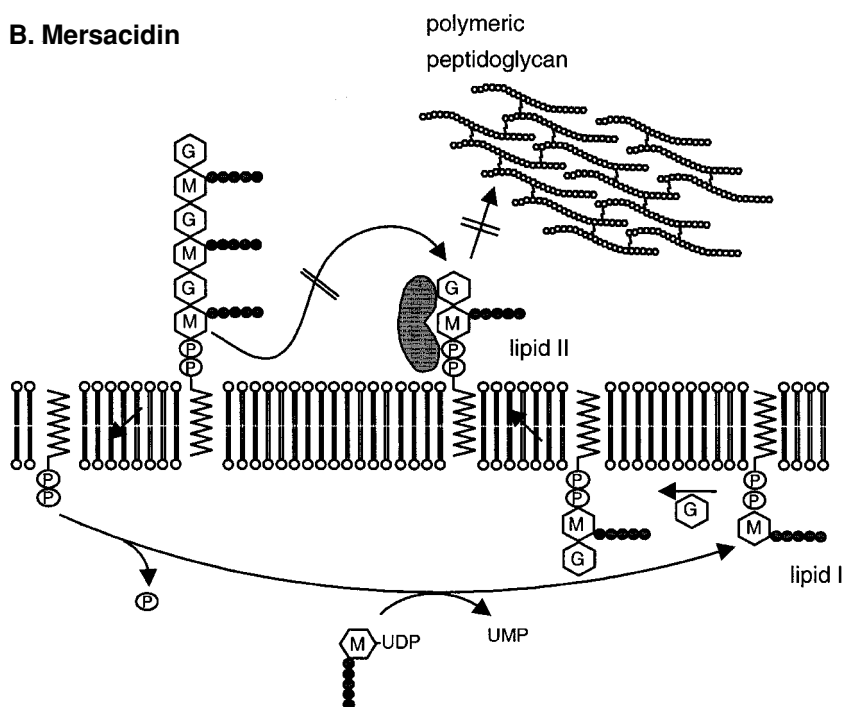
Figure 4 Mode of action of lantibiotics that interact with the membrane-bound peptidoglycan precursor lipid II (see text). (A) Hypothetical model for the formation of transmembrane pores by the type A lantibiotics nisin and epidermin. Both peptides (marked by shading) use lipid II as a docking molecule for binding to the membrane and subsequent pore formation. (B) Mechanism of action of the type B lantibiotic mersacidin. The peptide (marked by shading) binds tightly to lipid II, preventing its incorporation into polymeric peptidoglycan (Ref. 7).

concentrations, have no requirement for a specific membrane-associated receptor; this had been suggested for several channel-forming peptide bacteriocins, e.g., lactococcin A (101) and pediocin PA-1 (102). On the other hand, structure–function studies suggested a limited number of binding sites for nisin and specific antagonism of its activity by the inactive nisin1–12 fragment (103), which indicated the existence of a defined binding site. Subsequently, it was possible to demonstrate that nisin and epidermin use the membrane-bound peptidoglycan precursor lipid II [undecaprenylpyrophosphoryl-MurNAc(pentapeptide)-GlcNAc] as a specific target for binding to the cytoplasmic membrane. Lipid II serves primarily as a docking molecule, which energetically facilitates the formation of pores (64). Studies with isolated cytoplasmic membranes demonstrated that an increase in the lipid II content resulted in increased pore formation activity of nisin (104). Furthermore, upon incorporation of lipid II, the susceptibility of artificial membranes increased by three orders of magnitude, e.g., from the micromolar to the nanomolar concentration range. The interaction of nisin and epidermin with lipid II is highly specific, presumably involving parts of the lipid moiety and of the disaccharide-PP headgroup of the lipid intermediate (Fig. 4). More recently, nisin mutant peptides were used to define the structural elements for binding to lipid II and for pore formation activity, and the results clearly demonstrate that nisin is a dual-function antibiotic. Through interaction with lipid II, nisin inhibits peptidoglycan synthesis and forms highly specific pores. The N terminus appears to be necessary for the initial binding to lipid II, and mutations in this region (e.g., [S3T]nisin) equally affect cell wall biosynthesis and pore-forming activity. Peptides mutated in the hinge region were unable to form pores but retained considerable antimicrobial activity through blocking lipid II incorporation into peptidoglycan (105). Since many lantibiotics act on selected indicator strains at nanomolar concentrations, it seems reasonable to assume that docking molecules generally play an important role in interaction of lantibiotics with the cytoplasmic membrane. The identification of specific target molecules within bacterial membranes will greatly facilitate the rational optimization of the antibiotic activity.

In addition to forming pores in the cytoplasmic membrane, both nisin and Pep5 have been shown to induce autolysis of susceptible staphylococcal cells. The cationic peptides interact with the negatively charged teichoic and lipoteichoic acids in the cell wall and competitively release cell wall autolytic enzymes through an ion exchange-like process (80,106,107). This activation of autolysis occurs most markedly in the area of the septa between dividing daughter cells (107). Furthermore, nisin and subtilin inhibit the germination of bacterial spores; this activity obviously depends on the presence of the Dha residue in position 5 (76,108). Recently, nisin has been shown to function as a signal molecule for measuring the cell density of a population, a phenomenon called quorum sensing. Fully modified nisin induces transcription of the genes involved in its own biosynthesis in a pheromone-like manner in which the signal is transduced via the two-component regulatory system NisKR (28). The latter aspects also have to be considered when peptide engineering is discussed.

The type B lantibiotics mersacidin and actagardine both exert their bactericidal action by inhibition of peptidoglycan biosynthesis (109,110) at the level of transglycosylation (63); both also form a tight complex with the membrane-bound peptidoglycan precursor lipid II (64) (Fig. 4). In contrast to the glycopeptide antibiotic vancomycin, complex formation does not involve the C-terminal D-alanyl-D-alanine moiety of the pentapeptide side chain of the lipid intermediate; instead, mersacidin and actagardine bind to lipid II via the disaccharide-pyrophosphate moiety (64), a novel target binding site not used by any current antibacterial drug. Both lantibiotics contain one conserved ring structure (17,63,111), which is probably of central importance for their activity.

Although the cinnamycin-like type B lantibiotics show relatively modest antibacterical activity, these peptides display a number of effects on eukaryotic cells that are a result of specific binding to phosphatidylethanolamine (112–114), e.g., induction of hemolysis of erythrocytes (115) and inhibition of phospholipase A_2, which interferes with prostaglandin and leucotriene biosynthesis (111,116). Treatment of *Bacillus* strains resulted in impaired ATP-dependent protein translocation (117) and calcium uptake (118), as well as in increased membrane permeability (119).

VI. MAKING USE OF THE PEPTIDE-MODIFYING ENZYMES: POSSIBILITIES AND LIMITATIONS

Protein engineering of lantibiotics has shown that novel amino acids and novel bridging structures (73) may be introduced, indicating that substrate specificity of the modification apparatus is not strongly restricted to the wild-type peptides. To develop new peptide compounds without time-consuming and costly chemical syntheses, it has been proposed to isolate the modification enzymes from the producing strain and then to transform peptides in vitro into novel compounds of biotechnological interest. The establishment of such an in vitro modification system could provide a solution for the problems associated with the construction of expression systems (65,69) or with inadequate immunity systems when engineered peptides become toxic. Extensive in vitro research has been carried out with the flavoprotein EpiD, which is required for the oxidative decarboxylation of the C-terminal Cys residue of preepidermin (45,46,120). EpiD was tested in an in vitro system using synthetic peptide substrates (120), which resulted in transformed peptides carrying an enethiolate group (47). In contrast, in vitro assays using purified EpiB (41) or EpiC (121) for modifications of the epidermin precursor peptide EpiA did not result in modified peptides. Therefore, an in vivo approach has been developed by co-expressing $(His)_6$-labeled EpiA and EpiD in *E. coli*, which resulted in conversion of the precursor peptide (47). This experimental approach may be a valuable tool for the investigation of the posttranslational modification reactions involved in lantibiotic biosynthesis, as well as for applications of the modification enzymes.

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4

Unmodified Peptide-Bacteriocins (Class II) Produced by Lactic Acid Bacteria

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I. INTRODUCTION

Bacteria produce ribosomally synthesized antimicrobial peptides or proteins termed bacteriocins. This term was initially introduced by Jacob et al. (1) to designate antimicrobial substances produced by gram-positive bacteria. In 1976 Tagg et al. (2) modified the definition such that the term referred to proteinaceous substances that kill bacteria that are closely related to the bacteriocin-producing bacteria. Since then it has become evident that many bacteriocins also kill bacteria that are not related to the bacteriocin producer, hence the present definition that bacteriocins are simply ribososmally synthesized antimicrobial substances.

The differences between bacteriocins and classical antibiotics should be clarified because there is some confusion in the literature about this. The main differences between classical bacteriocins and antibiotics are:

1. Bacteriocins are ribosomally synthesized, in contrast to antibiotics, which are synthesized by unique enzymatic systems.
2. Bacteriocins have a much more narrow target specificity than antibiotics.
3. Each bacteriocin has its own dedicated immunity protein whose gene is linked to the bacteriocin gene, whereas genetic determinants for antibiotic resistance are not linked to, and are expressed independently of, the genes encoding the antibiotic synthesis apparatus.
4. Bacteriocins are usually produced in the growth phase, and production is frequently regulated by a two-component regulatory system, while antibiotics are secondary metabolites produced in the stationary phase.

Colicins produced by *Escherichia coli* were the first bacteriocins identified (3,4), but bacteriocins have since been found in a large number of bacterial species investigated. In particular, lactic acid bacteria (LAB) and their bacteriocins have been the focus of many research programs due to the industrial importance of LAB and their contribution to the well-being of humans. The production of an antimicrobial substance by LAB was first reported about 70 years ago (5,6). However, it was in 1971 that the structure of this substance, nisin, was elucidated (7). Since then, numerous other LAB bacteriocins have been isolated and characterized, particularly during the last 10 years (8–11). These LAB bacteriocins should be treated differently from antibiotics from a legislative point of view, since LAB (often referred to as food-grade bacteria) are part of the natural microbial flora of foods that humans have consumed for centuries and constitute a significant part of the indigenous flora of mammals, including humans. This is not the case for antibiotic-producing microorganisms.

Bacteriocins produced by strains of *Lactococcus, Lactobacillus, Pediococcus, Leuconostoc, Carnobacterium, and Enterococcus* will be discussed in this review. The LAB within these genera are the ones that are most frequently found in food and feed, and they serve an important function in industrial food and feed fermenta-

tion. These bacteriocins have been grouped into three different classes (10,11). Class I consists of the small, post-translationally modified peptides, often referred to as lantibiotics due to the presence of the modified residues lanthionine and methyllanthionine. Class II consists of peptide-bacteriocins without modified residues. Both Class I and Class II bacteriocins are relatively heat stable. The third class, Class III, consists of large, heat-labile antimicrobial proteins. Helveticin J and enterolysin A are the only Class III LAB bacteriocins that are thoroughly characterized biochemically and genetically (12,13). In this chapter we will limit our discussion to Class II bacteriocins produced by the genera mentioned above, but it should be pointed out that Class II–like bacteriocins have also been found in gram-negative bacteria such as *E. coli* and *Haemophilus influenzae* (14,15).

II. CLASSIFICATION AND GENERAL FEATURES OF CLASS II BACTERIOCINS

Class II bacteriocins found in LAB are small peptides about 30 to 70 amino acid residues long. They are cationic at neutral pH, and they have a hydrophobic and/or an amphiphilic region. Bacteriocins whose mode of action has been studied have been shown to permeabilize the membrane of target cells.

A large number of different Class II bacteriocins have been characterized (Table 1). From a practical point of view, it has been useful to subclassify these bacteriocins according to sequence similarities, mode of secretion, target specificity, and the number of peptides that constitutes the bacteriocin (10,11). However, it should be emphasized that this subclassification is merely a way to organize functionally bacteriocins that show some common features. This subclassification may eventually be changed when new bacteriocin sequences and information about the characteristics of these bacteriocins become available. Even today, some newly described bacteriocins can be placed in more than one subclass, while others, such as lactococcin A, lactococcin B, and enterocin B, do not fit easily into any of the subclasses.

Table 1   An Overview of Genetically and Biochemically Characterized
Class II Bacteriocins

Bacteriocin	Producer	Reference
Nonsubgrouped Class II bacteriocins		
Lactococcin A	*Lactococcus lactis*	16, 17
Lactococcin B	*Lactococcus lactis*	18
Carnobacteriocin A	*Carnobacterium piscicola* LV17A	19
Piscicolin 61	*Carnobacterium piscicola* LV61	20
Lactobin A	*Lactobacillus amylovorus* LMG P-13139	21
Divergicin 750	*Carnobacterium divergens* 750	22
Enterocin B	*Enterococcus faecium* T136/CTC492	23
Leucocin B-TA33a	*Leuconostoc mesenteroides* TA33a	24
Antilisterial pediocin-like bacteriocins (Class IIa)[a]		
Leucocin A	*Leuconsostoc gelidum* UAL 187	25
	Leuconostoc mesenteroides TA33a	24
Leucocin C-TA33a	*Leuconostoc mesenteroides* TA33a	24
Pediocin PA-1	*Pediococcus acidilactici* PAC 1.0	26–28
	Lactobacillus plantarum WHE 92	29
Pediocin AcH	*Pediococcus acidilactici* AcH	30
Sakacin P	*Lactobacillus sake* LTH673	31
	Lactobacillus sake Lb674	32
Bavaricin A	*Lactobacillus bavaricus* MI401	33
Curvacin A	*Lactobacillus curvatus* LTH1174	31
Sakacin A	*Lactobacillus sake* Lb706	34, 35
Mesentericin Y105	*Leuconostoc mesenteroides* Y105	36, 37
Carnobacteriocin B2	*Carnobacterium piscicola* LV17B	38
Carnobacteriocin BM1	*Carnobacterium piscicola* LV17B	38
Piscicocin V1b	*Carnobacterium piscicola* V1	39
Acidocin A	*Lactobacillus acidophilus* TK9201	40
Enterocin A	*Enterococcus faecium* CTC492	41, 42
	Enterococcus faecium DPC1146	43
Bavaricin MN	*Lactobacillus sake* MN	44
Piscicolin 126	*Carnobacterium piscicola* JG126	45
Piscicocin V1a	*Carnobacterium piscicola* V1	39
Mundticin	*Enterococcus mundtii* AT06	46
Divercin V41	*Carnobacterium divergens* V41	47
Sec-dependent secreted Class II bacteriocins		
Acidocin B	*Lactobacillus acidophilus* M46	48
Divergicin A	*Carnobacterium divergens* LV13	49
Enterocin P13[b]	*Enterococcus faecium* P13	50
Bacteriocin 31[b]	*Enterococcus faecalis*	51
Lactococcin 972	*Lactococcus lactis* IPLA 972	52
Two-peptide bacteriocins (Class IIb)		
Lactococcin G		
(LcnGα and LcnG-β)	*Lactococcus lactis* LMG2081	53

Table 1 Continued

Lactococcin M (LcnM and LcnN)	*Lactococcus lactis* 9B4,	17
Lactacin F (LafA and LafX)	*Lactobacillus johnsonii* VPI11088	54
Thermophilin 13 (ThmA and ThmB)	*Streptococcus thermophilus* Sfi13	55
Plantaricin S (PlsA and PlsB)	*Lactobacillus plantarum* LCP010	56, 57
Plantaricin EF (PlnE and PlnF)	*Lactobacillus plantarum* C11	58, 59
Plantaricin JK (PlnJ and PlnK)	*Lactobacillus plantarum* C11	58, 59
Enterocin 1071 (Ent1071A and Ent1071B)	*Enterococcus faecalis* BFE 1071	60
Lactocin 705 (705α and 705β)	*Lactobacillus casei* CRL 705	61
Leucocin H (Hα and Hβ)[c]	*Leuconostoc* MF215B	62
Class II bacteriocins without a leader sequence		
Enterocin L50[d]	*Enterococcus faecium* L50	63
Enterocin I[d]	*Enterococcus faecium* 6T1a	64

Note: The primary amino acid sequences are identical for the bacteriocins grouped together.
[a]Enterocin P13 and bacteriocin 31, listed among the *sec*-dependent secreted bacteriocins, are also pediocin-like bacteriocins (i.e., belong to Class IIa). See also note added in proof for two new pediocin-like bacteriocin.
[b]Enterocin P13 and bacteriocin 31 are pediocin-like bacteriocins (i.e. belong to Class IIa; see footnote[a]).
[c]Leucocin H has not been completely sequenced, and it is consequently not certain that it does in fact contain only unmodified residues and thereby belongs to Class II.
[d]Although the bacteroicin contains two peptides, it is not considered a bonafide two-peptide bacteriocin, since the two peptides have similar amino acid sequences and they have potent antimicrobial activity when assayed individually.

A. One-Peptide Pediocin-Like Bacteriocins

The strongly antilisterial one-peptide bacteriocins, frequently referred to as pediocin-like bacteriocins, have been allocated to Class IIa (10,11). The first of these bacteriocins to be identified and thoroughly characterized were leucocin A (25) and pediocin PA-1, from which the term pediocin-like bacteriocins has been derived (26–28,30). Since then, the subclass of pediocin-like bacteriocins has greatly expanded and presently contains at least 15 different well-characterized bacteriocins (Table 1). They are all between 37 and 48 amino acid residues long, cationic, and share amino acid sequence similarities, as first noted by Nieto Lozano et al. (28). The sequence similarity, ranging

from 40% to 60%, is especially pronounced in the N-terminal half, where one finds a "pediocin-box" motif containing two cysteine residues (in the form of cystine): Y G N G V X C X K/N X X C X V, where X represents a polar residue. In spite of sequence similarities, the pediocin-like bacteriocins have different target specificity (65,66). The structure and mode of action of these bacteriocins will be discussed more extensively later in the chapter.

B. One-Peptide Non-Pediocin-Like Bacteriocins

Several one-peptide bacteriocins that show no sequence similarity to the pediocin-like bacteriocins have also been identified and characterized (Table 1). The group includes the hydrophobic, cationic bacteriocins lactococcin A and B (16–18), divergicin 750 (22), carnobacteriocin A (19), and enterocin B (23), which is homologous to carnobacteriocin A. In addition, numerous one-peptide bacteriocins have been partly sequenced at the protein level. These include curvaticin FS47 (67), numerous bacteriocins from *Lactobacillus acidophilus* strains (68,69), four antimicrobial peptides obtained from *Lactobacillus gasseri* JCM 2124 (70) and one from *L. gasseri* LA39 (71), lactococcin 972 (52,72), and lactobin A (21), among others.

C. Two-Peptide Bacteriocins

The two-peptide bacteriocins are allocated to Class IIb. These bacteriocins are unique in that they consist of two complementary peptides, and optimal antimicrobial activity is obtained when both peptides are present in approximately equal amounts. A list of two-peptide bacteriocins that have been characterized is shown in Table 1.

The individual peptides of two-peptide bacteriocins have many of the same characteristics as one-peptide bacteriocins: they are cationic, contain hydrophobic and/or amphiphilic regions, and may have some antimicrobial activity. Nevertheless, two-peptide bacteriocins should, for the following reasons, not be thought of as two synergistically acting one-peptide bacteriocins. (a) In contrast to one-peptide bacteriocins, the peptides of two-peptide bacteriocins usually display very low, if any, bacteriocin activity when tested individu-

ally. When combined, however, their activity increases, often by more than 100-fold. The two peptides that constitute lactococcin G show no activity when assayed individually at concentrations as high as 50 μM, but when combined they are active at 50 pM (73). (b) The fact that the genes encoding the two complementary peptides of the two-peptide bacteriocins are found next to each other on the same transcriptional unit (11,17,54,56,59,74) indicates that the two complementary peptides function together as one entity. (c) Similarly, the fact that only one putative immunity gene is found for the two-peptide bacteriocins also indicates that the two complementary peptides function together as one antimicrobial entity. (d) Structural analysis of two-peptide bacteriocins indicates a direct physical interaction between complementary peptides when they exert their bactericidal effect (75,76).

Although two-peptide bacteriocins clearly should not be considered as being two synergistically acting one-peptide bacteriocins, two-peptide bacteriocins may have evolved from one-peptide bacteriocins. If two one-peptide bacteriocins act synergistically, there will be a selection pressure for the enhancement of the synergistic effect (probably at the expense of the activity of the individual bacteriocins) and for genetic linkage of the two bacteriocin genes. This could lead to the evolution of a two-peptide bacteriocin. It is interesting to note that one of the peptides (LafA) of the two-peptide bacteriocin lactacin F (54) shows sequence similarity to one of the peptides (PlsA) of the two-peptide bacteriocin plantaricin S (56). Thus, it appears that one bacteriocin-like peptide has on two different occasions merged genetically with another bacteriocin-like peptide, resulting in the formation of the two two-peptide bacteriocins, plantaricin S and lactacin F. Two of the two-peptide bacteriocins, lactococcin G (53) and enterocin 1071 (60), show about 60% sequence identity and thus are clearly evolutionary related.

D. *sec*-Dependent Bacteriocins

Most Class II bacteriocins are encoded in a pre-form with a double-glycine leader and are secreted by a dedicated ABC transporter (11). There are, however, a few exceptions. Presently, five different Class II one-peptide bacteriocins—acidocin B, divergicin A, enterocin P,

bacteriocin 31, and lactococcin 972—have been shown to be encoded in a pre-form containing a *sec*-dependent leader (48–52). It has been suggested that these should be classified in a special subclass consisting of the *sec*-dependent bacteriocins (11). However, except for their *sec*-dependent leaders, these five bacteriocins share the general characteristics of other Class II bacteriocins. Based on their primary structures, enterocin P and bacteriocin 31 are in fact pediocin-like bacteriocins (Class IIa) and are treated as such in this discussion, whereas acidocin B, divergicin A, and lactococcin 972 are one-peptide, non-pediocin-like bacteriocins. It has been shown that among Class II bacteriocins one may replace a *sec*-dependent leader with a double-glycine leader, and vice versa, without grossly affecting bacteriocin secretion (77,78). The N-terminal leader apparently specifies which secretion system is to be used for externalization of bacteriocins.

E. Bacteriocins Synthesized with No Leader

The bacteriocin enterocin L50, which is identical to enterocin I (64), is another exceptions to the rule that Class II bacteriocins are synthesized with a double-glycine leader and secreted by a dedicated ABC transporter (63). This bacteriocin, which is produced by *Enterococcus faecium*, consists of two peptides (L50A, with 44 residues, and L50B, with 43 residues) that are synthesized without a leader sequence. Although each of its two peptides has bacteriocin activity, the activity increases almost 100-fold, depending on the target strain, when the peptides are combined. Moreover, the genes encoding the two peptides are located next to each other in the same transcriptional unit (63). No immunity gene has been found in the vicinity (about 1 kb upstream and downstream) of the two structural genes.

Enterocin L50 is different from other two-peptide bacteriocins that have been characterized in that its two peptides are very similar, having more than 70% sequence commonality (63), suggesting that this bacteriocin has evolved via gene duplication. It is also different from other two-peptide bacteriocins in that both peptides (L50A and L50B) have significant antimicrobial activity when assayed individually (63). Enterocin L50 should, consequently, not be considered a bona fide two-peptide bacteriocin.

There is significant amino acid sequence homology and similarity in the gene organization of enterocin L50 and some staphylococcal antimicrobial peptides (63). The staphylococcal antimicrobial peptides, however, are also hemolytic, whereas enterocin L50 is not (63, 79, 80). The sequence homology between enterocin L50 and these staphylococcal, hemolytic antimicrobial peptides raises interesting questions concerning the development of and relationship between such peptides with and without hemolytic activity. It should also be mentioned that within Class I bacteriocins, the two-peptide lantibiotic, cytolysin, isolated from *E. faecalis,* has both antimicrobial and hemolytic activity (81).

III. STRUCTURE AND MODE OF ACTION OF CLASS II BACTERIOCINS

Mode of action studies show that most, if not all, Class II bacteriocins kill bacteria by rendering the membrane of the target cell permeable for small molecules. It should be pointed out, however, that many of these studies have been carried out with only partly purified bacteriocins or bacteriocins concentrated from the growth medium of the bacteriocin producer. This is not optimal, since many LAB produce more than one bacteriocin (11). Moreover, bacteriocin activity may be influenced by other components—for instance detergents, membrane and cell wall fragments and proteins—that may be present in crude bacteriocin extracts. The membrane-permeabilizing mode of action reflects the structural characteristics common to most Class II bacteriocins: their cationic character and the presence of hydrophobic and/or amphiphilic regions. The cationic properties are thought to enable the initial binding to the negatively charged cell wall and/or target cell membrane, whereas the hydrophobic or amphiphilic regions presumably enable penetration into, and permeabilization of, the target cell membrane.

A. One-Peptide Pediocin-Like Bacteriocins

As discussed earlier, the group of pediocin-like bacteriocins presently includes at least 15 different bacteriocins, 37 to 48 residues long, that

show 40–60% sequence similarity. Based on their primary structures, the polypeptide chains of these bacteriocins may roughly be divided into two regions: a hydrophilic, cationic, and highly conserved N-terminal half that contains the "pediocin box motif" discussed earlier and a somewhat less conserved hydrophobic or amphiphilic C-terminal half (65). Nuclear magnetic resonance structural analysis of leucocin A (82) and carnobacteriocin B2 (83) in the presence of a membrane-mimicking environment indicates that a segment spanning from about the middle to halfway toward the C terminus forms an amphiphilic α-helix, while the remaining C-terminal stretch is relatively unstructured. The presence in some pediocin-like bacteriocins of a disulfide bridge that connects the C-terminal end with the middle region (see discussion below) indicates that the unstructured C-terminal stretch folds back onto the helical segment.

It is thought that the well-conserved cationic N-terminal region mediates the initial binding of these bacteriocins to target cells through electrostatic interactions (84), and that the hydrophobic or amphiphilic C-terminal region penetrates into the hydrophobic part of the target cell membrane, thereby mediating membrane leakage (65,85). This hypothesis is consistent with the observation that chimeric pediocin PA-1, which has maltose-binding protein fused to its N terminus, displayed bacteriocin activity. Consequently, it is unlikely that the N-terminal region is the part of the bacteriocin that penetrates into the target cell membrane (85).

Despite similarity in primary structures, the pediocin-like bacteriocins differ in their target cell specificity (i.e., in their antimicrobial spectra) (65,66). The hydrophobic/amphiphilic C-terminal region, which is thought to penetrate into the target cell membrane, appears to play a central role in mediating specificity, since hybrid bacteriocins containing N- and C-terminal regions from different pediocin-like bacteriocins have antimicrobial spectra similar to that of the bacteriocin from which the C-terminal region is derived (65). Moreover, the bacteriocin activity of pediocin PA-1 has been shown to be specifically inhibited by a 15-mer fragment that spans the bacteriocin from the center toward the C terminus (86). The inhibition was specific in the sense that the fragment inhibited pediocin PA-1 to a much greater extent than other closely related pediocin-like bacteriocins. None of the other possible 15-mer fragments that span pediocin PA-1 inhibited bacteriocin

activity to the same extent. These results suggest that a region in the hydrophobic/amphiphilic C-terminal half participates directly or indirectly in a specific interaction with a component of the target cell.

Site-directed mutagenesis studies show that individual amino acid residues in the C-terminal half of the pediocin-like bacteriocins are important for target cell specificity. Pediocin PA-1 has a disulfide bridge in its C-terminal half; sakacin P does not. The introduction of a disulfide bridge by site-directed mutagenesis into sakacin P made it more pediocin PA-1-like with respect to target cell specificity and temperature stability; similarly, removal of the disulfide bridge in pediocin PA-1 made it more sakacin P-like (87). Mode of action studies with purified pediocin PA-1 revealed that it dissipated the transmembrane electrical potential and inhibited the amino acid transport in sensitive cells (88). Moreover, the bacteriocin interfered with the uptake of amino acids by cytoplasmic membrane vesicles derived from sensitive cells to a greater extent than vesicles derived from immune cells. Pediocin PA-1 also elicited efflux of small ions from liposomes fused with membrane vesicles. With increasing bacteriocin concentrations, efflux of molecules with increasing molecular weights, up to 9000, was observed (88). Chen et al. (89) demonstrated that pediocin PA-1 was able to permeabilize synthetic vesicles composed of only phosphatidylcholine, suggesting that the bacteriocin can function in the absence of a protein receptor. Thus, it is unclear if a receptor on target cells, which has previously been postulated (88), does in fact exist. It is particularly interesting from an applied point of view that the pediocin-like bacteriocins are very active against almost all *Listeria* strains tested (90, 91).

B. Other One-Peptide Bacteriocins

The other one-peptide bacteriocins form a heterogeneous group of peptides, although most, if not all, are cationic and are thought to kill cells by membrane permeabilization. We will limit our discussion to the lactococcal bacteriocin lactococcin A (16,17), which is perhaps the best characterized of the one-peptide non-pediocin-like bacteriocins. Lactococcin A has 54 residues and is initially synthesized as a 75-residue precursor with a 21-residue leader of the double-glycine type. Hydrophobicity analysis has revealed a 21-residue region in the

bacteriocin—from residue 30 to 50—which might form a membrane-spanning helix (92).

Lactococcin A appears to kill only lactococci (16). When whole lactococcal cells and membrane vesicles prepared from these cells are exposed to purified lactococcin A, they are permeabilized in a voltage-independent manner; this results in efflux of various low molecular weight substances, including amino acids (92, 93). Treating the vesicles with proteinase K rendered them insensitive to lactococcin A (94). Vesicles from similar cells that expressed the lactococcin A immunity gene were also insensitive to lactococcin A, as were phospholipid liposomes. It has, consequently, been suggested that lactococcin A must bind to a proteineacous membrane receptor in order to permeabilize cells and vesicles, although such a receptor has yet to be identified. It has also been proposed that the immunity protein acts by shielding the receptor, consistent with the localization of the immunity protein in the cell membrane (94–96).

Recently, it was shown that Class II bacteriocins can interfere with cell wall biosynthesis. Martinez et al. (97) provided evidence that lactococcin 972 may kill sensitive bacteria by interfering with septum formation. This is a remarkable observation, since all other Class II bacteriocins studied so far have been shown to act on the bacterial membrane. It will be interesting to discover if this finding is related to what has been observed with nisin, where it has been shown that a receptor-like entity is involved in the antibacterial mode of action (98).

C. Two-Peptide Bacteriocins

Lactococcin G, produced by a *Lactococcus lactis* strain, was the first two-peptide bacteriocin to be isolated and characterized (53) and is presently the most studied of the two-peptide bacteriocins. It consists of two peptides with 39 and 35 amino acid residues that are initially synthesized as pre-forms with double-glycine-type leaders. These are encoded by the two bacteriocin structural genes that are adjacent to each other in the same operon, which also contains a putative immunity gene and genes encoding an ABC transporter and an accessory protein (74,99).

Carbonate dehydratase (CD) structural analysis of the two lactococcin G peptides and various fragments derived thereof revealed that

the peptides were unstructured in water but became structured upon exposure to membrane-like entities, such as dodecylphosphocholine micelles and negatively charged (dioleoyl-L-α-phosphatidyl-DL-glycerol) liposomes (75). The membrane-like entities induced the formation of an amphiphilic α-helix in the N-terminal half of both peptides. Moreover, in the presence of liposomes, the two peptides interacted, resulting in enhanced α-helical structuring (75). This additional structuring occurred only when the two peptides were added simultaneously to liposomes, but not if one peptide was added before the other or if two liposome mixtures each containing a peptide were mixed (75). Thus, the individual lactococcin G peptides apparently interact in a structure-inducing manner prior to or upon arrival at the target membrane, resulting in the formation of amphiphilic α-helical structures. This interaction is presumably important for the formation of an optimal antimicrobial complex. The synergistic antimicrobial effect obtained with the two lactococcin G peptides is thus apparently due to interpeptide interactions rather than to the two peptides interacting separately with target cells. It is not clear at exactly what stage this interpeptide interaction occurs when peptides are exposed to sensitive cells, but presumably it occurs after the peptides come in contact with the target cells and before they become fully embedded in the lipids of the cell membrane.

There appears to be no structural interaction between the two lactococcin G peptides when they are free in aqueous solution, as judged by CD analysis. One might speculate that the peptides first bind individually to an entity on the cell wall or membrane, thereby allowing the two peptides to interact before penetrating further into the hydrophobic part of the cell membrane. Such a model is consistent with the following observations: antibacterial activity is observed when cells are first treated with one peptide, washed, and then treated with the other peptide, whereas no activity is observed when cells treated with one peptide are mixed with cells treated with the other peptide (100). This indicates that either peptide can separately interact stably with the target cell surface without losing its potential activity in this "dormant state," but they are unable to diffuse to another cell once bound to the cell surface.

CD structural studies similar to those done on lactococcin G were recently carried out with two other two-peptide bacteriocins, plantaricin EF and plantaricin JK, both produced by a strain of *Lac-*

tobacillus plantarum (58,76). Basically the same results were obtained for these two bacteriocins as for lactococcin G: membrane-like entities induced amphiphilic α-helices in the peptides constituting these two bacteriocins, and additional structuring was obtained when two complementary peptides were exposed simultaneously to the membrane entities. Thus, plantaricin EF and plantaricin JK might be expected to act somewhat similarly to lactococcin G (76).

Although three-dimensional structural studies of other two-peptide bacteriocins have not been conducted, the primary structure of all except lactococcin MN suggests that at least one of their peptides—often both peptides—contains a putative α-helical region with a relatively high degree of amphiphilicity. It therefore appears that the amphiphilic α-helix is an important structural motif in most, if not all, two-peptide bacteriocins, and this motif presumably enables their membrane-interacting mode of action (75,76).

For some two-peptide bacteriocins, such as lactococcin G and lactocin 705, the two complementary peptides display no activity when assayed individually (61, 73). At the other extreme one finds enterocin L50, where two complementary peptides are very active, although the activity may increase by about 100-fold when the two peptides are combined (63). In between, one finds the two-peptide bacteriocins, such as plantaricin EF and JK (58), plantaricin S (56,57), thermophilin 13 (55), and lactacin F (54), where one or both complementary peptides display some activity alone, but where the activity is greatly enhanced when two complementary peptides are combined. For some of the peptides that individually display activity, this might be due to contamination of the complementary peptide. One can, however, be certain that this is not the case for lactacin F, enterocin L50, and plantaricin EF and JK since individually synthesized or cloned peptides have been analyzed (54,58,63).

All two-peptide bacteriocins whose mode of action has been analyzed [lactococcin G (73, 100), plantaricin EF and JK (101), lactacin F (102), and thermophilin 13 (55)] render membranes permeable to various small molecules. The permeabilization of the membrane of sensitive cells destroys the proton motive force by dissipating the transmembrane electrical potential and/or the transmembrane pH gradient. Dissipation of the electrical potential is due to permeabilization of the membrane for

various ions. Lactococcin G permeabilizes the target cell membrane for a broad range of monovalent cations, such as Na^+, K^+, Li^+, Cs^+, Rb^+, and choline, but not for H^+, divalent cations (Mg^{2+}) or anions such as phosphate (73,100). Plantaricin EF and plantaricin JK also permeabilize membranes for monovalent cations, including H^+ (in contrast to lactococcin G), but not for divalent cations (Mg^{2+}) (101). It appears that plantaricin EF conducts cations more efficiently than plantaricin JK and vice versa for anions (101). Neither is able to conduct phosphate across the membrane, whereas lactacin F makes the membrane permeable to K^+ and phosphate (101). Thermophilin 13 dissipates transmembrane electrical potentials and pH gradients, although its specificity with respect to compounds it conducts across the membrane has not yet been characterized (55). Taken together, these characteristics show that two-peptide bacteriocins have some specificity with respect to which small molecules they conduct across the membrane, and that the specificity varies among the various two-peptide bacteriocins.

The detailed mechanism by which amphiphilic α-helical peptides permeabilize membranes has not been elucidated and is subject to current debate. There is general agreement that amphiphilic peptides, upon interacting with membranes, initially lie parallel to the plane of the membrane, with the hydrophobic side facing toward and shallowly penetrating the membrane. It is, however, uncertain whether this "carpet mechanism" is sufficient to destabilize the phospholipid packing of membranes, leading to membrane permeabilization, as suggested for cecropin P1 (103). Several facts suggest that the two-peptide bacteriocins permeabilize the membrane through pore-formation, rather than through the carpet mechanism. The fact that the two-complementary peptides function together synergistically through structure-stabilizing interactions indicates that the membrane permeabilization process is much more complex than simply the horizontal binding of amphiphilic structures to the cell surface, as suggested by the carpet model. Moreover, the high potency of the two-peptide bacteriocins (and of most other LAB bacteriocins) suggests that membrane permeabilization depends on a relatively low number of peptides, which would not be expected if permeabilization occurs through the carpet model. Finally, the fact that two-peptide bacteriocins are relatively specific with respect

to the molecules they conduct across membranes suggests that they form specific pores, rather than cause unspecific disintegration of the membrane through the carpet mechanism.

IV. GENES AND GENE PRODUCTS INVOLVED IN THE SYNTHESIS OF BACTERIOCINS

Four genes (five for two-peptide bacteriocins) are, with a few exceptions, the minimal number required to produce Class II bacteriocins (11,74): (a) the structural gene (two structural genes for two-peptide bacteriocins) that encodes the pre-form of the bacteriocin, (b) the immunity gene that encodes the immunity protein needed to protect the bacteriocin producer, (c) a gene that encodes a membrane-associated ABC transporter that transfers the bacteriocin across the membrane concomitantly with removal of the leader sequence, and (d) a gene that encodes an accessory protein that is also needed for secretion of the bacteriocin, but whose specific role is not known. The four genes are usually found in either one or two operons. Each of the four genes and their products will be discussed separately in the following sections.

A. Structural Genes

The structural genes for all genetically characterized Class II bacteriocins are located adjacent to the promoter in an operon that, with only a few exceptions, e.g., enterocin L50 (63), carnobacteriocin A (19), enterocin B (23), and probably divercin V41 (47), also contains an immunity gene downstream. For a few bacteriocins, such as pediocin PA-1 and lactococcin G, the immunity gene is followed by the genes encoding the ABC transporter and the accessory proteins, all within the same operon (27,74). However, for most Class II bacteriocins, such as lactococcin A, curvacin A, sakacin P, and plantaricin JK and EF, the genes encoding the ABC transporter and accessory proteins are on another operon, often nearby the operon that contains the structural and immunity genes (32,35,59,104).

Comparison of the amino acid sequence of mature bacteriocins with the nucleotide sequence of their structural genes has revealed that

the structural genes encode the pre-form of bacteriocins (enterocin L50 is an exception). The pre-form consists of an N-terminal leader sequence containing 15–31 residues followed by the sequence of the mature bacteriocin. The leader presumably functions to facilitate interaction with the transporter and/or to keep the bacteriocin inactive until it has been secreted from the cell. Most bacteriocin-leader sequences are of the so-called double-glycine type. They have a characteristic consensus sequence (see Fig. 1) and are cleaved at the C-terminal side of two glycine residues (10,12). Exceptions to this are the four Class II bacteriocins that have *sec*-dependent leaders and enterocin L50 without a leader (Table 1).

B. Immunity Genes and Proteins

Immunity genes encode relatively small proteins, ranging in size from 50 to 150 residues. The genes have generally been identified as immunity genes by rendering sensitive cells resistant to bacteriocins upon transfer of the genes into the cells (16,32,35,105–108).

The immunity proteins for lactococcin A and carnobacteriocin B2 have been isolated and characterized (95,106). The lactococcin A immunity protein is synthesized without leaders or any form of posttranslational modification. It is a major cell component, and it is distributed

Position		-12	-11		-9	-8	-7			-4		-2	-1
MK………..		L_{70}	S_{53}	-	K_{44}	E_{70}	L_{72}	-	-	I_{58}	-	G_{100}	G_{90}
		V_{14}	T_{17}		E_{16}		M_{21}			V_{35}			
		I_{14}											
		$*$	$\Diamond$	-	$\Diamond$	$\Diamond$	$*$	-	-	$*$	-	G	G
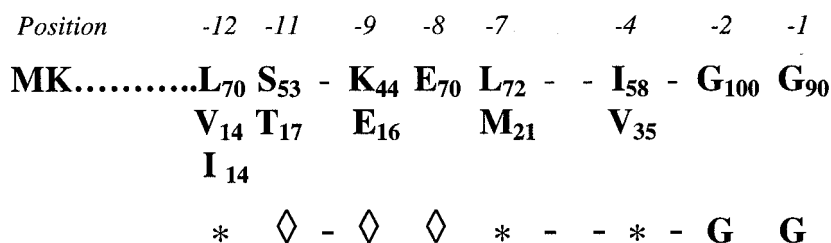
Figure 1   The consensus sequence of the double-glycine leader. The consensus is based on the alignment of 43 individual leader sequences that are between 15 and 30 amino acid residues long. The frequency (percentage) of specific amino acids in the various consensus positions is indicated in the suffix. The consensus sequence is also based on defined spacing (–) and hydrophobic ($*$) and hydrophilic ($\Diamond$) residues.

equally between the cytosol and the cell membrane (94,95). A minor fraction, less than 1% of the carnobacteriocin B2 immunity protein, was found associated with the cell membrane (106). Epitope mapping of the lactococcin A immunity protein in normal and inside-out vesicles suggested a model for the association of the protein with the cell membrane. The model proposes that a transmembrane α-helix traverses the cell membrane in such a way that the C-terminal part of the immunity protein is on the outside of the cell and that the protein somehow interacts with a putative receptor for lactococcin A, thereby preventing pore formation by the bacteriocin (94). However, exposing sensitive cells to either the lactococcin A or the carnobacteriocin B2 immunity protein did not protect the cells from the corresponding bacteriocin, suggesting that these immunity proteins do not protect cells by simply binding to the bacteriocins or to externally exposed domains on the cell surface (95,106).

Computer prediction programs indicate that many immunity proteins have transmembrane helices. The putative immunity proteins of the two-peptide bacteriocins lactococcin MN (17), lactococcin G (74), and plantaricin JK (59) may contain four or five transmembrane helices, while the putative immunity proteins of thermophilin 13 (55), plantaricin S (56), and brochocin C (109) appear to contain two transmembrane helices. Thus, the number of transmembrane helices seems to differ among immunity proteins, although a common mechanism for bacteriocin immunity involving interactions with membrane components may nevertheless exist.

C. ABC Transporter Genes

Secretion of bacteriocins containing a double-glycine leader is mediated by a membrane-associated ABC transporter, which belongs to the ATP-binding cassette (ABC) transporter superfamily (99,110). This superfamily is probably the largest and most diverse family of proteins that mediate the selective movement of solutes across biological membranes. Transporters that belong to this superfamily are found in both eukaryotic and prokaryotic cells. As a group, ABC transporters import or export a great variety of compounds, such as inorganic ions, sugars, drugs, amino acids, and polypeptides. Each individual ABC transporter is, however, very specific with respect to the substances it transports

across membranes. These transporters are, consequently, often referred to as dedicated transporters. All ABC transporters share a common domain organization, which may involve two or more separate polypeptides or one large multidomain polypeptide. A typical ABC transporter consists of a hydrophobic, multiple-spanning membrane domain, usually in the "two-times-six" format, and two ATP-binding domains (110).

The gene encoding the pediocin PA-1 transporter was the first LAB bacteriocin ABC transporter gene described (27). Since then, numerous bacteriocin ABC transporter genes have been characterized (99), and it appears that most of the more than 40 known LAB bacteriocins (including Class I bacteriocins) and bacteriocin-like peptides (including peptide pheromones) are secreted by ABC transporters (99). Exceptions are the five bacteriocins (enterocin P, bacteriocin 31, acidocin B, divergicin A, and lactococcin 972) that have been shown to be encoded with a *sec*-dependent leader and that presumably are secreted by the *sec*-dependent translocation system (see Table 1). The *sec*-dependent secretion of bacteriocins has not been studied in any detail and will not be discussed further.

The gene that encodes a bacteriocin ABC transporter is in a few cases located in the same operon as the bacteriocin structural gene. Most often, however, the transporter gene is part of a separate operon together with the gene encoding the accessory protein. This is the case in *L. plantarum* C11, which produces at least two bacteriocins, plantaricin EF and JK (59). The structural genes for these two two-peptide bacteriocins are in two separate operons together with their respective immunity genes, whereas a third operon contains the genes encoding the bacteriocin ABC transporter and the accessory protein together with at least four additional open reading frames (ORFs) of unknown function (see Fig. 2) (59). In this strain, the same ABC transporter apparently translocates six peptides, two two-peptide bacteriocins, one bacteriocin-like peptide, and a peptide-pheromone, all of which are synthesized in a pre-form with double-glycine leaders.

A unique feature of ABC transporters that translocate bacteriocins is an N-terminal extension of approximately 150 residues not found in other ABC transporters. Functional studies using the N-terminal region of the lactococcin G ABC transporter revealed that this region is able to specifically cleave off the lactococcin G leader
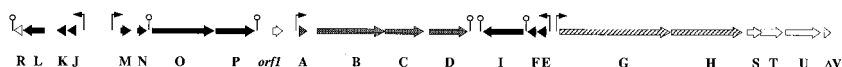
Figure 2 Genetic organization of the five operons involved in bacteriocin synthesis in *L. plantarum* C11 (59). The genes are denoted with capital letters. The bent arrow and the lollipop denote regulatory promoters and stem-loop structures, respectively. The *pln*ABCD genes that encode for the pheromone, histidine protein kinase, and the two response regulators, respectively, constitute a three-component regulatory system. The transport operon contains the *pln*GHSTUV genes that encode the ABC transporter, the accessory protein, and three putative proteins of unknown function, respectively. The three bacteriocin operons, each containing a potential immunity-encoding gene, are *pln*JKL, *pln*EFI, and *pln*MNOP.

sequence at the C terminus of the double-glycine motif (99). Moreover, in vivo studies on the pediocin PA-1 ABC transporter also indicate that the N-terminal region of this transporter participates in the proteolytic removal of the bacteriocin leader sequence (107). The proteolytic domain associated with these ABC transporters appears to belong to the cysteine protease family. It is of particular interest to note that within Archea, the organism *Methanobacterium thermoautotrophicum* has an *orf* that encodes a putative protein with approximately 32% identity with the proteolytic domain of the protease-containing dedicated bacteriocin ABC transporters (111). This finding suggests that these ABC transporters have been formed by the fusion of such a protease and an ABC transporter without a proteolytic domain.

It is thought that the bacteriocin ABC transporters exist as homodimers in the membrane, as proposed by Higgins (110), with each monomer having six or four transmembrane segments. When a reporter protein was fused to various parts of the ABC transporter of lactococcin A, it was shown that both the N-terminal proteolytic and C-terminal ATP binding domains are on the cytosolic side of the membrane (112). Results from the same study also suggested that there are only four transmembrane segments, not six as predicted by computer sequence analysis. Thus, the number of transmembrane segments is still somewhat uncertain. Considerable conformational changes may take place during the transport process, leading to two different topo-

logical states of the transmembrane domain. Additional experiments will be required to understand conclusively how bacteriocin ABC transporters function and are organized in the membrane.

D. Accessory Genes and Proteins

Another protein required for successful transport of bacteriocins with a double-glycine leader is the accessory protein, whose function has not been entirely clarified. It has been shown that both lactococcin A and pediocin PA-1 require a functional accessory protein to be externalized (104,113). The accessory proteins usually contain approximately 470 residues (11), one exception being the putative accessory protein for pediocin PA-1, which has only 174 residues (27). The gene encoding the accessory protein is located next to the ABC transporter gene and the two are transcribed together, either as part of a bacteriocin-immunity operon, as part of a regulatory operon (see the next section on regulatory operons), or as part of an operon that contains only these two genes.

Computer-assisted hydrophobicity analysis of the accessory protein for lactococcin A predicts an N-terminal transmembrane domain (113). The presence of this transmembrane domain was confirmed in a topological study using accessory protein for lactococcin A fused to a reporter protein (113). The results suggested the following model for the organization of the accessory protein in the membrane. A short stretch of the N-terminal part (about 20 residues) is on the intracellular side of the membrane, and this is followed by a transmembrane domain (from residue 22 to residue 43), while the remaining and major part of the protein is exposed to the extracellular side of the membrane (113). The model also suggests that two accessory proteins are included in a secretion complex with two ABC transporters.

V. PHEROMONE REGULATION OF BACTERIOCIN PRODUCTION—A QUORUM SENSING SYSTEM

The production of many Class II bacteriocins (such as sakacin A and P, carnobacteriocin B2, enterocin A and B, and plantaricin EF and JK) is transcriptionally regulated through a signal transduction system that

consists of a peptide-pheromone (frequently referred to as an inducing factor or induction peptide), a histidine protein kinase, and a response regulator. The two latter components constitute a two-component regulatory system (reviewed in refs. 11, 114, and 115). The pheromone is a bacteriocin-like peptide synthesized with a double-glycine leader. Examples are plantaricin A (26 amino acids), which induces the production of plantaricin EF and JK (116,117), and the 19-, 23-, 24-, and 25-mer peptides that induce the production of sakacin P, sakacin A, carnobacteriocin B2, and enterocin A, respectively (11,32,35,42,43,118,119). These bacteriocin-like pheromones are encoded by genes located in a regulatory operon that also contains the genes encoding a two-component regulatory system (a histidine protein kinase and a response regulator), which, together with the peptide pheromone, has been referred to as a three-component regulatory system. One Class II bacteriocin, carnobacteriocin B2, has been shown to possess some induction activity (119). In this context, it is interesting that the pheromone, plantaricin A, shows some antimicrobial activity (117). The production of plantaricin S may also be regulated (56). The DNA sequencing of plantaricin S operons revealed a two-component regulatory system, but no peptide pheromone gene was identified, and it was speculated that the regulation was more analogous to the *agr* system in *Staphylococcus aureus* (56, 120). However, no experimental data showing that bacteriocin production can be turned on and off by the dilution techniques used for other bacteriocins (116) have been presented. Presently, only the two regulatory genes in the vicinity of the bacteriocin genes support the theory that plantaricin S synthesis is regulated.

Bacteriocin production is activated through three-component regulatory systems when the concentration of the pheromone reaches a threshold value as a result of high cell density and/or changes in environmental conditions. In addition to activating bacteriocin production, the accumulated pheromone induces its own production. Thus, an autoinduction loop is activated, resulting in a rapid increase in the transcription of all genes involved in bacteriocin production and regulation. Signal transduction occurs through the histidine kinase (which is presumed to function as a receptor for the pheromone), which after autophosphorylation transfers a phosphate group to the response regulator. The phosphorylated response regulator then activates

transcription by binding to regulated promoters in front of the bacteriocin genes (121).

VI. PERSPECTIVE

In recent years the frequency of antibiotic-resistant bacteria causing human and animal diseases has increased dramatically. These antibiotic-resistant bacteria are found in our environment, including food, and they represent a serious health problem. There is increasing concern about the use of synthetic chemicals as food preservatives due to their toxicity. Consequently, there is a demand for new "antibiotic agents" and more natural preservatives. The LAB bacteriocins exert their bactericidal activity in a manner entirely different from those of antibiotics and preservatives. Thus, they might be developed as an alternative or a supplement to traditional antibiotics and chemical preservatives. The LAB bacteriocin, nisin, has been used prophylactically to prevent bacterial infection in the cow udder in order to avoid mastitis. Recently, another lantibiotic, lacticin 3147, has also been shown to efficiently prevent growth of mastitis pathogens and is presently undergoing further trials (122).

Several problems must be addressed before LAB and LAB bacteriocins can replace or supplement presently used preservatives and antibiotics. Although it has been demonstrated quite convincingly under laboratory conditions that many LAB bacteriocins are able to efficiently eliminate bacteria that represent a potential hazard to food safety and human health, the same bacteriocins do not efficiently kill these bacteria in food. There are at least two main reasons for this, and both are connected to the physicochemical properties of bacteriocins. Firstly, the bacteriocins are cationic and thus stick strongly to negatively charged material. Secondly, the bacteriocins are hydrophobic and/or amphiphilic and thus absorb strongly to lipid-like material that is quite common in food. Consequently, bacteriocins are absorbed to the food matrix and thereby removed from the water phase where the bacteria are found. The actual bacteriocin concentration in the environment of the bacteria is therefore much lower than expected. More optimal bacteriocin variants may possibly be constructed. Recently, a sakacin P variant that functions at higher temperatures was constructed

by connecting the C terminus and the middle region with a disulfide bridge (87), and a more stable pediocin PA-1 variant less prone to oxidative inactivation was constructed by replacing a methionine residue with an alanine, isoleucine, or leucine residue (123).

When it comes to LAB starter cultures and bacteriocin production, it has been shown that production of bacteriocins may be influenced by growth condition (87,124–127). The composition of the bacterial growth components, temperature, ionic strength, and pH all affect bacteriocin production. The synthesis of many bacteriocins is regulated at the transcriptional level and can be permanently turned off if the growth conditions do not comply with the conditions that are required for bacteriocin synthesis (116). In spite of these obstacles, experiments show that several bacteriocins and bacteriocin-producing starter cultures do reduce the number and/or inhibit the growth of many potential pathogenic and food-spoiling bacteria in food and feed and that the LAB bacteriocins have a great potential as antibacterial agents (128,129).

Note Added in Proof: Two new pediocin-like bacteriocins [coagulin (130) and sakacin 5X (131)] were recently reported.

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5
Insect Cationic Antimicrobial Peptides

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I. DISCOVERY OF INSECT PEPTIDES

Insects have been particularly successful in evolution, and current estimates are that they represent three-quarters of all extant animal species. With the marked exception of the seas, insects occupy nearly all ecological niches on earth and hence are confronted by innumerable potential pathogenic bacteria, viruses, fungi, and protozoan and helminth parasites. Not surprisingly, therefore, insects have developed efficient host defense mechanisms.

Studies by Metchnikow (1) and Cuénot (2) at the end of the nineteenth century highlighted the role of hemocytes in this resistance. A more complex picture evolved in the 1920s, when several investigators showed that insects could be immunized against lethal doses of bacteria by a previous injection of either heat-attenuated cultures or low doses of virulent cultures (3). The induced protection was apparent as early as 3 h after injection. It provided a relatively broad spectrum of protection and was paralleled by the appearance of a strong heat-stable bacteriolytic activity in the hemolymph (4,5).

Surprisingly, these promising studies almost fell into oblivion and the field of invertebrate host defense was all but deserted until the late 1970s, when H. G. Boman and associates in Stockholm began a series of investigations on the defense reactions in *Drosophila* (6) and, later, in the Cecropia moth (*Hyalophora cecropia*) (7). These authors observed that injection of bacteria into pupae of this moth resulted in the synthesis of immune proteins. They purified several of these proteins and characterized two novel classes of antibacterial peptides, which they called cecropins (7) and attacins (8). The time was then ripe for large-scale isolation of immune-induced molecules in various insect species, and, in the years that followed, an unexpectedly wide variety of inducible antimicrobial peptides were isolated in several laboratories from various insect sources; their number now exceeds 150. They are produced predominantly in the fat body, a functional equivalent of the mammalian liver, and their synthesis is induced within hours following a septic injury. They are secreted into the hemolymph, accounting for the inducible bacteriolytic activity evidenced by the earlier authors. Recent studies have shown that in addition to this defense reaction, now referred to as systemic immune response, a local immune reaction is observed in various epithelia, such as tracheal epithelia, gut epithelia, genital tracts and others (9–12).

The current view is that the insect host defense is multifaceted and involves, in addition to the rapid and transient synthesis of a battery of potent, small cationic antimicrobial peptides, cellular reactions, namely, phagocytosis and capsule formation by blood cells. Additional defense reactions in insects are blood coagulation and melanization, which occur at the sites of injury as a result of almost immediate activation of proteolytic cascades. We speculate that some of the products of these cascades can activate the synthesis of antimicrobial peptides in the fat body and blood cells.

II. STRUCTURAL CLASSES OF ANTIMICROBIAL INSECT PEPTIDES

The antimicrobial peptides of insects are frequently grouped for convenience into four classes: (a) peptides forming amphipathic α-helices;

(b) β-sheet peptides with intramolecular disulfide bridges; (c) proline-rich antibacterial peptides; and (d) glycine-rich polypeptides. In the following, we will restrict our presentation to those families for which new structural information has been obtained during the last three years. A complete list of antimicrobial peptide sequences is presented in the Trieste library (13). The reader is also referred to the recent reviews by Hetru et al. (14,15).

A. Peptides Forming Amphipathic α-Helices— Cecropins

The only known inducible insect antimicrobial peptides consisting of amphipathic α-helices are the cecropins. Cecropins are 31- to 39-residue peptides devoid of cysteines. They have a strongly basic N-terminal region and a long hydrophobic stretch in the C-terminal half. The three-dimensional structure of cecropins, as deduced from nuclear magnetic resonance (NMR) studies in water-trifluoroethanol solution (16), consists of two α-helices joined by a hinge region containing a glycine-proline doublet. The N-terminal α-helix is almost perfectly amphipathic. Since the initial report on *Hyalophora* cecropin in 1981 (7), up to 28 cecropin isoforms have been isolated from the blood of bacteria-challenged insects belonging to various species of Lepidoptera and Diptera (Table 1). In spite of several detailed investigations, cecropins have not been reported to date from other insect orders. Cecropins are highly active [minimal inhibitory concentrations, (MICs) in the low micromolar range] against several gram-positive and gram-negative bacteria. Recently a new cecropin has been isolated from a cell line of *Aedes albopictus* (17); the sequence of this molecule is significantly different from those of all other reported cecropins (cf. Table 1).

B. Peptides with Intramolecular Disulfide Bridges

Insects produce several types of antimicrobial peptides with disulfide bridges, namely, the insect defensins. Below we will describe thanatin, a peptide with a single disulfide bridge; androctonin, which contains two bridges, insect defensins, with three bridges, and, finally, drosomycin, which carries four bridges.

Table 1 Peptide Sequences of Cecropins

Peptide							
Hyalophora cecropia A[7]	KW - - KLF	KKI	EKV	GQNI	RDGIIKAGPAVAVVGQ	ATQI - - -AK	
Hyalophora cecropia B[7]	KW - - KVF	KKI	EKM	GRNI	RNGIVKAGPAAVLGE	AKAL	
Hyalophora cecropia D[18]	- W - - NPF	KEL	EKV	GQRV	RDAVISAGPAVATVAQ	ATAL - - -AK	
Antheraea pernyi B[19,20]	KW - - KIF	KKI	EKV	GRNI	RNGIIKAGPAVAVLGE	AKAL	
Antheraea pernyi D[19]	- W - - NPF	KEL	ERA	GQRV	RDAVISAGRPVATVAQ	ATAL - - AK	
Manduca sexta B2[21]	- W - - NPF	KEL	ERA	GQRV	RDAVISAAPAVATVSQ	AAAI - - AR	
Manduca sexta B3[21]	- W - - NPF	KEL	ERA	GQRV	RDAIISAGPAVATVGQ	AAAI - - AR	
Manduca sexta B4[21]	- W - - NPF	KEL	ERA	GQRV	RDAIISAAPAVATVGQ	AAAI - - AR	
Manduca sexta B5P[21]	- W - - NPF	KEL	ERA	GQRV	RDAVITSAAAVATVGQ	AAAI - - AR	
Bombyx mori CMIV[22]	RW - - KIF	KKI	EKV	GQNI	RDGIVKAGPAVAVVGQAATI		
Bombyx mori A[23]	RW - - KIF	KKI	EKV	GRNV	RDGLIKAGPAIAVIGQ	AKSL	
Bombyx mori B[23]	RW - - KIF	KKI	EKM	GRNI	RDGIVKAGPAIEVLGS	AKAI	
Bombyx mori moricin[24]	PW - - NIF	KEI	ERA	VART	RDAVISAGPAVRTVAA	ATSVAS	
Hyphantria cunea IIID[25]	RW - - KIF	KKI	ERV	GQNVRDGIIKAGPAIQVLGT		AKAL	
Hyphantria cunea IIIE[25]	RW - - KFF	KKI	ERV	GQNVRDGLIKAGPAIQVLGA		AKAL	
Hyphantria cunea IIIF[25]	RW - - KVF	KKI	EKB	GRNI	RDGVIKAGPAIAVVGQ	AKAL	
Hyphantria cunea IIIG[25]	RW - - KVF	KKI	EKB	GRHI	RDGVIKAGPAITVVGQ	ATAL	
Trichoplusia ni[26]	RW - - KFF	KKI	EKV	GQNI	RDGIIKAGPAVAVVGQ	AASIT	
Drosophila melanogaster A[27]	GWLK KIG	KKI	ERV	GQHT	RDATIQ - GLGIA - - QQ	AANVAATAR	
Drosophila melanogaster B[27]	GWLR KLG	KKI	ERI	GQHT	RDASIQ - VLGIA - - QQ	AANVAATAR	
Drosophila melanogaster C[28]	GWLK KLG	KRI	ERI	GQHT	RDATIQ - GLGIA - - QQ	AANVAATAR	
Sarcophaga peregrina IA[29]	GWLK KIG	KKI	ERV	GQHT	RDATIQ - GLGIA - - QQ	AANVAATAR	
Sarcophaga peregrina IB[29]	GWLK KIG	KKI	ERV	GQHT	RDATIQ - VIGVA - - QQ	AANVAATAR	
Sarcophaga peregrina IC[29]	GWLK KIG	KKI	ERV	GQHT	RDATIQ - VLGIA - - QQ	AANVAATAR	
Sarcophaga peregrina ID[30]	GWIRDFG	KRI	ERV	GQHT	RDATIQ - TIAVA - - -QQ	AANVAATLK	
Ceratitis Capitata A[31]	GWLK KIG	KKI	ERV	GQHT	RDATIQ - TIAVA - - -QQ	AANVAATARG	
Ceratitis Capitata B[31]	GWLK KIG	KKI	ERV	GQHT	RDATIQ - TIAVA - - -QQ	AANVAATLKG	
Aedes albopictus[17]	GGLK KLG	KKL	EGV	GKRVFKASEK - ALPV - - - - -AVGIKALG			

Gaps are introduced to maximize identities. Conserved residues are in shaded boxes. The superscript numbers indicate the references. All the cecropins are C-terminally amidated.

1. Thanatin

Thanatin is an antimicrobial peptide isolated from the hemipteran insect *Podisus maculiventris* (32). Thanatin has no sequence homology with other insect defense molecules but shows striking similarities to the brevinins, a family of antimicrobial peptides isolated from frog skin secretions (33). The overall sequence homology between thanatin and brevinin-1 is close to 50% (Table 2), and both contain a C-terminal loop of six (thanatin) or five (brevinin) residues formed by an intramolecular disulfide bridge. This loop

Table 2 Amino Acid Sequence Comparison of Thanatin from the Hemipteran *Podisus maculiventris* (32) and Brevinin - 1 from the Amphibian *Rana brevipoda* (33)

Thanatin	-	-	-	-	-	-	G	-	S	K	K	P	V	P	I	I	Y	C	N	R	R	T	G	K	C	Q	R	M
Brevinin-1	F	L	P	V	L	A	G	I	A	A	K	V	V	P	A	L	F	C	-	K	I	T	K		K	C		

Gaps are introduced to maximize identities. Common amino acids are in shaded boxes. Connectivities of cysteines are drawn.

carries a strong positive charge in both molecules and contains a central threonine residue, which separates two subgroups of electropositivity. A similar motif (two C-terminally located cysteines flanking a group of charged residues separated by a Thr or a Ser residue) has been described in other frog skin antimicrobial peptides, e.g., esculentins, gaegurins, and ranalexin, and has been dubbed the *Rana* box. The insect box differs from the *Rana* box by addition of an Asn residue within the loop (34).

Thanatin has a broad spectrum of activity against gram-positive and gram-negative bacteria and against fungi. The activity is observed at concentrations ranging from 0.5 to 10 µM, which correspond to those found in the blood of immune-challenged insects. Thanatin is both bactericidal and fungicidal at these concentrations. Thanatin is not hemolytic, as is also the case for brevinins.

Preliminary results indicate that the cytoplasmic membrane is not the target of thanatin and that this molecule, in contrast to insect defensins and cecropins, is not a pore-forming peptide. Moreover, results obtained with all D-thanatin strongly suggest that more than one mechanism of activity underlies the killing of bacteria and fungi by thanatin (32).

Two-dimensional ^{1}H-NMR spectroscopy and molecular modeling indicate that thanatin adopts a well-defined antiparallel β-sheet structure from residue 8 to the C terminus (Fig. 1) (residue 21), including the disulfide bridge (35). In spite of the presence of two proline residues, there appears to be a large degree of structural variability in the N-terminal segment.
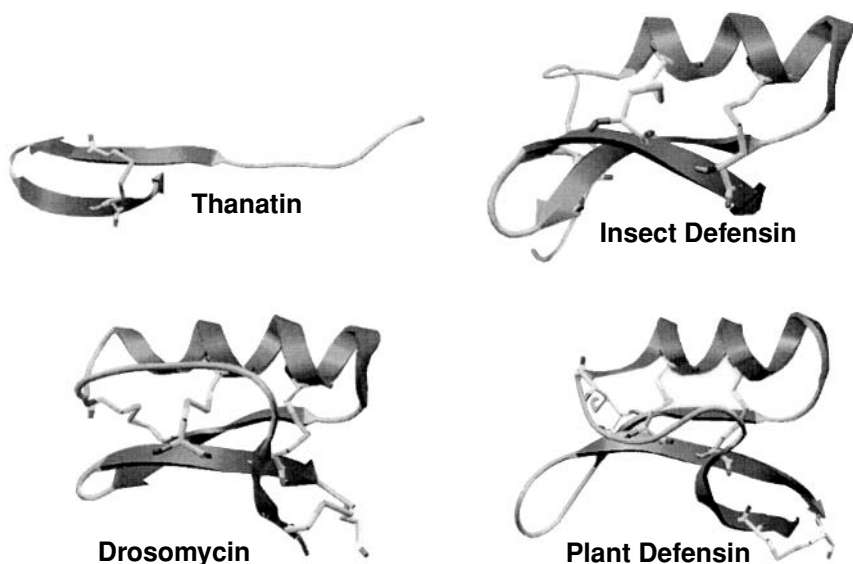
Figure 1   Ribbon diagram of the three-dimensional structures of insect-derived peptides that incorporate β-strands (arrows), and in three cases a single α-helix (coils), stabilized by one, three, or four disulfide bridges.

2.   Androctonin

Recently a 25-residue antimicrobial peptide has been isolated from the blood of the scorpion *Androctonus australis* (36). This peptide has four cysteines engaged in two intramolecular disulfide bridges with Cys 4 linked to Cys 20 and Cys 10 linked to Cys 16 (Table 3). Androctonin is highly cationic, with a calculated pI of 10.2, and contains eight positive charges including a cluster of three Arg residues linked to two Gly residues in the central part of the molecule. Interestingly, this motif is also observed in two defensins isolated from the blood of the scorpions *Leiurus* and *Androctonus* (Table 4). Androctonin has no sequence similarity to other insect defense molecules. However, it shows a high degree of similarity to tachyplesins, a family of cationic antimicrobial peptides from the Japanese horseshoe crab, *Tachypleus tridentatus* (37). In addition, androctonin presents some structural similarities to protegrins, which are antimicrobial peptides isolated

Table 3   Amino Acid Sequence Comparison of Androctonin from the Scorpion *Androctonus australis* (36), Protegrin 1 from Porcine Leukocytes (38), and Tachyplesin II from the Horseshoe Crab *Tachypleus tridentatus* (37)

```
Protegrin 1     R G G R L C – – – – Y C R R F – C V – – C V G R
Androctonin     R – – S V C R Q I K I C R R R G G C Y Y K C T N R P Y
Tachyplesin II  R – – – W C – – F R V C Y – R G I C Y R K C R
```

Gaps are introduced to maximize identities. Common amino acids are in shaded boxes. Connectivities, which are identical, are drawn.

from porcine leukocytes (38). The disulfide array (Cys 1–Cys 4 and Cys 2–Cys 3) is identical in the three families, suggesting a similar three-dimensional structure.

Androctonin is active against both gram-positive and gram-negative bacteria and also exhibits a broad spectrum of cidal activity against fungi. Even at high concentrations (150 μM), androctonin has no hemolytic activity against bovine or porcine red blood cells.

3. Insect Defensins

The name insect defensins was initially proposed by Lambert and associates (1989) (39) for two 4-kDa peptides, isolated from the blood of bacterial-challenged larvae of the fleshfly *Phormia terranovae*, that showed sequence similarity with mammalian defensins. In an independent study, Natori and associates showed that an embryonic cell line, NIH Sape-4, derived from another dipteran species, *Sarcophaga peregrina*, produces an insect defensin isoform that they named sapecin (30).

Most insect defensins are active against gram-positive bacteria but have little activity against gram-negative bacteria. They are not hemolytic. These molecules have a large distribution within the class of insects. Their presence has been reported from the orders of the Diptera, Coleoptera, Trichoptera, Hymenoptera, Neuroptera, Hemiptera, and Odonata (see Table 4 and references therein). They are also present in other invertebrates. Antibacterial peptides with

Table 4 The Insect Defensin Superfamily

Phormia terranovae	Defensin A[39]	– – – – – – AT CDLLS – – – GTGINHSA CAAHCL – LRGNRGGY CNG – – KGVC V C – RN

Species	Protein	Sequence
Phormia terranovae	Defensin A[39]	– – – – – – AT CDLLS – – – GTGINHSA CAAHCL – LRGNRGGY CNG – – KGVC V C – RN
	Defensin B[39]	– – – – – – AT CDLLS – – – GTGINHSA CAAHCL – LRGNRGGY CNR – – KGVC V C – RN
Sarcophaga peregrina	Sapecin A[30]	– – – – – – AT CDLLS – – – GTGINHSA CAAHCL – LRGNRGGY CNG – – KGVC V C – RN
	Sapecin B[a]	– – – – – – LT CE – ID – – – – – – – –RSL CLLHCR – LKGYLRAY CSQ – QK – VC R CVQ
	Sapecin C[a]	– – – – – – AT CDLLS – – – GIGVQHSA CALHCV – FRGNRGGY CTG – – K GIC V C – RN
Calliphora vicina	Defensin[43]	– – – – – – AT CDLLS – – – GTGANHSA CAAHCL – LRGNRGGY CNG – – KAV C V C – RN
Eristalis tenax	Defensin	– – – – – – AT CDLLS – – – FLNVNHAA CAAHCL – SKGYRGGY CDG – – KKVC N C – RN
Drosophila melanogaster	Defensin[44]	– – – – – – AT CDLLS – – – KWNWNHTA CAGHCI – AKGFKGGY CND – – KAVC V C – RN
Aedes Aegypti	Defensin A[45]	– – – – – – AT CDLLS – – – - GFGVGDSA CAAHCI – ARGNRGGY CNS – – K AVC V C – RN
	Defensin B[45]	– – – – – – AT CDLLS – – – - GFGVGDSA CAAHCI – ARGNRGGY CNS – QK – VC V C – RN
	Defensin C[45]	– – – – – – AT CDLLS – – – - GFGVGDSA CAAHCI – ARRNRGGY CNA – – KKVC N C – RNS
Stomoxys calcitrans	SMDI[10]	– – – –AAKPMGIT CDLLS – – – LWKVGHAA CAAHCL – VLGDVGGY CTK – – E GLC V C – KE
	SMD2[10]	– – – – – – – – AT CDLLS – – – MWNVNHSA CAAHCL – LLGKSGGR CND – – DAVC V C – RK
Anopeles gambiae	Defensin[46]	– – – – – – AT CDLLS – – – - GFGVGSSL CAAHCI – ARRYRGGY CNS – – K AVC V C – RN
Limnephilus stigma	Defensin[a]	– – – – – – AT CDLLS – – – GTGVGHSA CAAHCL – LRGNRGGY CNG – – KAVC V C – RN
Apis mellifera	Defensin[47]	– – – – – – VTCDLLS – – –FKGQVNDSA CAANCL – SLGKAGGH CE – – – KGVC I C – RKTSFKDLWDKRF
	Royalisin[48]	– – – – – – VTCDLLS – – –FKGQVNDSA CAANCL – SLGKAGGH CE – – – KGVC I C – RKTSFKDLWDKYF
Bombus pascuorum	Defensin[49]	– – – – – – VTCDLLS – – –IKGVAEHSA CAANCL – SMGKAGGR CE – – – N GIC L C – RKTTFKELWDKRF
Formic rufa	Defensin[50]	– – – – – – FT CDLLS – – – GAGVDHSA CAAHCI – LRGKTGGR CNS – – D RVC V C – RA
Zophobas atratus	Defensin A[51]	– – – – – – FT CDVLGFEIAGTKLNSAA CGAHCL – ALGRRGGY CNS – – K SVC V C – R
	Defensin B[51]	– – – – – – FT CDVLGFEIAGTKLNSAA CGAHCL – ALGRTGGY CNS – – K SVC V C – R

Tenebrio molitor	Tenicin 1[52] – – – – – – – – VT**C**DILSVEAKGVKLNDAA**C**AAHCL – FRGRSGGY **C**NG – – K S V**C** V **C** – R
Allomyrina dichotoma	Defensin[53]– – – – – – – – VT**C**DLLSFEAKGFAANHSL **C**AAHCL – AIGRRGGS **C**ER – – – GV **C** I **C**RR
Holotrichia diomphalia	Holotricin 1[54]– – – – – VT**C**DLLSLQIKGIAINDSA **C**AAHCL – AMRRKGGS **C**KQ – – – GV **C** V **C** – RN
	Holotricin 1A[55]– – – – – VT**C**DLLSKQIKGIAINDSA **C**AAHCL – AMRRKGGS **C**KQ – – – GV **C** V **C** – RN
	Holotricin 1B[55]– – – – VT**C**DFLSKQIKGIAINDSA **C**AAHCL – AMRRKGGS **C**KQ – – – GV **C** V **C** – RN
	Holotricin 1C[55]– – – – VT**C**DLLSFEILGVALNHSG **C**AAHCL – AITRRGGA **C**QD – – – GV **C** V **C** – RN
Pyrrhocoris apterus	Defensin[56] – – – – – – – – AT**C**DILSFQSWVTPNHAG **C**ALHCV – IKGYKGGQ **C**KI – – – –TV**C** H **C** – RR
Palomena prasina	Defensin[57] – – – – – – – – AT**C**DALSFSSKWLTVNHSA **C**AIHCL – TKGYKGGK **C**VN – – – T I **C** N **C** – RN
Podisus maculiventris	Defensin 1[32] – – – – – – – AT**C**DALSFSSKWLTINHSA **C**AIHCL – TKGYKGGR **C**MN – – – T I **C** N **C** – RN
	Defensin 2[32] – – – – – – – AT**C**DALSFSSKWLTINHSA **C**AIHCI – TKGYKGGK **C**VN – – – T I **C** L **C** – RR
Notonectes glauca	Defensin[58] – – – – – – – – AT**C**DLLSFSTPWFTANDAA **C**AGHCL – VKGYKGGK **C**RN – – – G I **C** H **C** – RN
Chrysopa perla	Defensin[a] – – – – – – – VT**C**DLMSVSTPIGSINHAA **C**AAHCL – LMGGGRRR **C**YY – – – NN**C** V **C**IRR
Aeschna cyanea	Deefensin [59] – – – – – – G F G**C**PLD – – Q – – – – – – MQ **C**HRHCQTITGRSGGY**C** S G P L K L**C** T **C**YR
Leiurus quinquestriatus	Defensin[60] – – – – – – – G F G**C**PLN – – Q – – – – – – G A **C**HRHCRSIR – RRGGY**C**AGFFKQT **C** T **C**YRN
Androctonus australis	Defensin[36] – – – – – – – G F G**C**PFN – – Q – – – – – – G A **C**HRHCRSIR – RRGGY**C**AGLFKQT **C** T **C**YR
Mytilus edulis	Defensin 1[61] – – – – – – G F G**C**PND – – – – – – – – – – YP**C**HRHCKSIPGRXGGY**C**GGRHRLR **C** T **C**
	Defensin 2[62] – – – – – – G F G**C**PND – – – – – – – – – - YP **C**HRHCKSIPGRRGGY**C**GGXHRLR **C** T **C**YR

Gaps are introduced to maximize identities. Common amino acids are in shaded boxes. Connectivity of cysteines are drawn. The superscript numbers indicate the references.
[a]S. Cherhysh, personal communication.

sequence similarities have also been found in scorpions and in the mollusc *Mytilus edulis*.

To date, 38 members of the insect defensin family have been characterized (Table 4). They are cationic peptides composed of 37 to 51 amino acid residues, and all contain a characteristic motif of six cysteines engaged in three intramolecular disulfide bridges (Fig. 1). The three-dimensional structure has been worked out in detail (40,41) for the defensin of *Phormia terranovae* (Fig. 1). The peptide has three distinct domains: (a) an N-terminal loop that has a certain degree of flexibility, (b) a central α-helix, and (c) a C-terminal antiparallel β-sheet with a turn involving three residues. The α-helix is stabilized via two disulfide bridges to one of the strands of the β-sheet and the N-terminal loop is linked via the third disulfide bridge to the other strand. The helix and the β-structure are connected by two of the three disulfide bridges forming the so-called cysteine-stabilized ββ (CSββ) motif. The structural organization of insect defensins differs markedly from that of mammalian defensins, which lack an α-helix.

Recently, two defensins have been characterized from the anterior midgut tissue of the blood-sucking fly *Stomoxys calcitrans*. The expression of these defense molecules is tissue specific: both are produced by the anterior midgut, and neither appears to be expressed in fat body cells or hemocytes (10).

4. *Drosomycin*

In response to a septic injury, larvae and adults of *Drosophila* produce considerable amounts of a 44-residue peptide containing eight cysteines engaged in four intramolecular disulfide bridges (62). This molecule exhibits potent antifungal activity but shows no activity against bacteria and has no hemolytic activity against erythrocytes. The peptide, named drosomycin, is active against a relatively broad spectrum of filamentous fungi at micromolar concentrations. Drosomycin shows striking similarities with plant defensins that are 5-kDa cysteine-rich plant antifungal peptides (63) (Table 5).

The three-dimensional structure of drosomycin in aqueous solution was determined using ^{1}H 2D-NMR (64). This structure, involving an β-helix and a twisted three-stranded β-sheet, is stabilized by four

Table 5 Amino Acid Sequence Comparison of Drosomycin (62) and the Plant Defensin Rs–AFP2 (63)

Drosomycine	D CL – – SGRYK G P C A V W D NET CRRV C – KE EGR S S GH C – – – SPSL K CWCEG – C
Rs–AFP2	ZKL CQRP SGTWS GV CG – – N NNA CKNQ C I RL EKARH GS CNYVF P A H K C I C Y F P C

Gaps are introduced to maximize identities. Common amino acids are in shaded boxes. Connectivities of cysteines are drawn.

disulfide bridges. Drosomycin, insect defensins, and plant defensins adopt a very similar architecture that results in part from the common presence of the CSββ motif (Fig. 1).

C. Proline-Rich Antibacterial Peptides

Small, proline-rich peptides, mainly active against gram-negative bacteria, have been isolated from insects of the orders of the Hymenoptera, Diptera, Hemiptera, and Lepidoptera. The hemolymph of bacteria-challenged honey bees (Hymenoptera) contains two proline-rich antibacterial peptide families, the apidaecins and the abaecins. Apidaecins form a homogeneous group of 16- to 20-residue peptides and contain up to 33% of proline residues with characteristic proline-arginine-proline or proline-histidine-proline repeats (Table 6). Abaecins are 34- to 39-residue peptides that show no obvious se-

Table 6 Peptide Sequences of the Apidaecin Family

Apis mellifera[65]	G N – – N R	P V Y I P – Q	P R P P H P R I
	G N – – N R	P V Y I P – Q	P R P P H P R L
	G N – – N R	P I Y I P – Q	P R P P H P R L
	G N - – N R	P V Y I S – Q	P R P P H P R I
Bombus pascuorum[49]	G – – – N R	P V Y I P – P	P R P P H P R L
Bombus terrestris[66]	A – - – N R	P V Y I P – P	P R P P H P R L
	– – – – N R	P V Y I P – P	P R P P H P R L
Sphericius speciosus[66]	– – – – N R	P T Y V P – A	P R P P H P R L
	– – – – N R	P T Y V P – P	P R P P H P R L
Vespula maculata[66]	G – K P – R	P Q Q V P – –	P R P P H P R L
	– – – – – R	P Q Q V P – –	P R P P H P R L
Vespula maculifrons[66]	S N K P – R	P Q Q V P – –	P R P P H P R L
	– N K P – R	P Q Q V P – –	P R P P H P R L
Coccgominus disparis[66]	G – K P N R	P R P A P I Q	P R P P H P R L
	– – – – N R	P R P A P I Q	P R P P H P R L
	G – K P N K	P R P A P I K	P R P P H P R L
	– – – – N K	P R P A P I K	P R P P H P R L
	G – K P S K	P R P A P I K	P R P P H P R L
	– – – – S K	P R P A P I K	P R P P H P R L

Gaps are introduced to maximize identities. Conserved residues are in shaded boxes. The superscript numbers indicate the references.

quence homology with apidaecins (Table 7). The *Drosophila* proline-rich peptide, metchnikowin, shows sequence similarities with abaecins. In contrast to the majority of the proline-rich peptides, metchnikowin is active against gram-positive bacteria and fungi (68). Hemiptera contain another family of proline-rich peptides named metalnikowins (Table 8). These form a group of closely related anti-gram-negative peptides that differ slightly in their three C-terminal residues. Other proline-rich peptides include indolicidin, Bac5, and Bac7 from cattle neutrophils. These peptides have characteristic circular dichroism spectra in lipidic environments similar to that observed with a poly-L-proline type II helix (basically an extended helix structure). NMR structural studies on indolicidin have indicated that it forms an extended boat-shaped structure in membrane mimicking solvents (94).

Of particular interest are proline-rich antibacterial peptides that carry *O*-glycosylated substitution(s). The first member of this group

Table 7 Peptide Sequences of the Abaecin Family

Apis mellifera abaecin[67]	YVPLPNVPQPGRRPF PTFPGQ – G PFNPKIKW – PQ– G – – Y
Bombus pascuorum abaecin[49]	FVPYNPRPGQ – SKPF PSKPGH – G PFNPKIQWY PL P NXGF
Metchnikowin A[68]	– – – – – – – – – –HRHQGP IFDTRPS PFNPN – Q – PR P GPIY
Metchnikowin B[68]	– – – – – – – – – –HRRQGP IFDTRPS PFNPN – Q – PR P GPIY

Gaps are introduced to maximize identities. Conserved residues are in shaded boxes. The superscript numbers indicate the references.

Table 8 Peptide Sequences of the Metalnikowin Family

Palomena prasina[57]	Metalnikowin I	– – V DKPD YRPRPRP P– N M
	Metalnikowin IIa	– – V DKPD YRPRPW– PRPN
	Metalnikowin IIb	– – V DKPD YRPRPW– PRNMI
	Metalnikowin III	– – V DKPD YRPRPW– PRPN M
Podisus maculiventris[12]	Metalnikowin I	– – V DKPD YRPRPW– PRPPSM
	Metalnikowin IIa	– – V DKPD YRPRPG– PRPII
	Metalnikowin IIb	– – V DKPD YRPRPG– PRPIR
	Metalnikowin III	– – V DKPD YRPRPG– PRPIIVM
Riptortus clavatus[67]	λdiff2–1	EAG DKPV YLPRPTP PRPIHPRLARE
	λdiff2–2	SPV DKGG YLPRPTP PRPVYRSRRD

Gaps are introduced to maximize identities. Conserved residues are in shaded boxes. The superscript numbers indicate the references.

Table 9 Peptide Sequences of the *O*-Glycosylated, Proline Rich Molecules

Drosocin[70]	– – – –G K P R P Y S P R	PT S H P R P I – – R V
Pyrrhocoricin[56]	– – – –V D K G S Y L P R	PT P– P R P I Y N R N
Formaecin 1[72]	– – – –G R P N P V N N K	PT P H P R – L
Formaecin 2[72]	– – – –G R P N P V N T K	PT P Y P R – L
Lebocin 1[71]	D L R F L Y P R G K L P V	PT P P P F N P K P I Y I D M G N R Y
Lebocin 3[71]	D L R F L Y P R G K L P V	PT L P P F N P K P I Y I D M G N R Y
Lebocin 4[71]	D L R F W N P R E K L P L	PT L P P F N P K P I Y I D M G N R Y

Gaps are introduced to maximize identities. Conserved residues are in shaded boxes. The superscript numbers indicate the references.

was characterized from *Drosophila* and named drosocin (70). It carries an *N*-acetylgalactosamine-galactose on a threonine residue in position 11. Other members of this family are pyrrhocoricin from the hemipteran *Pyrrhocoris* (57), lebocins from *Bombyx mori* (71), and formaecins from *Myrmecia gulosa* (72).

D. Glycine-Rich Antibacterial Polypeptides

Several large antibacterial polypeptides isolated from bacteria-challenged insects have a higher than normal percentage of glycine residues in common. These molecules include the attacins, sarcotoxins II, diptericins, and several other glycine-rich polypeptides. They are active against gram-negative bacteria, with their activity spectrum sometimes also including gram-positive cells. The grouping of these polypeptides under one heading here may be artificial. Our information on the structural aspects and the mode of action of the glycine-rich antibacterial polypeptides of insects is still poor.

III. THE ROLE OF ANTIMICROBIAL PEPTIDES IN THE HOST DEFENSE OF INSECTS

The fact that insects produce significant amounts of antimicrobial peptides in response to a septic injury has led most authors to consider that these molecules play a major role against invading microbes. Similar assumptions are made with regard to mammalian and plant antimicro-

bial peptides, although experimental evidence was lacking until recently. Because of its powerful genetics, *Drosophila* appeared to be a good model to address the question of the relevance of antimicrobial peptides in the host defense of an organism. In *Drosophila*, seven distinct antimicrobial peptides (plus their isoforms) have been characterized. They belong mostly to peptide families frequently found in the Diptera and other insect orders. Five of the peptides (attacin, cecropins, defensin, diptericin, drosocin) are antibacterial and two are antifungal (drosomycin and metchnikowins).

A genetic analysis has established that, in adults, the antifungal peptide genes are controlled by a regulatory cascade similar in part to that which controls dorsoventral patterning in the *Drosophila* embryo. In short, in the embryo an extracellular proteolytic cascade cleaves a 40-kDa cysteine-containing protein, Spaetzle, to its active form, which then interacts with a transmembrane receptor, the product of the gene *toll*. Activated *toll* induces, via the products of the *tube* and *pelle* genes, the dissociation of the inhibitor Cactus from the transactivator Dorsal. As a consequence, Dorsal, which has a nuclear localization signal, is translocated into the nucleus and activates expression of genes involved in dorsoventral patterning. This regulatory cascade shows striking similarities to the cytokine-induced activation of NF-κB during the immune response in mammals: in particular, dorsal and NF-κB belong to the Rel family of inducible transactivators and the proteins through which they are retained in the cytoplasm, i.e., IκB and cactus, show significant sequence similarities. Lemaitre and colleagues (73) showed that the immune-induced expression of drosomycin is mediated in adult insects through the Tollreceptor and via the Tube and Pelle products, leading to the dissociation of Cactus. Transactivation of the gene then can occur via the Rel proteins Dorsal or Dif. In *toll*-deficient mutants, the level of inducibility of the drosomycin gene is dramatically decreased. Antibacterial peptide genes are not strongly affected in a *toll* mutant background, but rather depend on a functional *imd* (for immune deficiency) gene. This gene is located on the right arm of the second chromosome, and its cloning is in progress. Taken together, the results achieved with *toll* and *imd* mutants indicate that different pathways lead to the activation of antibacterial and antifungal responses.

These results provided a welcome model to investigate the role of the antimicrobial peptides in the host defense under in vivo conditions. In experiments performed in wild-type and mutant adults of *Drosophila* infected with either gram-negative *Escherichia coli* or the filamentous fungus *Aspergillus fumigatus* (74), the following observations were made. Mutant *imd* flies, in which the infection-induced synthesis of antibacterial peptides is dramatically lowered, have a severely reduced survival rate when injected with *E. coli* compared to *toll*-deficient or wild-type flies; however, their resistance to infection with the fungus *A. fumigatus* is similar to that of wild-type flies. Conversely, *toll*-deficient flies, in which induction of drosomycin but not of the antibacterial peptides is severely compromised, are poorly resistant to infection by *A. fumigatus*, but show no alteration in survival rate when injected with *E. coli* compared to that observed with wild-type adults. Significantly, in *imd* mutants, the number of *E. coli* per fly increases by three magnitudes within 24 h following an infection, whereas no bacterial growth is observed in wild-type or *toll*-deficient adults. These results demonstrate that the *imd* and *toll* pathways are both essential for full antimicrobial resistance. They establish a correlation between the impairment of antifungal gene induction and reduced resistance to fungal infection and, conversely, between the impairment of antibacterial gene induction and reduced resistance to bacterial infection.

IV. ANTIBACTERIAL MECHANISM OF ACTION

Most antimicrobial peptides have been assumed to act on the bacterial cytoplasmic membrane as their primary target of action (75,76), based on the following lines of evidence obtained with both insect peptides and analogous peptides from other sources. Model membrane studies with liposomes demonstrate that these peptides cause the dispersal of liposome contents as long as the lipids constituting this membrane are negatively charged (like the cytoplasmic membranes of bacteria). Planar bilayer model membrane studies demonstrated that these peptides will insert into lipid membranes and cause discrete increases in conductance, although unlike the channels produced by formal channel-

forming proteins, the magnitude and duration of these channels are highly variable. Such studies have been criticized because they are usually performed at very high peptide to lipid ratios. An alternative "carpet" model has been proposed suggesting that the peptides align across the surface of the negatively charged membrane and, when they achieve a high enough local concentration (a carpet), cause electrostatically driven collapse of the cytoplasmic membrane barrier (77). Consistent with this latter model, it has been demonstrated that the α-helical peptides change conformation when they interact with membrane bilayers or environments mimicking that of the bilayer interior. This occurs even at low peptide to lipid ratios, and the difference in α-helicity between aqueous and membranous environments (called the Helican parameter) (78) was found, in one study on lactam-bridged peptides derived from insect-peptide templates, to be directly proportional to the antimicrobial activity of the peptide. Examination of the orientation of similar peptides at low peptide to lipid ratios has indicated that they lie parallel to the plane of the membrane (79), and orient perpendicularly only at very high concentrations (80).

However, one observation that disagrees with both models is that the action of cationic antimicrobial peptides at their minimal bactericidal concentration usually does not involve the lysis of cells (e.g., 67,81–84), or the emptying out of cell contents, as is obvious from electron micrographs (83,85,86). Indeed, there have been relatively few studies addressing the mechanism of action from the intact bacterial cell perspective. One study that addressed this problem directly involved investigation of the action of silkmoth cecropin A on lipid vesicles and on *E. coli* (81). These authors concluded that "cecropin A was potently bactericidal at concentrations which dissipated ion gradients in lipid vesicles, but much higher concentrations were required to cause release of cytoplasmic contents," even though cecropin A had been previously referred to as a lytic peptide (87). This suggests that we can discount the possibility of massive damage of cytoplasmic membranes, as suggested in some studies that used the peptides at concentrations far exceeding the minimal bactericidal concentration. Thus, if interaction with the cytoplasmic membrane did lead to lethality, it would have to be as a result of more modest changes to the membrane, for example, dissipation of cytoplasmic membrane

electrochemical gradients, as suggested by Silvestro et al. (81). How-
ever, it is known that compounds that cause mild perturbations of the
cytoplasmic membrane barrier [uncouplers, such as carbonylcyanide
m-chlorophenylhydrazone (CCCP) or 2,4–dinitrophenol, which de-
stroy membrane potential] are bacteriostatic (i.e., cause reversible in-
hibition of growth), whereas most antimicrobial peptides are
bactericidal. Furthermore, we have recently demonstrated (88,89) that
various classes of cationic antimicrobial peptides, including insect ce-
cropin–melittin hybrids, vary substantially in their ability to break
down the membrane potential of *E. coli* and have made the following
observations: (a) the depolarization of bacteria occurs gradually over a
range of concentrations, rather than precipitously at a specific concen-
tration as predicted by the carpet model, and (b) there is no absolute
correlation between the concentration of peptide causing a maximal
change in membrane potential and the MIC for killing bacteria, since
some peptides depolarize cells below the MIC, while others do not de-
polarize cells at all at the MIC. Thus it is possible that the cytoplasmic
membrane is not the final killing target for some or even most cationic
antimicrobial peptides, although the preferential interaction of such
peptides with bacterial membranes, and their ability to disrupt these
membranes at high concentrations, are well-established observations.
Another relevant observation by Matsuzaki and colleagues (90) is that
the peptides are actually able to traverse membranes. We have recently
put these observations together in a micellar aggregate model (89).
This model (Fig. 2) suggests that peptides insert and fold into the
membrane interface between the headgroups and fatty acyl chains of
the membrane phospholipids. This is assisted by the net negative
charge on the lipid headgroups of the bacterial phospholipids and by
the amphipathic nature of the folded peptides. Under the influence of
the transmembrane electrical potential gradient and at a sufficient con-
centration, they reorient perpendicular to the membrane to form trans-
membrane micelles (basically, informal aggregates that contain
irregular aqueous passages for ion movement across the membrane).
Such passages would have variable lifetime and ion permeability, as
demonstrated for most peptide studies to date in planar bilayer model
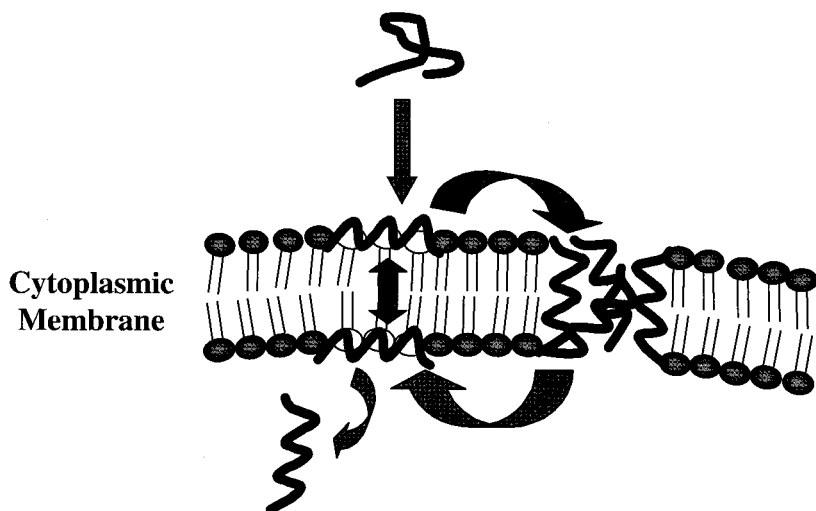
Figure 2 Micellar aggregate channel model. The peptides are proposed to bind to the surface (interfacial region) of the cytoplasmic membrane of bacteria and fold into their membrane-associated structures (for the α-helical structures or remain in their native structures for the β-stranded peptides) in such a way that the hydrophobic surface of the folded peptide inserts into the lipidic domain of the membrane and the hydrophilic and positively charged domain interacts with the negatively charged phospholipid headgroups. Given a high enough concentration and transmembrane voltage, the peptide is driven into the membrane to adopt a quasi-micellar aggregate arrangement. Such a structure may be accompanied by the formation of a hexagonal phase and is favored by the high dielectric constant of protein-containing membranes. These peptide structures are proposed to contain passageways for the movement of ions across the membrane as well as larger molecules. The structures would be unstable and, as observed in planar bilayer experiments, would have variable lifetimes, dissociating into monomers that could then reassociate with the interfacial region of either monolayer. An equilibrium would then be established between free and membrane-associated peptides. An alternative method of trans-bilayer movement of the peptides would involve flip-flop. Moderating effects on these mechanisms would involve efflux and the action of proteases.

membrane systems. This aggregate would then disperse to the interior of the membrane under the influence of the concentration gradient and the electrochemical gradient, which is internally negative. Cationic antimicrobial peptides have also been shown to cause lipid "flip-flop" from one side of the membrane to the other, and it is possible that peptides could themselves transit across the membrane without channel formation being required, explaining how some bactericidal peptides do not disrupt membrane potential at the inhibitory concentration (88,89). It is proposed that the major target could be the cytoplasmic membrane if the propensity to form these micellar aggregate channels is very high, but that for many peptides the lethal action of the peptide would be in the bacterial cytoplasm. Other targets have been suggested, including DNA or RNA synthesis (84,91), and the anionic nature of these polynucleotides makes them ideal substrates for cationic peptide binding.

For gram-negative bacteria, there is another membrane barrier to cross, the outer membrane, which is an asymmetric lipopolysaccharide:phospholipid barrier. This has been shown to occur by self-promoted uptake that involves binding to the polyanionic surface lipopolysaccharide, disruption of the outer membrane barrier, and subsequent transit of the cationic antimicrobial peptide through the weakened outer membrane barrier (92). Two consequences of this action are (a) that the outer membrane becomes permeabilized to other antimicrobial molecules, including other peptides, and (b) that the lipopolysaccharide becomes neutralized and can no longer signal by the NFκB pathway (93), at least in mammalian cells.

V. CONCLUSIONS

Insects offer an amazing variety of cationic antimicrobial peptides that they have developed as part of their arsenal of weapons against microbial pathogens. While the insect immune system has often been referred to as primitive, we prefer to think of it as a primary defense system. Clearly, the strategy of an insect is to react immediately with an arsenal of induced peptides that have overlapping spectra of activity, allowing most pathogens in modest numbers to be taken care of.

Higher eukaryotes utilize a similar system to protect themselves against daily exposure to microbes but have overlayed this with a system of exquisite specificity, the mammalian immune system, which is very effective in handling larger amounts of the pathogens but requires days to weeks to become fully operative.

One of the potential assets provided by this chemical diversity of peptide antimicrobials is a potentially novel source of antibiotics. Insect peptides serve as a template for the design of novel antibiotic peptides with very exciting potential (93). This will further encourage the continuance of efforts to discover and characterize novel peptides.

ACKNOWLEDGMENTS

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6
Mammalian Antimicrobial Peptides

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I. INTRODUCTION

A. Peptides in Innate Defense

Despite continuous challenges by a wide variety of microbes, mammals remain remarkably free from infectious diseases. The relatively long life span of mammals, compared to those of many other species in the animal kingdom, highlights the exceptional efficiency of their defensive capacity. By convention, mammalian antimicrobial defenses are grouped into the acquired (clonal) and the innate immune systems. The acquired immune system uses white cells, largely of lymphocytic lineage, to mediate highly specific antigen-directed humoral and cellular responses. These responses may lead to immunological memory providing efficient defense against subsequent infection by the same microorganism. However, on first exposure to an offending microbe the response requires days to weeks to achieve maximal activity, leaving the host vulnerable were it not for other defensive capabilities. Innate immunity, a system encompassing a complex array of defense elements mediated by both local and circulating effector cells, provides these crucial first-line host

defenses. The innate system remains ever ready or immediately inducible. The system includes receptors for recognition of microbial organisms, effector molecules for the incapacitation and elimination of pathogens, and signaling molecules that coordinate the various defensive responses, including communication with the acquired immune response. Important elements of the innate defensive system were first described many decades ago independently by Élie Metchnikoff, Alexander Fleming, and other scientific pioneers. Much current investigation is focused on elucidation of the complex details of the integrated innate immune response in mammals, but these studies have lagged behind those in lower organisms and behind experimental scrutiny focused on the lymphocyte-mediated mammalian defense.

The effector molecules of innate immunity include reactive oxidants, proteins that sequester limiting nutrients, enzymes that cleave cell walls and membranes, and peptides that can form pores in microbial membranes. The last group, collectively termed antimicrobial peptides, is evolutionarily recognized as a very old element of innate immunity. Antimicrobial peptides are found in organisms as diverse as unicellular prokaryotes and multicellular plants and animals. This chapter will focus on the antimicrobial peptides of mammals.

B. General Properties of Antimicrobial Peptides

Mammalian antimicrobial peptides have striking similarities to those discovered in plants and invertebrates by a number of criteria (for other reviews see refs. 1–5). They are gene-encoded and are initially synthesized as a prepropeptide, containing an N-terminal endoplasmic reticulum targeting sequence (also termed the signal or pre sequence), the adjacent precursor sequence (also termed the propeptide sequence), and the mature peptide at the C terminus. The mature, active peptide is liberated from the precursor by proteolytic cleavage and typically has activity against a wide array of microbes, including bacteria, fungi, and some parasites. Structurally, the peptides are generally amphipathic, and most are cationic, although there are some recently described anionic peptides. Most of the peptides have cidal activity, and the target killing is thought to be a consequence of their disruption of

membrane structure and function (6–8). The expression of many of the peptides are inducible, with signaling pathways bearing a striking similarity to those described in lower organisms. Although the possible evolutionary linkages of the ancestral genes remain highly speculative, the dramatic parallels in the structure, function, and regulation of antimicrobial peptides from the plant and animal kingdoms underscore that antimicrobial peptides are highly effective defense molecules in a wide range of biological contexts.

II. CLASSIFICATION OF ANTIMICROBIAL PEPTIDES

Mammalian antimicrobial peptides are commonly grouped according to structural features of the peptides, by the anatomical site of their expression, or by the gene family in which they are encoded. The number of mammalian antimicrobial peptides identified at the peptide and/or gene level currently exceeds 100. The complexity of this group of peptides is accentuated by the striking species-to-species variation in the peptides with respect to the aforementioned three means of classification. Given the common evolutionary origin of mammals, the complexity of antimicrobial peptide structure and distribution suggests that these host defense molecules are part of a multifaceted defensive system that is under intense selective evolutionary pressures.

A. Groupings Based on Structure of Peptides

Most mammalian antimicrobial peptides are cationic and amphiphilic. The peptides can be grouped into the structural categories first proposed by Boman (2) to classify more broadly antimicrobial peptides found throughout nature (Table 1). Many mammalian antimicrobial peptides have disulfide linkages. Defensins, the best characterized of the mammalian peptides, contain three intramolecular disulfide bonds. Each subgroup of mammalian defensin peptides, termed α-, β-, and θ-, contains six cysteines, but they differ in the pairing of the cystine bonds (9–11). Two intramolecular disulfide bonds are found in protegrins isolated from pig neutrophils (12), and a single disulfide bond characterizes bovine dodecapeptide (13).

Table 1 Structural Grouping of Mammalian Antimicrobial Peptides

Chemical class	Example(s)	Primary structure	Source (reference)
Linear helix	Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	Porcine (237)
	CRAMP	ISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE	Mouse (73)
	LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Human (14)
Linear	Bac-5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLRFP	Bovine (15)
Disproportionately rich in certain amino acids	Histatin-1	DSHEKRHHGYRRKFHEKHHSHKEFPFYGDYGSNYLYDN	Human (16)
	PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPRFP	Porcine (238)
	Prophenin	AFPPPNVPGPR(FPPPNFPGPR)$_3$FPPPNFPGPP	Porcine (17)
	Indolicidin	ILPWKWPWWPWRR	Bovine (18)
Disulfide containing	Dodecapeptide	RLCRIVVIRVCR (1–2)	Bovine (13)
	Protegrin-1	RGGRLCYCRRRFCVCVGR (1–4, 2–3)	Porcine (239)
	HNP-1 (α-defensin)	ACYCRIPACIAGERRYGTCIYQGRWAFCC (1–6, 2–4, 3–5)	Human (30,31)
	TAP (ß-defensin)	NVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK (1–5, 2–4, 3–6)	Bovine (35)
	RTD-1 (π-defensin	–GFCRCLCRRGVCRCICTR– (Macrocyclic) (1–6, 2–5, 3–4)	Macaque (11)

Source: After Refs. 2 and 210

Other mammalian antimicrobial peptides are linear. An example of a peptide that has a largely alpha-helical structure is LL-37, a peptide isolated from human neutrophils and epithelial cells (14). Other peptides are grouped because they have a preponderance of one or two amino acids. Examples are proline-rich bactenecins from bovine leukocytes (15), histidine-rich histatins (16), proline-rich prophenin from porcine leukocytes (17), and tryptophan-rich indolicidin from bovine neutrophils (18). An additional group of antimicrobial peptides that has recently been reported are strongly anionic (19,20).

Although the number of mammalian peptides within these groupings is limited, the value of this structural classification gains significance when combined with similarly classified peptides from plants, lower vertebrates, and invertebrates (2,21). Grouping antimicrobial peptides according to the gene family with which they are associated is currently very useful for the mammalian peptides. According to this classification scheme, the vast majority of mammalian peptides fall within two families: the defensins and the cathelicidins.

B. Defensins

Mammalian defensins, with over 80 peptides described to date, are characterized principally by the presence of three intramolecular disulfide bonds (for more detailed reviews see refs. 4,22, and 23). The defensins can be subdivided into two general classes, the α-defensins (22,23) and the β-defensins (24), based on (a) differences in the spacing of their six cysteines and pairing of the cysteines for disulfide bridges (9,10), (b) variation in the length of the pro segment, and (c) gene structure and chromosomal gene positioning. For α-defensins, invariant disulfide bonds form between cysteines C1–C6, C2–C4, and C3–C5 (9). For β-defensins, the pairing occurs between C1–C5, C2–C4, and C3–C6 (Figure 1A) (10). Although their disulfide linkages differ, the three-dimensional structure of both groups of peptides is very similar (25), and both types of defensins have comparable antimicrobial activities. The structural conformation of the defensins consists of three β-strands, and they have essentially no α-helical content (25–28).

First discovered in mammalian leukocytes (29–31), α-defensins also have been isolated from intestinal (32,33) and reproductive mu-

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RABBIT   Def-1      V V C A C R R A L C L P R E R R A G F C R I R G R I H P L C C R R
         Def-2      V V C A C R R A L C L P L E R R A G F C R I R G R I H P L C C R R
         Def-3a     G I C A C R R R F C P N S E R F S G Y C R V N G A R Y V R C C S R R
         Def-3b     G R C V C R K Q L L C S Y R E R R I G D C K I R G V R P P F C C P R
         Def-4      V S C T C R R F S C G F G E R A S G S C T V N G V R H T L C C R R
         Def-5      V F C T C R G F L C G S G E R A S G S C T I N G V R H T L C C R R
         RK-1       M P C S C K K Y   C D P W E V I D G S C G L F N S K Y I   C C R E K

MOUSE    CRYPT-1    L R D L V C Y C R S R G C K G R E R M N G T C R K G H L L Y T L C C R
         CRYPT-2    L R D L V C Y C R T R G C K R R E R M N G T C R K G H M L Y T L C C R
         CRYPT-3    L R D L V C Y C R K R G C K R R E R M N G T C R K G H M L Y T L C C R
         CRYPT-4    G L L C Y C R K G H C K R G E R V R G T C   G   I R F L Y C C P R R
         CRYPT-5    L S K K L I C Y C R I R G C K R R E R V F G T C R N L F L T F V F C C R
         CRYPT-6    L R D L V C Y C R A R G C K G R E R M N G T C R K G H L L Y M L C C R

HUMAN    HNP-1              A C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
         HNP-2                C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
         HNP-3              D C Y C R I P A C I A G E R R Y G T C I Y Q G R L W A F C C
         HNP-4                Y C S C R L V F C R R T E L R V G N C L I G G V S F T Y C C T R V
         HD-5   l r t s g s q a r a t C Y C R T G R C A T R E S L S G V C E I S G R L Y R L C C R
         HD-6   l r a l a s t r a f t C H C R R   S C Y S T E Y S Y G T C T V M G I N H R F C C L

α – defensins   - c - c - - - - - c - - - - - - - - - - - c - - - - - - - - - - - c c -

β – defensins   - c - - - - - - c - - - - - c - - - - c - - - - - - - - c - - - - - - c c -

HUMAN    HBD-1   L T G L G H R S D H Y N C V S S G G Q C L Y S A C P I F T K I Q G T C Y R G K A K C C K
         HBD-2           G I G D P V T C L K S G A I C H P V F C P R R Y K Q I G T C G L P G T K C C K K P

MOUSE    MBD-1   l t s l g r r t d q y k C L Q H G G F C L R S S C P S N T K L Q G T C K P D K P N C C K S
         MBD-2   l k s i g y e a e l d h C H T N G G Y C V R A I C P P S A R R P G S C F P E K N P C C K Y M K
         MBD-3             n n p v s C L R K G G R C W N R   C I G N T R Q I G S C G V P F L K C C R R K

BOVINE   TAP               N P V S C V R N K G I C V P I R C P G S M K Q I G T C V G R A V K C C R K K
         LAP      p E G V R N S Q S C R R N K G I C V P I R C P G S M R Q I G T C L G A Q V K C C R R K
         EBD      q g i s n p l s C R L N R G I C V P I R C P G N L R Q I G T C F T P S V K C C R W R
         BNBD-4   p E R V R N P Q S C R W N M G V C I P F L C R V G M R Q I G T C F G R P V C C C R R
         BNBD-13  S G I S G P L S C G R N G G V C I P I R C P V P M R Q I G T C F G R P V K C C R S W

(A)
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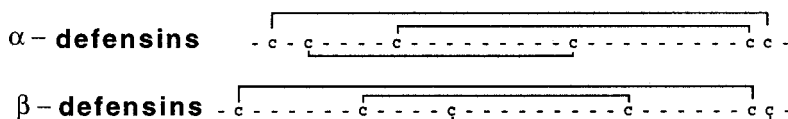
Figure 1 Primary structure of α-, β- and θ-defensins. (A) Sequence alignment of selected α- and β-defensins from rabbit, mouse, human, and bovine sources. Rabbit peptides are from hematopoietic cells (Def-1–5; ref. 190) and kidney tissue (RK-1; ref. 94). Mouse crypt defensins (cryptdins) were isolated from intestinal extracts (166–168). Human peptides were from neutrophils (HNP-1–4; refs. 30, 99, and 240) and intestine (HD-5 and -6; ref. 164). Human β-defensins were from hemofiltrate and urine (HBD-1; refs. 132, 157, and 158) and skin (HBD-2; ref. 39). Mouse β-defensins were from deduced cDNA sequences (refs. 110, 130, and 139). Bovine sequences were from trachea (TAP; ref. 35), tongue (LAP; ref. 38), colon (EBD; ref. 129), and neutrophils (BNBD-4 and -13; ref. 36). Lowercase letters indicate deduced sequences for which peptide data are unavailable or incomplete. Disulfide linkages are from refs. 9 and 10). (B) Primary structure of θ-defensin from rhesus macaque neutrophils (11).

(B)
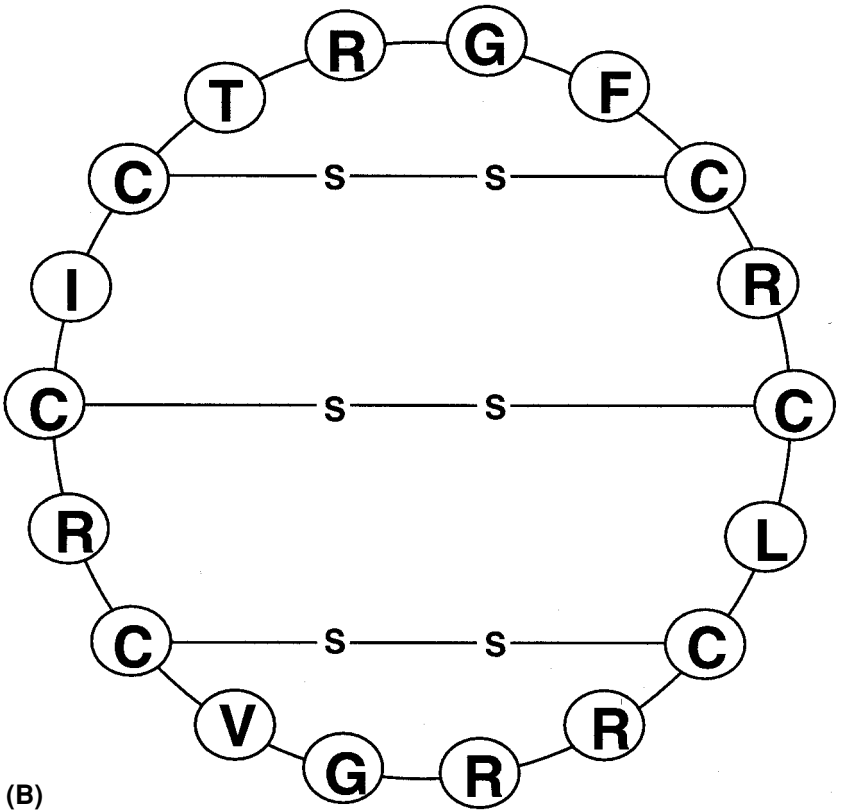
Figure 1 Continued

cosa (34). β-Defensins have been identified in several mammalian and avian species (35–37). They appear to be expressed by a greater variety of cell types than are the α-defensins (for review see ref. 24). For example, β-defensin peptides have been detected in kidney, skin, oral mucosa, and airway epithelium (35,38–43). β-Defensin mRNA has been detected in a wide array of tissues and leukocytes (44–47). Recently, a new member of the defensin family has been discovered in rhesus macaque neutrophils (11). This peptide is remarkable in that its amide-linked backbone forms a cyclic structure and represents the first of a new subfamily category, named θ-defensin (Fig. 1B)

C. Cathelicidins

By chemical structure, the cathelicidins are a remarkably diverse group of molecules. They are classified together because they are derived from prepropeptides sharing a highly conserved N-terminal propeptide segment (for reviews see refs. 48–50). The conserved propeptide segment of approximately 100 amino acids is very similar in amino acid sequence to the protein cathelin, a cysteine protease inhibitor isolated from pigs (51,52). The active antimicrobial molecule, ranging in size from 12 to 100 amino acids, is located at the C terminus of the precursor (Fig. 2). The cathelicidin gene family characterized in pigs (53–61), cattle (62–66), and sheep (61,67–69) is large, while that in humans, mice, horses, and rabbits appears to be more limited (70–74).

III. SPECTRUM OF ACTIVITY

A. Antimicrobial Activity

Many of the mammalian antimicrobial peptides have activity against a wide array of microbes, including gram-negative and gram-positive bacteria, fungi, enveloped viruses and protozoa (5,49,75). Some peptides are more restricted in their activity profiles. In some cases, small variations in peptide structure can result in notable changes in antimicrobial activity, but a clearer understanding of structure–function relationships awaits further investigation. Sensitive microassay methodologies have been developed to allow extensive assessment of antimicrobial activity using conservative amounts of peptides (76,77). The activity of antimicrobial peptides can synergize with that of other antimicrobial factors, such as bactericidal permeability-inducing protein (78). This general property may be of particular significance given the complex array of various antimicrobial factors detected in many biological contexts.

Mammalian antimicrobial peptides whose mechanism of action has been studied are thought to kill target microbes through disruption of membrane integrity (for reviews see refs. 2, 23, and 79). The peptides are cationic and amphipathic, properties that promote favorable
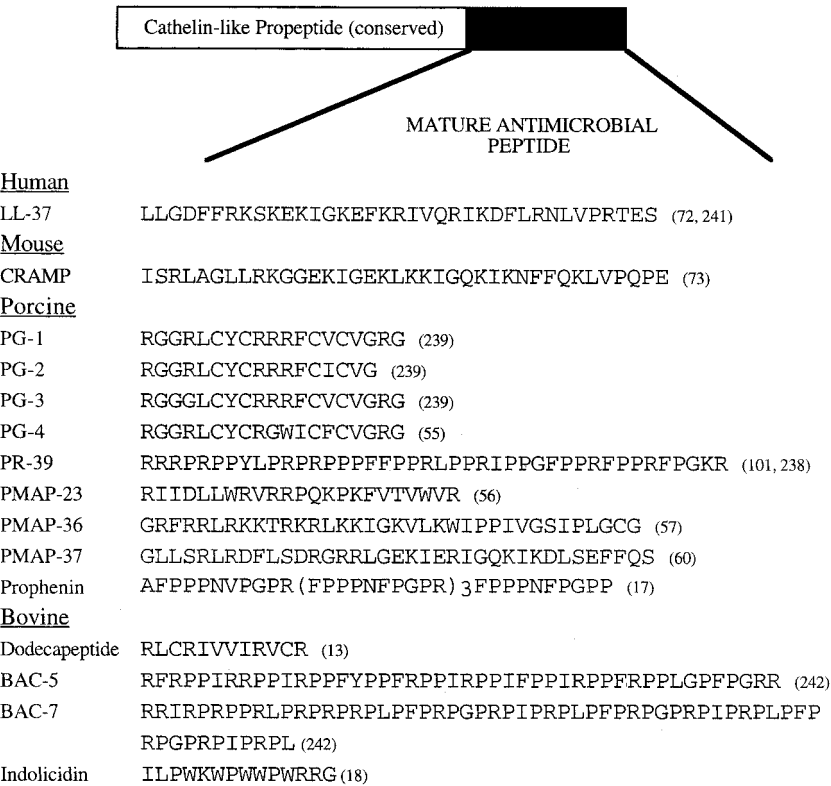
Figure 2   Primary structure of selected cathelicidin antimicrobial peptides from human, mouse, porcine, rabbit, and bovine species.

interactions with biological membranes. A leading hypothesis to explain the lethal event associated with the membrane interaction is that the loss of viability is due to the dissipation of electrochemical gradients across the disrupted membrane. An alternative possibility is that the peptide traverses the membrane and associates with a yet unidentified molecule(s), which leads to impaired function. Either of these two possibilities would provide an opportunity for the microbe to recover if the peptides were present at low concentrations or if the exposure time was brief. A more prolonged exposure to higher concentrations would be lethal, as repair processes would be overwhelmed.

Structural differences among defensins can alter their membrane activity. Analysis of the crystal structure of the human neutrophil α-defensin HNP-3 reveals a noncovalent, amphipathic dimer (80). The N and C termini of each peptide are clustered on the pole of the dimer opposite a largely hydrophobic opposing surface in the dimer, and the arginine residues extend equatorially above the hydrophobic face (80). The human neutrophilic defensin peptides induce the formation of multimeric pores in lipid bilayers whose pore structure is estimated to be 20 (81). A model in which six human neutrophil defensin dimers intercalate in the bilayer to form the pore annulus has been proposed (28). In contrast, the solution structure of rabbit neutrophil α-defensin, NP-1, reveals monomeric peptides (82). Furthermore, in contrast to the pore induced by the human α-defensins, the monomeric rabbit defensin causes graded leakage of dextran from phospholipid vesicles with the same composition (82,83). This suggests that NP-1 does not induce stable multimeric pores, but rather permeabilizes the membrane by generating large, short-lived defects in the phospholipid bilayer.

B.   Other Activities

In addition to their activity on microbes, some antimicrobial peptides have activities on host cells and may thereby impact on wound repair, inflammation, and the adaptive immune response. However, at high concentrations, antimicrobial peptides may also be cytotoxic to host cells (84). Although often emerging as attributes discovered after their initial characterization as antimicrobial agents, these properties of antimicrobial peptides may be critical to their biological role(s) in many biological responses.

Certain defensins and cathelicidins have been shown to possess chemoattractant activities (85–88). For example, human neutrophil α-defensins-1 and -2 have attractant activity for lymphocytes (88). Human neutrophil α-defensins also enhance the systemic IgG antibody response (89). The human β-defensin (hBD)-2 has chemoattractant activity for dendritic cells through interaction with the chemokine receptor CCR-6 (90). These activities highlight exciting avenues for future studies on the integration of the innate and adaptive host defense responses.

Further activities reported for defensins include inhibition of plasmin-mediated fibrinolysis and the enhancement of binding plasminogen and lipoprotein a to endothelial cells (91,92), certain ion fluxes in epithelial cells (93–95), mitogenic activity of epithelial cells and fibroblasts (96), histamine release from mast cells (97), and inhibition of adrenocorticotropin hormone–mediated cortisol release from the adrenal gland (98–100). The porcine cathelicidin PR-39 can induce upregulation of syndecans, heparan sulfate proteoglycans involved in the repair process at the site of skin wounds (101), and can inhibit neutrophil NADPH oxidase (102). Some of these functions may prove important in the coordination of a host defense response, combining direct bactericidal functions with recruitment of inflammatory cells and wound-healing activity.

IV. GENES AND TRANSCRIPTIONAL REGULATION

Antimicrobial peptides are encoded by prototypical genes (103), a feature that differentiates them from many other antibiotics in nature that are produced via multienzyme cascades. Throughout the plant and animal kingdoms, the genes encoding antimicrobial peptides typically have two or more exons and often exist as members of tightly clustered gene families. In mammals, the two predominant gene families, cathelicidins and defensins, each map to syntenic chromosomal segments, suggesting that the genes of each family have evolved divergently from their respective ancestral genes (69,104–115). In some cases the genes are adjacent to repetitive DNA sequence elements, suggesting that gene family expansion and diversification may have occurred through homologous but unequal crossover during meiosis (14,69,116–118).

A. Defensins

The defensin genes have selective tissue expression patterns. In humans and rodents, the close adjacent positioning of the genes encoding α- and β-defensin peptides (Fig. 3) is consistent with their derivation from a common ancestral sequence (109–112,115,119).
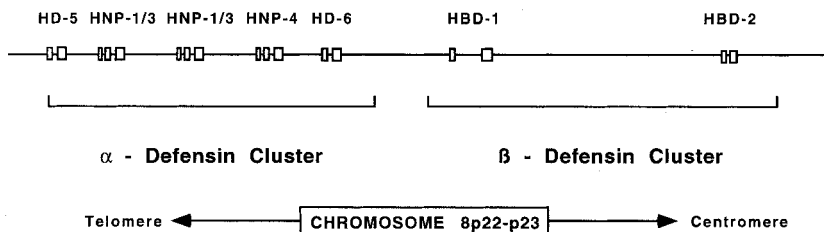
Figure 3   Genomic organization of the human defensin locus on chromosome 8p22–23. The data are from Refs. 109, 111, 114, and 119. The region represented spans approximately 0.8–1 megabase. The relative positioning of the genes is accurate, but the distances indicated are approximate. The orientation of the locus with respect to the centromere and telomere is based on experimental data, but the orientation of the individual genes is not known. The number of HNP-1/3 genes may vary among individuals (122).

The genetic mapping assignments of the human defensins is chromosome 8p22–p23 (104,107,109,111,114,120). Rodent defensins similarly map to corresponding (syntenic) chromosomal positions (105,110,112,113,115). The recently described θ-defensin of rhesus macaque neutrophils is encoded by two genes that are highly similar in structure and nucleotide sequence to the α-defensins (11). Some of the defensin-encoding genes are constitutively expressed, while others are inducible upon stimulation with infectious and inflammatory agents.

While many α-defensin genes are expressed in cells of myeloid lineage, others are expressed in epithelial cells. To date, no single gene has been found to be highly expressed in both types of cells. Interestingly, all α-defensin genes expressed in cells of hematopoietic origin have three exons, whereas those genes expressed in epithelial cells have two (Fig. 4). The θ-defensins are encoded by genes with three exons and nucleotide sequences highly similar to those of hematopoietic α-defensin genes. However, an astonishing aspect of these 18 amino acid peptides is that half of the peptide is encoded by one gene and the other half by an entirely distinct gene (11). The ligation of the two peptides to form the active peptide may reveal novel biochemical pathways for mammalian cells. The two exons of the epithelial α-defensin

α−Defensin (wbc)

Exon 1 Exon 2 Exon 3

5' 3'

Signal Propeptide Antimicrobial

θ−Defensin (wbc)

Exon 1 Exon 2 Exon 3

5' 3'

Signal Propeptide Hemi-peptide

α−Defensin (epithelial)

Exon 1 Exon 2

5' 3'
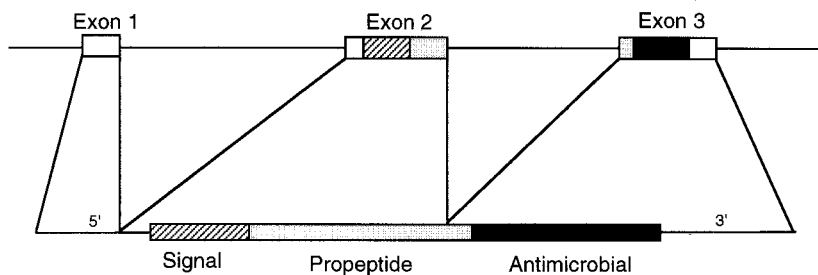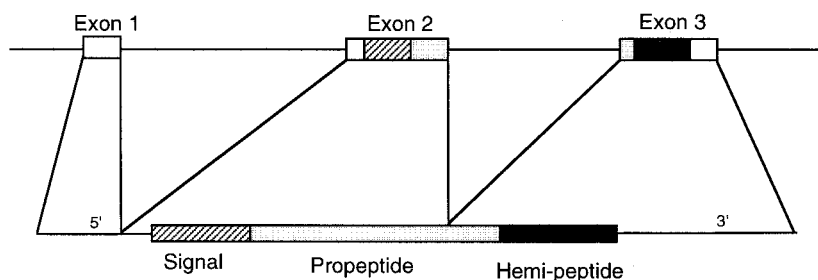
Signal Propeptide Antimicrobial
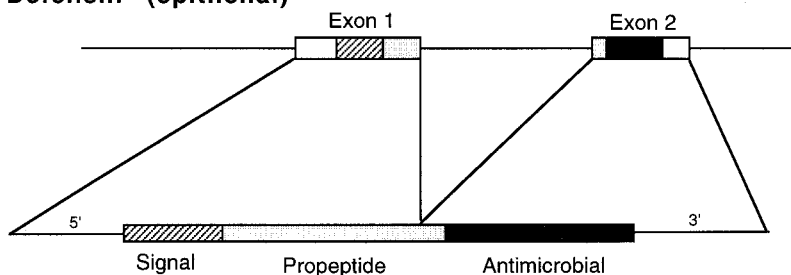
(A)

Figure 4 Genomic organization of α- and θ-defensin (A) and β-defensin (B) genes. The organization of the exons with respect to translated and untranslated regions is indicated. The hematopoietic genes (α- and θ-defensins) have three exons (11,243,244). The epithelial α-defensin and the β-defensin genes have two exons (33,116,117,131).

ß - Defensin (HBD - 1 like)

Exon 1

Exon 2

5'

3'

Signal

Antimicrobial

ß - Defensin (TAP & HBD -2 like)

Exon 1

Exon 2

5'

3'

Signal

Antimicrobial
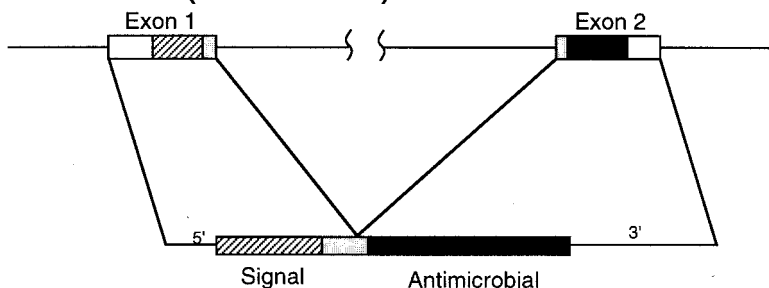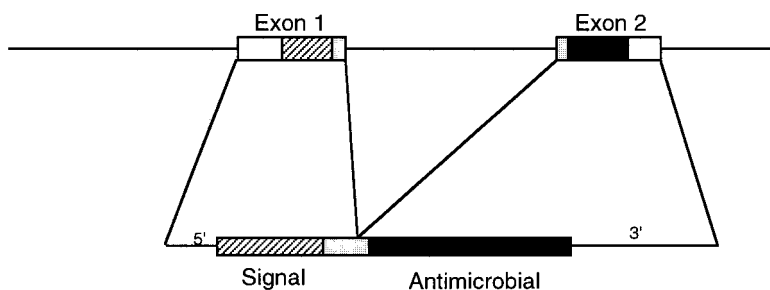
(B)

Figure 4 Continued

genes are similar in organization and sequence to the second and third of the hematopoietic defensin genes, with the most distal exon encoding the mature peptide. The highly similar exon composition of the respective hematopoietic and epithelial α-defensin genes of several mammalian species suggests that corresponding ancestral genes of each type existed prior to the evolutionary divergence of these species. Analysis of defensin sequences from five species indicates that following duplication of some defensin genes, amino acid changes favoring diversification of the mature peptides have been subject to positive Darwinian selection (121). A model for a possible evolutionary history of the human α-defensin gene family has been proposed (107). So-

matic cell hybrid mapping data indicate that the number of defensin genes on isolated copies of chromosome 8 is variable, indicating that the inheritance of defensin genes may be complex (122).

The transcription regulation of epithelial α-defensin genes has been addressed by analysis of transgenic mice. Lines of mice were produced that express transgenes consisting of 6.5 kb of DNA 5′- of the mouse intestinal α-defensin 2 (cryptdin-2) gene transcription start site upstream of a human growth hormone reporter gene. In these transgenic mice, the putative α-defensin gene promoter is capable of directing transcription of reporter genes in Paneth cells (123). Thus, 6.5 kb of DNA flanking the α-defensin gene is sufficient to direct transcriptional expression in Paneth cells. More recently, transgenic mice carrying the 3 kb of human DNA encompassing the *HD-5* gene, with 1.4 kb of 5′-flanking DNA, were found to express the peptide in Paneth cells in the same developmental sequence as the endogenous mouse genes (124). The transcription regulation of hematopoietic α-defensin genes also has been studied in cell culture (125–128).

Analysis of gene structure and expression suggests that β-defensins may be sub-divided into two groups based on sequence similarity, intron size, site of expression, and elements of genetic regulation (24). The first group is most similar to tracheal antimicrobial peptide (TAP) and includes lingual antimicrobial peptide, enteric β-defensin, human β-defensin-2, rat β-defensin-2, and mouse β-defensin-3 (38,115,119,129,130). These genes are all similar in both amino acid and nucleotide sequence, contain an intron about 2 kb in size, are expressed in various epithelial cells, and show inducible expression in response to inflammatory mediators such as lipopolysaccharide (LPS). The bovine neutrophil β-defensins have many features similar to those of these epithelial defensins (36,131). The second group includes human β-defensin-1, mouse β-defensin-1, and rat β-defensin-1 (109,110,112,113,115). These genes are similar to one another in sequence, have large introns (about 10 kb), are expressed principally in the genitourinary tract, and are not induced following challenge with inflammatory mediators. These genes are expressed at relatively lower levels in many epithelia, including the airway, gingiva, and skin (44,132–138). However, two recently identified β-defensins, mouse β-defensin 2 (139) and porcine β-defensin 1 (43,118, 140), have a mixture of features not clearly falling into either group.

This suggests that the proposed classification may require refinement as more β-defensins are characterized

The transcriptional induction of defensins was examined in greatest detail in studies focused on bovine TAP. The expression of TAP, a β-defensin in the bovine airway (35,116), was found to be upregulated by infectious agents and inflammatory mediators in primary culture systems. A 15–fold increase in the steady-state levels of mRNA encoding TAP was observed upon incubation of tracheal cells with bacterial LPS (141). The transcriptional induction occurs via activation of the p65/p50 heterodimer of the transcription factor nuclear factor-κB (NF-κB) (142), which binds to an NF-κB recognition sequence upstream from the TAP gene (143). These cells also upregulate the expression of TAP in response to other bacterial products (muramyl dipeptide and lipoteichoic acid; (see ref. 143), phorbol 12–myristate 13–acetate (PMA) (141), and several cytokines including, tumor necrosis factor-α (TNF-α) (144), IL-1β (143), and interferon- γ (G. Diamond and C. L. Bevins, unpublished results). Instillation of *Pasteurella haemolytica* into a lobe of a cow lung led to an increase in β-defensin expression in the airway epithelium that was not seen in the adjacent lobe instilled with saline (145). These data demonstrate that inflammation and infection mediate a peptide-based host response in tracheal epithelial cells through transcriptional regulation, and suggest that similar regulation of this response may occur in other mucosal tissues.

The studies on β-defensin induction were extended to other mammalian systems. In human airway cells, hBD-2 mRNA was also induced in response to *Pseudomonas aeruginosa* LPS, as well as to TNF (146) and IL-1β (40). Mouse homologues β-defensin-2 and -3 (mBD-2 and mBD-3) also undergo upregulation in response to LPS (130,139). Moreover, intratracheal instillation of *P. aeruginosa* led to increased expression of mBD-3 in the tracheal epithelium (130).

B. Cathelicidins

The cathelin domain is encoded across all four exons of the cathelicidin genes (Fig. 5), whereas the mature antimicrobial peptide (at the C-terminus) is encoded in a segment of the fourth exon (48,49,58,66, 69,106). There is a report of alternative splicing of cathelin genes that may provide a mechanism of diversification of gene expression (147).
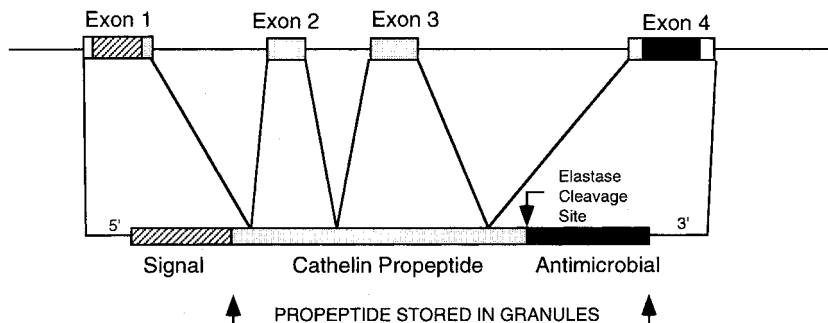
Figure 5 Genomic organization of the cathelicidin genes (48,49,58,66, 69,106). The organization of the exons with respect to translated and untranslated regions is indicated.

The nucleotide sequences encoding the cathelin domain are highly similar among cathelicidin genes (48,49). This similarity has enabled researchers to identify new cathelicidin genes in numerous species (148). To date, only one cathelicidin-like gene has been characterized in humans, LL-37 (72,106) (also termed hCAP18; (see ref. 149). Expression of LL-37 has been detected in neutrophils (14), testes (72), respiratory epithelia (150,151), and a variety of squamous epithelia (152,153). The genetic mapping assignment of the human cathelicidin LL-37 gene is chromosome 3p21.3 (106).

V. BIOSYNTHESIS AND PROCESSING

Characterization of mRNA-encoding mammalian antimicrobial peptides indicate that the peptides are synthesized as precursor molecules, which include an N-terminal endoplasmic reticulum targeting (signal) sequence. Adjacent to this signal peptide is a prosegment, which may be important for intracellular processing, charge neutralization, and/or folding of the cationic C-terminal peptide. Some peptides are stored as propeptides with posttranslational cleavage occurring extracellularly, whereas others are cleaved intracellularly and stored in lysosomal-type granules. In some cases, other posttranslational processing steps in-

clude formation of intramolecular disulfide bonding, C-terminal amidation, and N-terminal formation of pyroglutamate.

A. Defensins

The α-defensin precursor is 90–100 residues and includes a 19–20 amino acid hydrophobic signal peptide and a 40–50 residue pro-segment. Removal of the propeptide segment appears to be necessary for expression of antimicrobial activity (154,155). The principal posttranslational modifications of defensin peptides are formation of three intramolecular disulfide bonds (9,10), and proteolytic processing (4,156). The mature defensins from mouse small intestine have amino termini that are four to six residues longer than those of the various leukocyte-derived defensins, but the role of the N terminus has not been systematically examined. The θ-defensin isolated from rhesus monkey neutrophils is remarkable in that it (a) is a cyclic peptide, (b) is the product of two distinct genes, with nine amino acids from one gene and the other nine from a separate gene, and (c) contains three disulfide bonds, with one of these linkages connecting cysteines encoded by the two different genes (11). The processing steps for this peptide should prove fascinating. The β-defensins have overall structural characteristics similar to those of the α-defensin precursors, but their prepropeptides are significantly shorter, totaling 60–65 amino acids. They have a combined prepro-segment of 26–32 amino acids adjacent to the mature peptide (24). The N terminal glutamine in some mature β-defensin peptides is converted to a pyroglutamate residue (36,38). For epithelial β-defensins, generation of forms with variable N-termini was observed with renal human β-defensin-1 released into urine (157,158). Details of the processing pathways β-defensins have not been examined to date, but a chymotrypsin-like protease has been suggested to account for the N-terminal variants of hBD-1 present in urine.

In human neutrophils, defensins are stored in primary (azurophilic) granules as active, mature molecules (159,160). These granules are destined to fuse with phagolysosomes, delivering their abundant repertoire of antimicrobial factors to engulfed micobes (see section VI,A). The propeptide segment has been shown to be necessary for accurate processing and transport of the neutrophil defensin peptides to the azurophilic granules (154,160–163). The anionic characteristics of the

human neutrophil defensin propeptide segment have led to the hypothesis that it has a role in neutralization of the cationic mature peptide, thus inhibiting the activity of the peptide during processing (154).

The posttranslational processing of epithelial α-defensins has not yet been fully investigated, but it may be an important regulatory step in the generation of bioactive peptides. In the human small intestine, evidence indicates that α-defensins are stored in tissue as a propeptide(s), supporting the belief that proteolytic cleavage is a key aspect of regulating the liberation of active peptide (164). A matrix metalloproteinase, matrilysin, has been identified as an essential enzyme in the processing of mouse small intestinal defensins (cryptdins) (165). A mouse strain deficient in matrilysin was unable to process properly the α-defensins to the mature, active forms. These knockout mice had increased susceptibility to infection by orally introduced virulent *Salmonella typhimurium* (165), possibly because of a lack of biologically active defensins.

In mice, six α-defensin peptides have been purified to homogeneity from the small intestinal lumen (166–169). Peptide recoveries from intact small bowel showed that levels of intestinal isoforms differed, with approximately equivalent levels of cryptdins 1, 2, 5, and 6 and lower overall levels of cryptdins 3 and 4 (167). Humans express small intestinal defensin genes at levels equivalent to those in the mouse (33,170), but in contrast to the numerous defensin isoforms in mice, there are only two in the human (170). These two defensins are termed human defensin (HD)-5 and -6.

Isolation of HD-5 peptides from human specimens has revealed unexpected complexity in the N-terminally processed forms of this peptide (164). In studies of small intestinal tissue, at least three isoforms were detected by Western blot analysis. These studies, which used immunological detection of HD-5 rather than activity assays, indicate that HD-5 propeptides are the predominant forms of the peptide in small intestinal tissue (164). Two forms were chemically characterized in this study, which correspond to amino acids 24 to 94 and 29 to 94. The predominance of these larger forms suggests that, in contrast to neutrophil defensins, α-defensins of epithelial cells may be stored as propeptides. The HD-5 propeptides are presumably processed to mature peptides either during or after exocytic secretion, but details of this process are not yet understood. In surgically constructed neobladders from small intesti-

nal tissue, secreted forms of HD-5 were detected in the voided urine (164). Three forms, corresponding to amino acids 36 to 94, 56 to 94, and 63 to 94, were chemically characterized. The shortest form (63 to 94) is similar to a recombinant HD-5 peptide (64 to 94) that has broad-spectrum antimicrobial activity (171). The precise details of the complex processing of HD-5 are under investigation.

B.   Cathelicidins

Cathelicidins are stored in neutrophil granules as propeptides (nonantimicrobial), which are released into the extracellular space (172,173). Endoproteolytic cleavage then liberates the C-terminal antimicrobial peptide. In many cases the cleavage enzyme is neutrophil elastase (58,174,175). In bovine neutrophils, cathelicidins in inactive propeptide form have been co-localized to the same granules containing fully processed β-defensins (131).

VI.   STRATEGIC DEPLOYMENT OF MAMMALIAN ANTIMICROBIAL PEPTIDES

In mammals, antimicrobial peptides have been grouped by Ganz and Lehrer into four biological contexts (Fig. 6). As discussed below, these four categories are helpful in discussing the strategies that may be employed in utilizing antimicrobial peptides as mediators of innate host defense. Other sites of antimicrobial expression suggest that additional strategies will become evident. These sites include platelets (176), gingival epithelium (137,138), placenta (177), kidney (94,157,158,178) and reproductive tissues (34,157,179). Antimicrobial peptides have also been reported in plasma (42,132,180,181), and elevated levels in plasma and cerebrospinal fluid have been reported during the course of acute infection (42,180,182–184).

A.   Myeloid

Mammalian phagocytic cells contain various morphologically and functionally heterogeneous types of membrane-enveloped storage
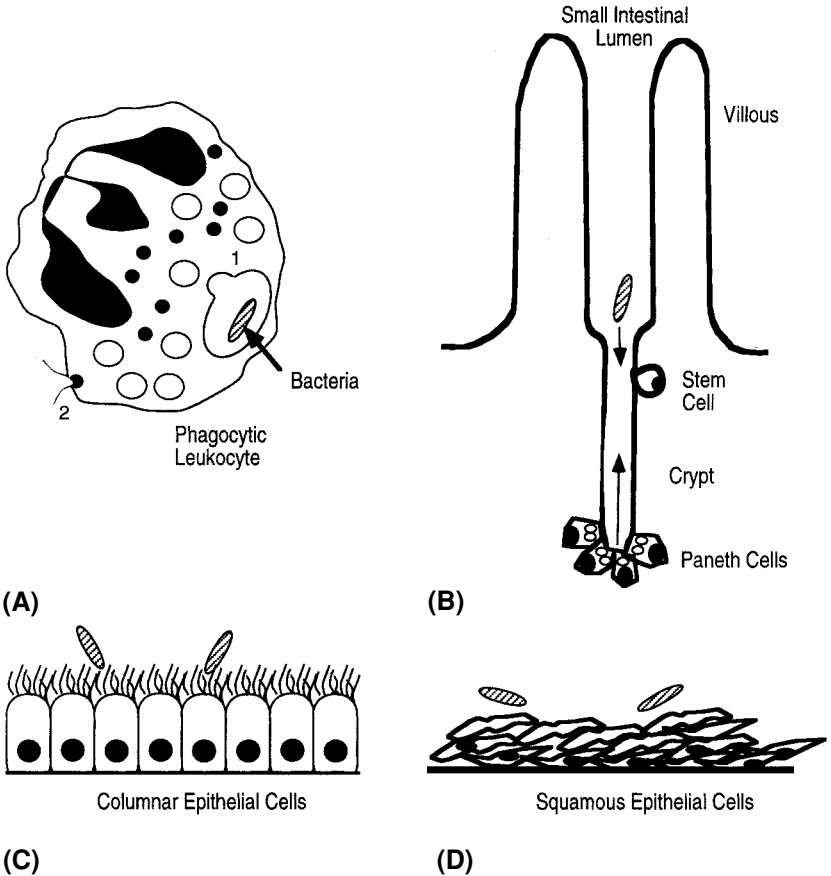
Figure 6 Four biological contexts for mammalian antimicrobial peptides (after Ref. 245). (A) Phagocytic leukocytes with release of peptides into phagolysosome (1) or extracellular space (2). (B) Small intestinal crypt lumen with release of peptides from Paneth cell granules. Epithelial stem cell at neck of crypt continuously repopulates villous and crypt epithelia. (C) Columnar epithelia cells that lack granules but are capable of secretion. Diagrammed here are cells of the respiratory tract with transcriptional induction of peptides upon challenge. (D) Squamous epithelia where peptide accumulates intracellularly. See text for details.

granules. These granules can be grouped according to biophysical properties, biochemical composition, stage of myelopoietic differentiation marking their biogenesis, and intracellular trafficking (185–187). The majority of these granules contain various antimicrobial factors (159). Early analysis of neutrophil extracts identified arginine- and cysteine-rich peptides that had broad-spectrum antimicrobial activity (188,189). Seminal studies by Lehrer and colleagues (29,30,190) identified defensins as the predominant cysteine-rich basic peptides in these cells. Microbicidal defects in neutrophils are observed in two human diseases in which the respiratory burst of these phagocytes appears normal but the microbicidal granule components are missing or defective, Chediak-Higashi syndrome and specific granule deficiency (191).

The azurophilic granules of mammalian neutrophils deliver their contents into phagolysosomes following cellular phagocytic engulfment of a microbe (Fig. 6A). High concentrations of antimicrobial substances, including α-defensins, accumulate in these vacuoles in an effort to kill the ingested microbe (192). In human neutrophils, the concentration of α-defensin that is delivered from azurophilic granules to phagolysosomes is estimated to be in excess of 10 mg/ml, three orders of magnitude greater than the mean inhibitory concentration for defensins against many bacteria (50). Only a small proportion of these peptides are released into the extracellular space during phagocytosis or in response to secretagogues (193). A second class of granules in mammalian neutrophils, specific granules, deliver their antimicrobial constituents into the extracellular space, where they may combat microbes in the local vicinity of the cell (187). For example, specific granules of pig neutrophils contain high concentrations of cathelicidins, stored as propeptides, and the cathelin-like propeptide is cleaved from the C-terminal mature antimicrobial peptide in the extracellular space (175,194). Neutrophil elastase is a protease that is reported to mediate this activation (174,175). Bovine neutrophils contain an abundance of a third type of granule, large cytoplasmic granules, which are a distinguishing characteristic of neutrophils from ruminants compared to those from nonruminant mammals (195,196). Activation of neutrophils with phorbol 12–myristate 13–acetate leads to release of their contents into the ex-

tracellular space. These granules contain the cathelicidins Bac5 and Bac7 as inactive propeptides, and their conversion to mature microbicidal peptides requires proteolytic processing (172). Recent data indicate that β-defensins exist as fully processed mature peptides in bovine neutrophils and are stored in the same large cytoplasmic granules (131). The colocalization of members of the cathelicidin and β-defensin families in the same granules suggests that there may be important synergistic and/or regulatory interactions as these molecules are released into the extracellular space (131).

B. Small Intestine

Paneth cells are granulated secretory epithelial cells residing in the bases of the crypts of Lieberkühn and are implicated in host defense of the small intestinal mucosa (197–199). The α-defensins are localized to apical granules in these cells (167,200). In addition to defensins, other antimicrobial molecules, including lysozyme and sPLA2, reside in the same granules (201–203). Paneth cell granules are released to the luminal mucosal surface consistent with their proposed functions in the crypt lumen (Fig. 6B). Intraluminal bacteria, bacterial products, and cholinergic agonists can stimulate Paneth cell secretion (201,204). The expression of Paneth cell α-defensin genes is induced in the course of pathological processes including necrotizing enterocolitis (205) and hemorrhagic shock (206).

The location of Paneth cells at the bottom of a narrow crypt suggests that a key function of their antimicrobial secretions may be protection from microbial invasion of the epithelial stem/progenitor cells, which reside at the neck of the crypts (207). If Paneth cell granules contain as much defensin peptide as do those of neutrophils, the concentration of defensins secreted into the crypt lumen could reach millimolar levels, and the defensins could serve as powerful antimicrobial agents at this site. However, to better understand their function in the crypt lumen, it will be important to determine the details of their posttranslational processing, as this seems to be a key level of regulating the liberation of active peptides (164,165). In addition, since the activity of antimicrobial peptides and proteins can often create synergistic combinations, it will be very important to determine

how the individual components of Paneth cell granules may interact if their expression is differentially regulated.

As for other peptides, the crypt defensins may have multiple biological functions. As the defensins emerge from the crypts and become more dilute (perhaps to the micromolar range), their antimicrobial activity may influence the resident flora in the intestinal lumen. Also, at even more remote locations from their site of secretion, very dilute (nanomolar) secretions could function as chemoattractants or provide other signals for host defense cells. In each of these compartments, the specific proteolytic enzymes present could potentially modify the N terminus of the α-defensins and modulate their function(s).

C. Airway

A working model suggests that transcriptional induction of β-defensins represents part of a host defense response of certain mucosal epithelial cells (Fig. 6C). The inducible expression of TAP in the respiratory epithelium is an example of such a defensive response. The TAP gene is expressed in vivo in the ciliated airway epithelium (116), and its expression levels in vivo are dramatically increased following experimentally induced bacterial infection (145). In vitro incubation of tracheal epithelial cells with heat-killed bacteria or bacterial LPS led to marked transcriptional induction of TAP mRNA levels involving the activation of the transcription factor NF-κB (141,143). The induction by LPS was mediated via epithelial cell–expressed CD14 (141), a mammalian receptor for LPS (208). Although initially characterized as a cell surface marker for cells of the monocyte/macrophage lineages (208,209), CD14 is also expressed by epithelial cells, and probably provides these cells with the capacity to detect and respond to bacteria at their luminal surface (141,146,210). These experiments demonstrate that tracheal epithelial cells challenged with bacterial products, such as LPS, respond via CD14–mediated transcriptional induction of an antibiotic peptide gene. This suggests that respiratory epithelial cells, and possibly those at other mucosal sites, can autonomously detect bacteria and responsively mount a direct antimicrobial action.

The lower respiratory tract of mammals is generally relatively free of bacteria (211). The local defensive responses of epithelial cells,

such as the TAP induction, may be important in host defense by preventing colonization and/or subsequent infection. The kinetics of this inducible response suggests that it could contribute to host defense in an important time window, that prior to the development of a clonal immune response. Epithelial β-defensins are thus predicted to participate in the initial prevention of microbial colonization, especially in the airway, utilizing bacterial recognition as part of an inducible innate immune response(141).

A number of genes encoding insect antimicrobial peptides are induced by injury, bacterial infection, and bacterial LPS (see ref. 212 and references therein). The signal transduction pathway mediating the insect response involves *dif* (213), a homologue of the mammalian transcription factor NF-κB, highlighting a striking parallel to the TAP induction detected in mammalian respiratory epithelial cells.

D. Skin and Other Squamous Epithelia

Squamous epithelia, both keratinized and nonkeratinized, provide barrier surfaces in a variety of locations in mammals. Recently, epithelial cells from both types of surfaces have been identified as sites of mammalian antimicrobial peptide expression (Fig. 6D; for review, see ref. 214). Nonkeratinized squamous epithelium of the tongue, esophagus, and vaginal epithelium produces β-defensins (38,43,157,215) and cathelicidins (153). High levels of expression of lingual antimicrobial peptide (LAP) were observed at sites of inflammation in the bovine tongue adjacent to regions that were not inflamed (38). Similarly, keratinocytes from healthy skin showed induced expression of hBD-2 mRNA when exposed to microorganisms in vitro (39,216). High levels of hBD-2 and LL-37 peptides were found in extracts from scale lesions of psoriasis, a noninfectious inflammatory skin disease that compromises the normal barrier function of skin; less of either peptide was recovered in the stratum corneum extracts of healthy skin (39,152). In situ hybridization identified high levels of hBD-2 and LL-37 mRNA in keratinocytes from areas of psoriatic inflammation but little expression in keratinocytes from healthy skin. A similar β-defensin response was observed in bovine skin adjacent to inflammation (145). Together, these findings suggest that induction of antimicrobial peptides might

bolster the effectiveness of the barrier function of these surfaces in response to local challenges.

VII. ANTIMICROBIAL PEPTIDES IN INNATE DEFENSE OF MUCOSAL SURFACES

In mammals and other higher animals, the initial encounter between the host and potential pathogens usually occurs at surface boundaries, especially the skin and wet mucosal surfaces of the gastrointestinal, respiratory, and genitourinary tracts. Not only is infection relatively uncommon, but remarkably, overt signs of inflammation are rare. These observations imply that highly effective host defense mechanisms, capable of dealing with the vast majority of microbial encounters, are operative at these sites.

Huttner and Bevins have recently proposed a working model of the host defense of mucosal surfaces (217). According to this model (Fig. 7), defense of mammalian mucosal surfaces includes inducible and constitutive expression of antimicrobial peptides (4,32,35,145,157), inorganic molecules with antimicrobial activity (218,219), and proteins that can directly inhibit microbial survival (220). These antimicrobial factors are made locally by epithelial cells at mucosal surfaces, but in some cases may also be derived from circulating cells and plasma. These factors, coupled with barrier properties and clearance mechanisms, constitute the essential elements of innate mucosal immunity. These elements of mucosal immunity are the aforementioned means of dealing with the vast majority of microbes to prevent infectious disease.

Only if the local innate defenses are overwhelmed will additional mechanisms of defense be called into action. The model suggests that inflammation and the acquired immune response are backup systems, which counter persistent or invasive challenges. Microorganisms may overwhelm innate defenses if they have virulence factors to evade defense mechanisms. In addition, deficits in defense mechanisms could allow microbes to gain an infectious foothold. Such deficiencies may result from genetic mutations, developmental immaturity, concurrent systemic disease, or toxic environmental exposures. Possible conse-
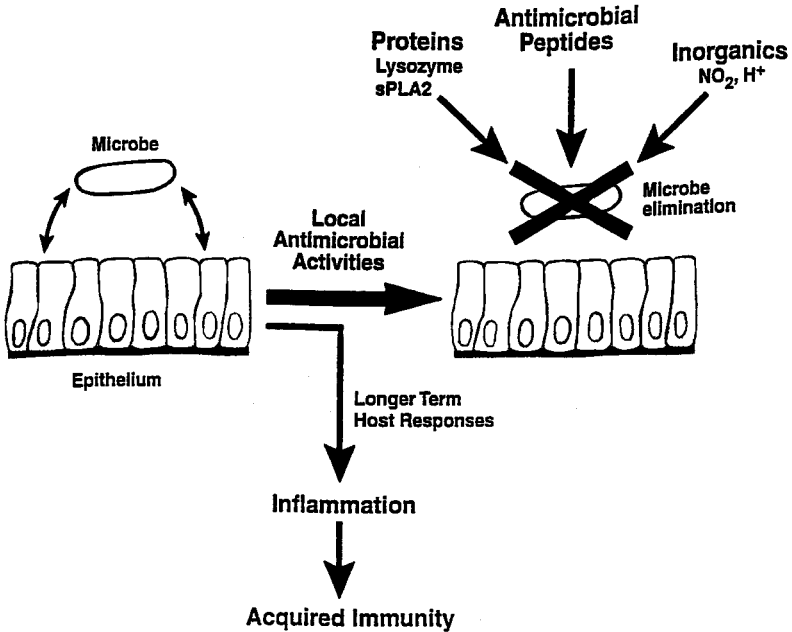
Figure 7   A model of mucosal host defenses, as proposed by Huttner and Bevins (217).

quences of long-term deficits of these innate defenses would be recurrent and/or chronic infections, or chronic mucosal inflammation.

Cystic fibrosis (CF) is a common genetic disorder caused by mutations in the CF transmembrane regulator (CFTR) (221). Chronic, recurrent infection is a devastating aspect of the disease, but infection is remarkably limited to the respiratory tract. This suggests that either directly or indirectly CFTR mutations cause a deficiency in lung host defense (222). In vitro studies of airway epithelial cells (AEC) grown in primary culture have suggested that CFTR mutations impair the capacity for airway surface fluid to kill *Pseudomonas* bacteria (223). The model proposed by Smith and colleagues holds that low molecular weight factors are secreted by AEC (both from controls and from CF patients) but that defective CFTR function in CF cells impairs the antimicrobial activity of these factors. The possible involvement of

antimicrobial peptides was first suggested by Zasloff (224). Studies of human AEC maintained in a xenograft model system have implicated hBD-1 as a candidate low molecular weight factor secreted by AEC (133). Antisense oligonucleotides directed specifically against hBD-1 inhibited antimicrobial activity of epithelial secretions, yet control oligonucleotides had no inhibitory effect. Recently, other lung antimicrobial factors [for example, hBD-2 (39–41), LL-37 (150,151), anionic peptides (225), and others (226,227)] have been discovered in lung secretions and may prove important in the pathogenesis of CF. An important area of future investigation will be to elucidate more clearly the local host defense factors of the airway epithelium. New therapeutic strategies to treat the lung infections of CF patients may emerge from these studies.

VIII.   FUTURE PERSPECTIVES

Recent attention has focused on the important contributions of innate immune mechanisms in mammalian host defense. Among the mechanisms of innate immunity in mammals is the production of a variety of antimicrobial peptides. The structure–function relationships, biological activities, and regulation of these peptides remain productive areas for further investigations. Evidence for the specific roles of antimicrobial peptides in host defense has been provided by experiments that assess the impact of ablation or augmentation of antimicrobial peptide production. Augmentation of antimicrobial peptide production in plants has been shown to increase their resistance to plant pathogens (228–230), while ablation of the pathways that induce the production of the antifungal peptide drosomycin in *Drosophila* dramatically reduces survival after fungal infections (231). Analogous experiments in transgenic mice will undoubtedly contribute to our understanding of their role(s) in host defense. These experiments may prove challenging because of the multiplicity of antimicrobial peptides and redundancies in the innate and adaptive immune systems.

It will be interesting to learn how the altered expression of antimicrobial peptides, related to genetic deficiencies, developmental immaturity, chronic inflammation, environmental toxins, or concurrent

systemic disease, impacts on innate host defense. Huttner and Bevins have discussed the possibility that insufficient expression of antimicrobial peptides will predispose to various diseases (217). For example, a deficiency in antimicrobial peptide levels (or activity) may contribute to patient subpopulations being at higher risk for neonatal sepsis, otitis media, periodontal disease, nasopharynx carriage of potential pathogens, urinary tract infections, etc. Alternatively, changes in the local environment where these peptides should function may impair antimicrobial activity and compromise host defense, as proposed in CF airway surface fluid (133,223,232).

A better understanding of innate host defense mechanisms may lead to therapeutic strategies that augment the effectiveness of the host defense. Antimicrobial peptides may prove effective as topical or systemic agents used therapeutically in treating infections (233,234). As these peptides are encoded by conventional genes, their expression could be induced at selected sites following transfer via somatic cell gene therapy. As proof of the concept, overexpression of LL-37/CAP-18 increases the antimicrobial capability of the mouse airway, which supports this conclusion (235). Clinical trials of an antimicrobial peptide from frog skin, magainin (224), and a pig cathelicidin, protegrin, as novel therapeutic agents (236) are underway.

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7
Exploitation of Lantibiotic Peptides for Food and Medical Uses

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I. INTRODUCTION

In recent years, there has been increased interest in the exploitation of natural microbial inhibitors such as bacteriocins for the biopreservation of food and as possible alternatives to antibiotics for medical applications. Owing to the success of the lantibiotic bacteriocin nisin as a biopreservative in food applications, interest in the potential use of other bacteriocins has increased considerably. Lantibiotics represent a class of bacteriocins that are extensively modified posttranslationally, resulting in the biologically active moiety. Members of the group include nisin, epidermin, gallidermin, Pep5, mersacidin, and actagardine, as well as the duramycin-type lantibiotics. Undoubtedly, the most fully documented and well characterized of the lantibiotics is nisin, which was discovered in 1928 by

Rogers and Whittier (1) and is now approved in over 40 countries worldwide for use in a variety of food applications. There are a number of features associated with nisin that make it very attractive for such applications. The bacteriocin has a broad target range and kills a wide spectrum of gram-positive bacteria including food pathogens such as *Listeria monocytogenes* and spoilage bacteria such as *Clostridium* species. Nisin is produced by the food grade bacterium *Lactococcus lactis* subsp. *lactis,* a strain commonly used in cheesemaking, and thus can be directly introduced into some products simply by using nisin-producing starters. Furthermore, nisin-producing strains occur naturally in raw milk. Nisin is commonly used as a preservative in processed cheese and cheese spreads in the United States, for which it has Food and Drug Administration (FDA) approval (2).

Early attempts to use nisin for biomedical applications, such as in the treatment of mastitis in cows (3) or tuberculosis (4) in humans, proved unsuccessful, however, and consequently interest in its exploitation for such uses diminished. However, more recently, interest in the biomedical applications of lantibiotics has been renewed, with potential applications proposed for nisin (5), lacticin 3147 (6), gallidermin (7), and mersacidin (8–10). Such interest has undoubtedly been fueled by the alarming rate of increase in the incidence of resistance to antibiotics among human pathogenic strains (11), which is now considered to be a major public health problem throughout the world (12). Consequently, natural alternatives such as bacteriocins for inhibition of pathogens are becoming more attractive. The World Health Organization (WHO) recommends global programs to reduce the use of antibiotics in animals, plants, and fish, for promoting livestock growth, and in human medicine. It also recommends alternative therapeutic procedures, including bacterial interference (12).

Given the widespread use and acceptance of nisin as a biopreservative, this chapter will focus on the food applications and properties of this well-characterized lantibiotic. In addition, the potential exploitation of emerging lantibiotics for both food and health uses will be discussed.

I. PROPERTIES OF NISIN AND OTHER LANTIBIOTICS

Lantibiotics are ribosomally synthesized peptides characterized by the presence of modified thioether amino acids such as lanthionine, β-methyllanthionine, and α,β-unsaturated amino acids such as didehydroalanine and α,β-didehydroaminobutyric acid. These unusual amino acids are formed posttranslationally by the action of specific modification enzymes. Such criteria as the stability, inhibition spectrum, and mode of action are important when considering lantibiotic peptides for particular applications, as these will influence the efficacy of the bacteriocin in different environments. On the basis of their ring structures, lantibiotics can be divided into two distinct groups (13), the Type A nisin-like peptides and the Type B duramycin-like lantibiotics. Type A lantibiotics display greater potential in biopreservative applications, as they have potent, if somewhat variable, antimicrobial activity, while Type B globular lantibiotics display weak antimicrobial activity. The Type A nisin-like peptides act by forming voltage-dependent pores in the bacterial cytoplasmic membrane, ultimately resulting in cell death, while the Type B duramycin-like lantibiotics act by binding to specific phospholipids and inhibiting membrane-associated enzyme functions (14). A third mode of action has also been described for mersacidin and actagardine that involves inhibition of cell wall biosynthesis by interfering with peptidoglycan biosynthesis (8–10). Such peptides are generally regarded as Type B lantibiotics due primarily to their flexible globular structures.

Nisin is a 34 amino acid peptide with a molecular mass of 3353 Da, and although extensively modified posttranslationally, it can be degraded in the gastrointestinal tract (GIT) by proteolytic enzymes such as α-chymotrypsin (15). Consequently, ingested nisin does not interfere with the natural intestinal microflora of the GIT. This is a significant point, as alteration of the complex collection of GIT microorganisms may be detrimental to human health. Nisin has a bactericidal mode of action when conditions are optimal, e.g., temperature, pH, water activity, redox potential, and nutrient availability (16), which significantly reduces the risk of intestinal flora acquiring resistance. However, when conditions are suboptimal or when sublethal concen-

trations of nisin are used, complete killing is not achieved and strains that survive can undergo membrane and/or cell wall changes, thus rendering them resistant to the bacteriocin. This risk of acquired resistance, therefore, must be taken into account when developing new applications for nisin.

Nisin is highly active in a wide variety of food environments and is particularly effective at the low pH levels found in fermented dairy products. However, there are a number of disadvantages associated with the properties of nisin when considering it for use in nonacid foods as well as in some pharmaceutical applications. These include its instability and low solubility at or above neutral pH values (5). Approaches to improve stability have been addressed in a number of patent applications (17,18). Nisin has a solubility that ranges from 57 mg/ml at pH 2 to 0.25 mg/ml at pH 8.5 (19), and in pH environments greater than 7, the bacteriocin becomes irreversibly inactivated. Furthermore, nisin is active against gram-negative bacteria only under conditions in which the outer cell membrane has been sensitized through the use of chelating agents such as ethylenediaminetetra acetic acid (EDTA) and citrate (20–22). Nisin Z, a natural variant of nisin also produced by a lactococcal strain, contains a single amino acid substitution of His to Asn at position 27 that results in improved solubility of the peptide at higher pH values, while biological activity or antibacterial specificity is unaffected (23). This natural variant has been patented by De Vos et al. (24) for application in food preservation. Further naturally occurring nisin variants may also exist in nature with different specificities, which may further broaden the applications of nisin in the future.

Bacillus and clostridial spores are commonly recognized as food spoilage and pathogenic organisms. An important feature of nisin is its ability to prevent the outgrowth of such spores by inhibiting the step between spore germination and preemergent swelling (25). The effectiveness of nisin in preventing the outgrowth of spores of toxin-forming pathogenic *Clostridium botulinum* is of particular significance since the botulinum toxin is the most deadly toxin known, with only 0.5 ηg/kg body weight required as the lethal dose (26). A number of patent applications have been filed for the use of nisin in inhibiting *C. botulinum* spore formation (27–29). The amount of nisin required to

prevent outgrowth of spores is dependent upon a number of factors including spore load, temperature and length of time of heat processing, pH, and moisture content (30–32). The degree of spore sensitivity varies somewhat depending on spore type, and the quantities of nisin required for inhibition have been shown to vary from 50 to 2500 IU/ml (30,31). The action of nisin against spores is generally sporostatic, which means that the bacteriocin must remain stable and active throughout storage of the product in order to maintain a preventive role. A number of studies have also shown that spores undergoing sublethal injury due to heating are more sensitive to nisin (30,31,33,34). This represents an important advantage for the use of nisin in heat-processed foods such as canned vegetables that may undergo long-term storage. Furthermore, thermophilic spores such as *Bacillus stearothermophilus* and *Clostridium thermosaccharolyticum* are particularly sensitive to nisin, so that its addition to foods destined for canning and long-term storage in warm climates has allowed the control of thermophilic spoilage.

As more lantibiotics are discovered, some of the limitations observed with nisin may be obviated. For example, lacticin 3147 is effective over a wide pH range and has been shown to be effective in nonacidic environments (6). Moreover, the limitations presently associated with lantibiotics may be overcome with the development of novel protein engineering strategies that have the potential to alter their biological properties.

II. FOOD APPLICATIONS OF NISIN

The antagonistic activity of nisin to bacteria was first discovered in the late 1920s (1) and was later observed by Whitehead in New Zealand in 1933 (35). The antimicrobial activity was attributed to a secreted protein that was subsequently concentrated and found to be inhibitory to several pathogenic bacteria (36). Nisin is produced by the lactic acid bacterium *L. lactis,* an organism used by the dairy industry for the manufacture of dairy fermented products for millennia and that consequently acquired GRAS (generally regarded as safe) status. Nisin has found widespread applications as a preservative in the food industry,

and in 1969 the joint Food and Agriculture Organization/World Health Organization (FAO/WHO) recommended its acceptance for food use. In 1988, nisin was approved by the FDA for use in processed cheese and is now approved for food use in at least 50 countries including those of the European Union (EU), where it has the designated food additive number E234.

A. Cheese Products

The most straightforward and economic approach to incorporation of nisin into fermented dairy products is to use nisin-producing starters during fermentation rather than to add nisin during processing, when it would be considered an additive. Much of the earlier work on nisin-producing starters related to their use in the retardation of late gas blowing in Swiss-style cheeses caused by clostridia. Detailed evaluation of nisin-producing strains by Hirsch et al. (37) showed that although they were effective against clostridial spoilage, nisin was found to interfere with starter performance and ripening of cheese. In general, nisin-producing strains have a slower rate of acid development, and limited proteolytic activity that directly affects their performance as starter cultures in milk (25,38). In addition, they are more sensitive to bacteriophage and thus may be problematic in commercial production (25,39). For these reasons, considerable effort has been devoted to producing multiple-strain starters composed of a nisin producer in combination with nisin-resistant "fast-acid" efficient starter strains. Although they have been used successfully for the production of Edam and Kostromski cheese, the selection of efficient nisin-resistant starters that produce sufficient acid proved too complex for routine use. Even so, research in this area has continued, as the use of nisin-producing starters remains an attractive alternative to the direct addition of nisin to cheese milk, which is costly, is inefficient, and, most importantly, is prohibited as an additive in most natural cheeses.

The inability of nisin-producing strains to produce sufficient acid for successful cheese manufacture is thought to be due to the lack of a proteinase enzyme system (40). Efficient selection techniques for the identification of naturally occurring, proteinase-positive, lactose-positive, nisin-producing strains were employed by

Roberts et al. (41) but resulted in the identification of only one such strain. The use of this strain in combination with a nisin-producing transconjugant allowed the successful manufacture of Cheddar cheese, with concomitant production of sufficient acid. Zottolla et al. (42) used these same strains to manufacture Cheddar cheese that was subsequently incorporated as an ingredient in pasteurized processed cheese and in cold processed cheese spreads, where significant reductions in *Clostridium sporogenes, Listeria monocytogenes,* and *Staphylococcus aureus* were observed.

In Gouda cheese, the outgrowth of butyric acid–producing clostridial spores is a major problem, leading to excessive gas formation and a foul-smelling product. While the addition of sodium nitrate combats clostridial growth successfully, the formation of carcinogenic nitrogen-containing compounds presents an obstacle to their continued use. As no naturally occurring nisin-producing strain has been isolated that is capable of introducing characteristic properties such as "eye-formation" and correct flavor, Hugenholtz et al. (43) developed a mixed strain starter that included two industrial strains normally used for Gouda manufacture, which had been genetically manipulated using food-grade approaches. This involved the introduction of nisin production and immunity into the citrate utilizer while nisin immunity was transferred separately to the proteolytic strain by conjugation (5,43). Nisin was produced in sufficient amounts in the final product to prevent the outgrowth of clostridial spores and also protected against *S. aureus* infection throughout ripening. However, cheese made with the nisin immune strain was found to have a bitter flavor. To investigate the possible role of this transconjugant in bitterness, Meijer et al. (44) investigated the biochemical composition of the cell wall. Interestingly, a difference in peptidoglycan composition between the nisin immune transconjugant and the parent strain was observed. This may give rise to a more rigid cell wall in the transconjugant, thereby resulting in decreased susceptibility to lysis, with a concomitant decrease in the release of debittering enzymes.

One problem that may be overcome during cheese manufacture through the use of nisin-producing starters is that caused by biogenic amines. Due to the activity of bacterial decarboxylases, some amino acids can be converted to amines during cheese ripening. Generally,

oral administration of these amines does not provoke adverse reactions unless the human body is saturated by ingestion of a high dose. Several cases of cheese-related outbreaks of amine poisoning have been reported, and histamine has been implicated in the majority of them. In an effort to prevent this problem, Joosten et al. (45) employed bacteriocin-producing starters, including a nisin producer to manufacture cheese to which a histamine-producing strain, *Lactobacillus buchneri* was added. The histamine producer was almost completely inhibited, and no histamine formation was detected in the cheeses made with the bacteriocin starters.

One of the principal applications of nisin in cheese products is protection from contamination with *L. monocytogenes,* a pathogenic bacterium that is capable of growing at refrigeration temperatures (46). In addition, *Listeria* has the ability to survive the acidic conditions of cheese manufacture (47) and to resume growth in cheeses exhibiting a pH rise during ripening, such as on the surface of mold-ripened cheese. It is also one of the most heat-resistant vegetative bacterial cells (48) and has been shown to survive the manufacturing process of cottage cheese, Camembert, and Cheddar (49). Listeriosis outbreaks linked to the consumption of contaminated dairy products are well documented. For example, consumption of contaminated Jalisco brand Mexican-style cheese (*queso blanco*) manufactured in California was directly linked to 142 cases of listeriosis including 48 deaths in 1985 (50). As a result of recurring outbreaks caused by *L. monocytogenes* in ready-to-eat foods, the U.S. federal regulatory agencies have adopted a zero tolerance policy (51,52). Like most other gram-positive bacteria, *Listeria* display sensitivity to nisin, and various studies have addressed the differing degrees of sensitivity of subspecies (53–57). The effect of Nisaplin® has been investigated in long-life cottage cheese (54,56), where it has been found to significantly reduce *L. monocytogenes* contamination.

Nisin-producing strains may also have potential use in controlling *L. monocytogenes* in Camembert cheese. Listerial growth in Camembert is common due to a rise in pH during ripening, thus allowing *Listeria* to survive. Maisnier-Patin et al. (58) showed the potential use of nisin-producing starters for *L. monocytogenes* inhibition in Camembert cheese. This study utilized a proteinase-positive lactococ-

cal strain together with a proteinase-negative variant for manufacture of the cheese. With the use of this strain combination, *L. monocytogenes* populations were reduced by over 3 log cycles during the first 2 weeks of ripening; however, regrowth of *Listeria* occurred, particularly on the surface, as the pH increased. Similar observations were made by Richard (59) when using a nisin-producing starter to manufacture Camembert cheese. The more pronounced regrowth observed on the crust may have been due to a significant decrease in the local nisin concentration caused by the strong proteolytic activity exhibited by the mold *Penicillium caseoculum.*

B. Processed Cheese and Cheese Spreads

Although this review will discuss the many potential applications of nisin, it is in the area of processed cheese and cheese spreads that nisin has realized much of its commercial value and has received approval for use in the United States by the FDA. In 1952, McClintock et al. (60) showed that nisin was effective in preventing clostridial spoilage in pasteurized processed cheese. The introduction of Nisaplin, a commercially available nisin concentrate with high and consistent activity (1 million IU/g), allowed its commercial application in such products. Nisaplin consists of approximately 2.5% nisin A in addition to milk solids and salts resulting from the fermentation process and is produced by Aplin and Barrett Ltd., Applied Microbiology Inc. Further advances by this company have led to more active preparations with an activity of up to 40 million IU/g, which should allow further applications with requirements for a higher specific activity. Anaerobic spore formers particularly associated with processed cheese are *Clostridium butyricum, C. tyrobutyricum,* and *C. sporogenes,* which can survive high processing temperatures and consequently grow and produce gas in the product, causing "blowing" of the product. Additionally, the composition of the processed cheese in terms of higher pH (5.4 to 6) and moisture content (50–54%) favors spore outgrowth, with subsequent detrimental effects. Addition of nisin can enhance flexibility in the formulation of processed cheese spreads, allowing reduced sodium and/or higher moisture levels for increased spreadability to be used (61). Another potential danger of

great significance is the outgrowth of *C. botulinum* spores in processed cheese, resulting in toxin formation. Addition of 500 to 10,000 IU/g nisin can prevent the outgrowth of *botulinum* spores, even when present in spreads with higher moisture or reduced sodium chloride and phosphate levels. Product innovation in the cheese industry includes the development of processed cheese with added flavors such as onion, chive, horseradish, prawn, and smoky bacon. Such product diversification, while necessary, can compromise the microbial quality of the resulting cheese product by carrying with it an inherent additional flora that may make it more susceptible to spoilage (62). These types of products can benefit from nisin addition, which offers both extended shelf life and improved safety. Interestingly, a study carried out by Plockova et al. (63) indicated that while the addition of Nisaplin to typical Czech-made processed cheese resulted in reduced bacterial counts, the resulting flora consisted mostly of aerobic *Bacillus* spores that exhibited increased tolerance to nisin.

Soft white fresh cheeses, such as Ricotta, Panir, Domiati, and Latin American cheeses made without starter culture, also pose a major risk of contamination, as they are subjected to only minimal processing prior to packaging and consequently have a short shelf life. Addition of Nisaplin at a level of 2.5 mg/l to Ricotta-type cheese has been shown to be effective in inhibiting the growth of *L. monocytogenes* for at least 8 weeks, as illustrated in Figure 1 (64). The pathogen was controlled for even longer periods when nisin was added in the presence of either acetic acid or potassium sorbate and is a good example of a situation in which nisin can provide an extra barrier using the "hurdle" concept of food safety (52,65). Similarly, Shaker et al. (66) observed that the addition of Nisaplin to Domiati cheese inhibited anaerobic spore formers, thereby preventing blowing of the cheese tins. The above examples serve to demonstrate the versatility of nisin and/or nisin-producing strains in extending the shelf life of a wide variety of cheese products.

C. Milk Products

Milk products cannot be subjected to full sterilization without damaging the appearance, taste, or texture of the product (e.g., ultra-heat-
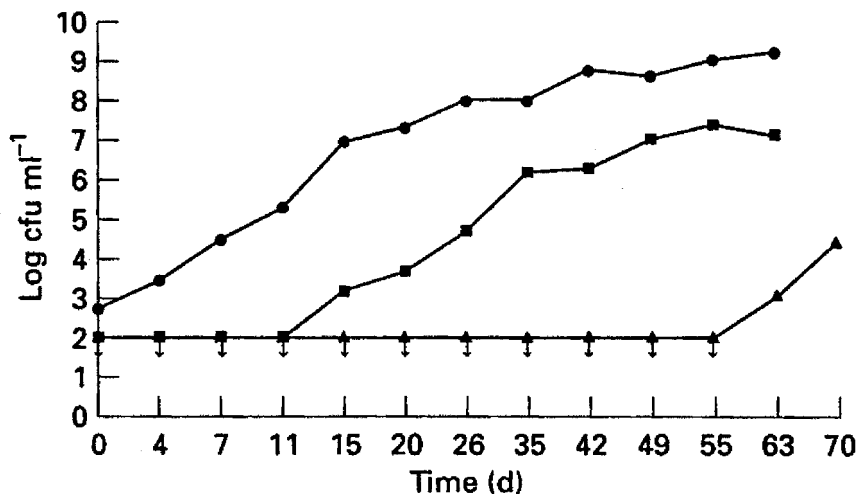
Figure 1 Nisin can be used to improve the safety of a wide variety of fermented foods. The above example demonstrates the effect of Nisaplin (Aplin and Barrett, Applied Microbiology, Inc.) in Ricotta-type cheese to control the food-borne pathogen *L. monocytogenes* at 6 to 8°C. All cheeses contained an initial inoculum level or approximately 5×10^2 colony-forming units per gram (CFU/g) of *L. monocytogenes*. The pathogen grew to 10^7 CFU/g in the control cheese (●), while the onset of growth was delayed for 11 days when 1.25 mg/1 nisin (■) was added and for 55 days when 2.5 mg/1 nisin (▲) was added. (From Ref. 64.)

treated milk generally has a "burnt" off-flavor), and as a consequence, they often contain spores. Several studies, as reviewed by DeVuyst and Vandamme (33), have demonstrated the beneficial effects of adding nisin to milk products, thus allowing less severe heat processing conditions. Nisin addition to milk products (10 to 50 mg/l Nisaplin) is commonplace in the Middle East, where shelf life problems are experienced due to the warm climate, inadequate refrigeration facilities, and the necessity to transport milk over long distances (2). In these countries, reconstituted and recombined milk are used in the preparation of sterilized and flavored milk drinks. Problems may occur if the milk powder and butter oil used in these preparations

contain heat-resistant spores. In this regard, Nisaplin addition has proven to be successful in controlling thermophilic spoilage microorganisms, thereby reducing the heat treatment required following reconstitution (67–69).

Nisaplin has also been shown to extend substantially the shelf life of dairy desserts (33,70). For example, studies conducted by Gupta and Prasad (71), and more recently by Kumar and Prasad (72), indicate that incorporation of nisin in lassi (a type of stirred yogurt) can extend its shelf life significantly. The inclusion of additional ingredients has been shown to act as a source of contaminating spores in milk-based desserts. A study carried out by Plockova et al. (73) showed that the cocoa powder and the stabilizing agent were the most acute sources of bacilli in a thermized quark dessert but that addition of Nisaplin at a rate of 50 IU/g or of a nisin-producing strain during manufacture of the dessert was successful in extending the shelf life by up to 16 days. The presence of *L. monocytogenes* in frozen desserts also poses a major potential health threat. The zero tolerance policy adopted by the U.S. federal regulatory agencies for such products means that incorporation of natural antimicrobials may become necessary to meet these demands. A study carried out by Dean and Zottola (74) found that nisin can be effective in inhibiting *L. monocytogenes* in both full-fat (10%) and reduced-fat (3%) ice cream.

D. Canned Products

Another area where nisin has received widespread use is in canned foods to prevent the outgrowth of *Bacillus* and *Clostridium* species. Although regulations in countries such as the United Kingdom dictate that low-acid canned foods (pH > 4.5) should be sufficiently heat treated to destroy *C. botulinum,* heat-resistant spoilage spores can sometimes survive. The two spoilage organisms especially associated with canned foods are *B. stearothermophilus* and *C. thermosaccharolyticum,* which are common contaminants associated with flour, sugar, and spices. By incorporating nisin, it is possible to control spoilage organisms while simultaneously allowing for a reduction in heat processing. This results in improved nutritional value, texture, and appearance of the product and an overall more economical process

(33). The many canned products in which nisin has been used are outlined in refs. 25, 33, 34, 75, and 76 and include canned peppers, where *Clostridium pasteurianum* spores can be controlled, and asparagus for the prevention of *C. sporogenes* spore outgrowth. Although up to 80% of nisin activity may be lost in the heat processing of low-acid foods, sufficient activity may be retained to ensure destruction of heat-damaged spores. In products such as semolina, rice sago, tapioca (67), kheer, and pumpkin (75), heat treatment results in product thickening, which may complicate efficient heat transfer in the product. The addition of nisin allows a reduction in heat treatment, thus reducing the problem and resulting in a product with improved nutritional and organoleptic properties.

E. Fish

Deterioration of fresh fish at chill temperatures is generally caused by gram-negative microorganisms, and relatively few attempts have been made to evaluate the potential of nisin in such products. However, the results of some studies suggest that nisin can be effective in extending the shelf life of fish such as dehydrated (77) or gutted bolti fish (78). There is much evidence to suggest that fresh fish may contain *C. botulinum* type E as a result of contamination after catching. If the fish is packaged either under vacuum or in a modified atmosphere life (by reduction of the oxygen level in order to expand storage) and stored above refrigeration temperatures, the growth of *C. botulinum* may occur. The application of nisin-containing sprays has been shown to have an inhibitory effect on toxin development in inoculated cod, herring, and smoked mackerel fillets that were packaged in 100% CO_2 atmosphere and stored at 10°C and 26°C (79).

The association of *L. monocytogenes* with seafood as a result of postprocessing contamination has led to several product recalls (80), thus indicating a further area where bacteriocins may play a role. *Listeria monocytogenes* in seafood is difficult to control, particularly in cooked, ready-to-eat products such as crabmeat, as it can grow at refrigeration temperatures, survive in brine solutions, and tolerate extremes of heat and pH. The potential of a variety of antimicrobial agents to inhibit this pathogen in blue crabmeat was investigated by

Degnan et al. (81). Nisin application at a rate of 10,000 to 20,000 IU/g resulted in a 10- to 100-fold decrease in *Listeria,* and although a resurgence of the organism was subsequently observed, the degree of this regrowth was lessened with increasing nisin concentrations and levels did not return to the initial inoculum level. Encouraging results have also been obtained by Einarsson et al. (82) when it was observed that nisin Z could extend the shelf life of brined shrimp from 10 to 31 days. These results indicate that nisin Z could be used as a natural alternative to replace the benzoate-sorbate mixture for similar refrigerated brined products requiring a 3- to 4-week shelf life. Finally, Nilsson et al. (83) determined that growth of *L. monocytogenes* in cold smoked salmon could be prevented by a combination of CO_2, nisin, NaCl, and low temperature.

F.   Brewing and Wine Making

Nisin has found practical application in beer fermentations and wine making. Beer is an inherently hostile environment for bacterial growth. Consequently, spoilage of beers is limited to only a few species of bacteria, with *Lactobacillus* and *Pediococcus* being the most prevalent contaminants in fermentations. Various studies have indicated that nisin can inhibit beer spoilage organisms without exhibiting detrimental effects on the brewing yeasts (84,85). Importantly, nisin addition during the fermentation process appears to have no adverse effect on the taste of the beer (86). Nisin may be added at various stages of the brewing process to prevent bacterial contamination. For example, nisin could be used as an alternative to acid washing of yeasts, the main source of bacterial contamination in the brewing process, because nisin does not affect the viability and performance of the yeasts, as occurs during acid washing (87). For future application in fermentations, the effects of postfermentation treatments on nisin activity such as fining and filtration are also of consequence. The effects of different filter aids and finings on the activity of nisin in beer have been determined, and only an 8–10% loss of activity was observed (88).

   Like beer, wine can spoil as a result of undesirable lactic acid bacteria (LAB) growth and subsequent production of detrimental com-

pounds (89). However, certain red wines benefit from LAB, such as *Leuconostoc oenes* and *Pediococcus damnosus,* which are capable of decarboxylating malic acid to monocarboxylic lactic acid, also known as malolactic fermentation (MLF). In contrast, for many white wines, MLF is detrimental. Although sulfur dioxide is widely used for the suppression of unwanted LAB in wine making, its use is strictly regulated. Experiments have indicated that nisin has potential in combatting contamination in wines, as most strains of *Leuconostoc* and *Pediococcus* are sensitive to it, with *Leuc. oenes,* the most important strain for MLF, exhibiting particular sensitivity (89–91). A study carried out to investigate the use of nisin in applications where MLF is required utilized nisin-resistant mutants of *Leuc. oenes* to conduct MLF in wines containing nisin. Significantly, nisin does not appear to affect the sensory characteristics of wine (89,91).

Nisin may also have a possible application in the ethanol fermentation industry for the control of LAB contamination (92–94). One further area in brewing where nisin may be of benefit is as a preservative in sake products to inhibit *Lactobacillus sake* Hiochi-type bacteria (the major spoilage organisms associated with sake) (95).

G. Salads

Freshly cut vegetables are becoming a popular convenience food and generally rely on refrigeration for their shelf life. However, the cutting of vegetables results in a nutrient-rich environment that can support the growth of food-borne pathogens including *L. monocytogenes.* The difficulty in maintaining cold temperatures throughout the handling and packaging of these foods gives rise to further opportunities for bacteriocins as extra hurdles to these pathogens.

Although salad dressings are reasonably safe due to their low pH, they are prone to spoilage by aciduric microorganisms such as lactobacilli and yeasts. With a growing consumer demand for less tart salad dressings, formulations with a lower concentration of acetic acid may result in a greater risk of spoilage by LAB during storage (5). Out of a total of 30 bacterial contaminants obtained from a range of commercial salad dressings, nisin was found to inhibit 27 (96). In further experiments carried out in spoonable buttermilk ranch dressing, Nisaplin

was found to inhibit the challenge organism, *Lb. brevis,* during the subsequent 90-day shelf life period.

H. Pasteurized Liquid Egg Products

Another significant market for nisin is its use in pasteurized liquid egg products of the type used by restaurants and fast food outlets. Even though it is a statutory requirement to pasteurize liquid egg product for 2 to 3 minutes at approximately 65°C to kill *Salmonella,* organisms such as spore-forming bacilli and other gram-negative organisms often survive. Many of them remain viable during subsequent refrigeration and, as a result, pasteurized liquid egg products generally have a shelf life of 10 to 11 days. Trials carried out by Delves-Broughton et al. (97) have indicated that nisin at a level of 5 mg/l is effective in significantly extending the shelf life of such products by up to 14 days. Although nisin does not inhibit gram-negative organisms alone, the combined effects of heat and the presence of other antimicrobials such as lysozyme in the egg may damage the cell wall of these organisms, thus allowing nisin to act on the cell membrane (98).

I. High-Moisture Bakery Products

Crumpets are flour-based bakery products that are particularly popular in the United Kingdom and Australia. They have a short shelf life of 5 to 6 days due to their nonacid pH (6 to 8) and high moisture content. Crumpets have been implicated in a number of food poisoning outbreaks due to growth of and subsequent toxin production by *B. cereus,* which contaminates the flour. Since *Bacillus* isolates from naturally contaminated crumpets were found to be particularly susceptible to nisin, a number of trials have been carried out to evaluate its use as a preservative in such products (99). Although nisin losses are quite high during cooking and storage of crumpets, the results obtained indicate that nisin can be an effective antimicrobial agent in this product. Addition of Nisaplin at a concentration of 3.75 µg/g was sufficient to prevent the outgrowth of spores to levels capable of causing food poisoning. Other studies have been carried out on wholemeal crum-

pets, pikelets, and flapjacks with similar results. A higher level, 6.25 µg/g, was necessary for the wholemeal crumpets due to the higher spore count in wholemeal flour. Significantly, the data obtained from these trials resulted in regulations in Australia allowing the use of nisin in high-moisture, flour-based products (99).

J. Fermented Vegetables

In Europe, vegetable/sauerkraut fermentations are followed by a process of packaging and pasteurization, whereas in the United States, fermentations are generally carried out in bulk tanks where it is difficult to maintain a predictable product. In addition, fermentations depend on the natural microbial populations present, thus adding to the difficulty of maintaining a consistent, marketable product. Even though the use of defined starters would assist in alleviating this problem, their selection is difficult, as vegetable fermentations are dependent on a complex succession of LAB throughout the process. Heterofermentative *Leuconostoc mesenteroides* dominate in the early stages, while homofermentative *Lactobacillus plantarum,* which produce large amounts of lactic acid, dominate in the latter stages (100). The correct sequence of organisms is essential for a stable product with a correct flavor and aroma.

Nisin-producing lactococcal strains have found application in combination with a naturally occurring nisin-resistant *Leuc. mesenteroides* strain in sauerkraut fermentations (101,102). When this paired starter culture system was evaluated in cabbage broth juice, sufficient nisin was produced to suppress the development of homofermentative *Lb. plantarum* without completely eliminating its presence. However, as further studies indicated that nisin loses activity in brined cabbage, it was determined that higher initial concentrations of nisin were required to ensure sufficient activity throughout the process. Breidt et al. (103) developed starter strains that were resistant to levels of nisin of up to 25,000 IU/g, and the addition of purified nisin (12,000 IU/g) with this resistant *Leuconostoc* starter was effective in controlling the progression of the indiginous flora (104). Nisin addition has also been used to delay the onset of homolactic fermentation during kimchi production. A low concentration, 100 IU/g, is sufficient to retard the

growth of indigenous LAB, thereby slowing the rate of acid production, which is undesirable in kimchi (105).

K. Meat

The use of nisin as a preservative in meat has been extensively examined but to date has found little success. Problems such as low solubility and heat sensitivity at neutral pH (30,31,106,107) have reduced the potential of this lantibiotic in meat applications. Nisin appears to be unstable in meat, particularly at ambient temperature, which may be due to binding of the bacteriocin to sulfhydryl groups or meat particles (108).

Traditionally, sodium nitrite has been added to meat to fix the color and to inhibit any possible *C. botulinum* contamination. However, concern over the potential formation of carcinogenic nitrosamines (33) led researchers to consider nisin as an alternative to sodium nitrite. Although studies have shown that nisin displays modest adjuvant activity with nitrite in the control of botulinum formation in pork slurries, bacon, and chicken frankfurter emulsions (109–111), Rayman et al. (111) concluded that nisin is not a suitable alternative to nitrite for protecting against *C. botulinum* in cured meats, given the higher pH environment. Synergistic activity, however, was observed against spoilage organisms such as *C. sporogenes* in meat slurries (112) and *Clostridium perfringens* in frankfurters (113). In contrast to cured meats, nisin can be useful for extending the shelf life of vacuum packaged meat, in which LAB are the main spoilage organisms (114).

As with other food products, there have been reports of an association between listeriosis outbreaks and the consumption of meat products. Measures to control *L. monocytogenes* in meat include inactivation of the pathogen or prevention of its attachment to the meat surface. To investigate the potential of nisin in this application, El-Khateib et al. (115) treated meat samples with nisin (40,000 IU/ml) and then immersed them in a cell suspension of *L. monocytogenes.* An immediate antilisterial effect was observed, as well as a slight reduction in its capacity to bind to the meat surfaces. A further study by Mahadeo and Tatini (116) showed that heat displays a synergistic activity with Nisaplin against *L. monocytogenes* in poultry, although this effect

was greater when the cells were suspended in the scald water rather than when attached to the turkey skin. The effect of replacing sulfur dioxide (sulfites are thought to have toxic effects) with organic acids and nisin to reduce microbial counts in fresh pork sausage was determined by Scannell et al. (117). The results indicate that a combination of sodium lactate and nisin provides increased protection against *S. aureus* and *Salmonella* species.

III. NISIN IN COMBINATION WITH FOOD PACKAGING MATERIALS

Vacuum packaging and modified atmosphere packaging are routinely used to prevent spoilage of fresh red meats by suppressing the growth of organisms following oxygen deprivation or inhibition with CO_2. In the United States, approximately 93% of beef is distributed this way. Although this procedure is beneficial in extending the shelf life of fresh meat products, organisms such as lactobacilli, *Brochothrix thermosphacta,* and *L. monocytogenes* are capable of growth under controlled packaging conditions. A number of studies have indicated that nisin combined with efficient packaging is capable of suppressing these organisms (118,119). Cutter and Siragusa (118) have demonstrated that a nisin spray (5000 IU/g) followed by vacuum packaging at refrigeration temperatures results in significant reductions in *Listeria innocua* and *B. thermosphacta.* As spray washing with antimicrobials such as trisodium phosphate and organic acids has already been approved for use in processing plants for the decontamination of meat and poultry, this approach may be of particular commercial interest. The combination of a modified atmosphere (CO_2) and nisin has also been used successfully against *Pseudomonas fragi* and *L. monocytogenes* on cooked pork (120). Recently, Szabo and Cahill (121) noted that *L. monocytogenes* could be controlled for prolonged periods following a combination treatment of modified atmosphere, refrigeration, and nisin application.

While vacuum packaging in combination with nisin application has been found to be effective in controlling gram-positive bacterial contamination, there is now growing emphasis on packaging that is

more "environmentally friendly." Hence, packaging research in more recent years has focused on biodegradable films (122). Although environmental pollution is a major concern, the issue of food safety must also be borne in mind in the development of these new packaging techniques. Recent studies have tested nisin incorporation in films for inhibition of bacterial growth (123–125). Natrajan and Sheldon (124) reported a 4.3 log reduction in the number of *Salmonella typhimurium* using a protein-based agar film coated with nisin on fresh poultry meat products and a 4.6 log reduction with a nisin-coated polyvinyl chloride film, while Dawson et al. (125) observed inhibition of *Lb. plantarum* when both nisin and lauric acid were incorporated into cast corn zein films. A study carried out by Padgett et al. (122) used two packaging film-forming methods (heat press and casting methods) to incorporate nisin or lysozyme into bidegradable protein films, and both antimicrobials retained their inhibitory activity against *Lb. plantarum.* Nisin has been incorporated into a packaging film patented by the Viskase Corp. (126).

The inactivation of nisin by proteolytic enzymes in foods such as fresh meats has led to limitations in its application. Microencapsulation technology has proven to be effective in the protection, stabilization, and controlled release of agricultural and pharmaceutical chemicals as well as food ingredients (122) and has been considered as a nisin delivery system. A number of studies have investigated the use of calcium alginate as an incorporation matrix, as it has food grade status, is low in cost, and facilitates a simple incorporation process. Cutter and Siragusa (127) immobilized nisin in a calcium alginate gel and observed a 2.5 log reduction in *B. thermosphacta* on beef carcass tissue. Wan et al. (128) also incorporated nisin into a calcium alginate gel and achieved an 87–93% incorporation efficiency. Full nisin activity could be recovered from the formulations, and the calcium alginate microparticles containing nisin were quite resistant to inactivation by proteolytic enzymes, thereby offering a potential delivery technology where indigenous proteolytic enzyme activity exists. Finally, Cutter and Siragusa (129) have demonstrated that nisin can be successfully incorporated into a binding system such as Fibrimex®, which has been approved in the United States as a means of adhering high-quality or lean trimmings from meat, poultry, and fish into one piece. However,

these restructured products may contain contaminating bacteria that can become internalized in the process. It was determined in this recent study that the inoculated strain *B. thermosphacta* can be suppressed by the addition of nisin. Therefore, Fibrimex may form a potential delivery system for nisin and other bacteriocins.

Food spoilage and pathogenic microorganisms may have the ability to adhere to inert surfaces and hence be less susceptible to the activity of sanitizers (130). When these colonize and create a biofilm, they can be a source of potential food contamination (131). One approach that has been examined is the prevention of initial adhesion of contaminants to food contact surfaces by coating them with a layer of nisin (131–133). Daeschel et al. (133) treated silicon-coated surfaces with nisin and demonstrated that nisin can retain antimicrobial activity, while Bower et al. (131) demonstrated that nisin adsorbed to silica surfaces could decrease cellular adhesion of the pathogen *L. monocytogenes*. These results suggest that nisin may have potential for use as a food-grade antimicrobial agent on food contact surfaces.

IV. CONCLUSION ON THE USE OF NISIN AS A BIOPRESERVATIVE

Although nisin has found much application to date in biopreservation, repeated exposure of pathogenic and spoilage organisms to the bacteriocin may result in the emergence of nisin-resistant strains that pose difficulties for the development of new food applications. Spontaneous resistant mutants of *L. monocytogenes* are reported to occur at relatively high frequencies of 10^{-6} to 10^{-8} colony-forming units per milliliter (CFU/ml) (134–136). A number of studies have implicated changes in the cell membrane and, in particular, changes in the phospholipid content as being responsible for acquired resistance (135, 137–139). In addition, evidence from other studies suggests that cell wall adaptations may also be responsible (134,140,141).

Other factors that limit the use of nisin include those already mentioned, i.e., interactions with food components (142–144), an inability to inhibit gram-negative organisms, and poor solubility and stability at neutral pH. Therefore, it may be more appropriate to con-

sider nisin as an alternative to be used in conjunction with other preservatives and preservative techniques, as outlined by Leistner's "multiple hurdle" theory. This theory suggests that combinations of sublethal levels of stresses such as salt, heat, and pH work synergistically to inhibit or kill cells (65). However, reductions can vary widely, depending on the strain and the stress endured (145). Alternatively, other antilisterial bacteriocins that are active against nisin-resistant cells of *L. monocytogenes* can be utilized to inhibit the onset of mutant cells.

Of particular interest is the use of other agents to extend the host range of nisin to include gram-negative organisms. It is suggested that any method of sublethal injury to the LPS layer of gram-negative bacteria should render them more susceptible to the inhibitory activity of bacteriocins (146). Studies have shown that nisin in combination with food-grade chelators such as EDTA and citric acid can inactivate *Salmonella* species and other gram-negative bacteria (20–22,147). Sublethal damage may also occur during pasteurization and other heat treatments, thus inducing nisin sensitivity. Consequently, inclusion of nisin could contribute to a reduction in heating time, thereby reducing costs as well as maintaining the characteristics of the product. Boziaris et al. (142) determined that addition of nisin to liquid egg resulted in a reduction in the required pasteurization time for inhibition of *Salmonella* by approximately 35%, due primarily to thermal injury.

Although nisin in combination with mild heat treatment has been shown to be effective against *L. monocytogenes,* enhanced inhibition is more pronounced against this pathogen when nisin is combined with an increased sodium chloride concentration (148,149). Studies by Jaquette and Beuchat (150) on the combined effects of pH, nisin, and temperature on the growth and survival of *B. cereus* indicate that nisin is more effective at 8°C than at 15°C and as the pH is decreased from 6.57 to 5.53. Similarly, lower temperatures were found to enhance nisin inhibition of *S. aureus* (149). Interestingly, it has been observed that a low concentration of nisin renders *L. monocytogenes* more acid sensitive, which may increase the sensitivity of the organism to the low pH of the stomach as well as in acid foods.

Ultrahigh hydrostatic pressure (UHP) and pulsed electric field (PEF) may also be used as nonthermal methods of sublethally injuring cells. Both UHP and PEF destroy microbial cells by destabilizing the structural and functional integrity of the cytoplasmic membrane, and the amount of inhibition is proportional to the treatment exerted. Kalchayanand et al. (151) reported that when either of the bacteriocins nisin or pediocin AcH was added in combination with UHP or PEF to gram-negative (*Escherichia coli* 0157:H7 932 and *S. typhimurium*) and gram-positive (*L. monocytogenes*) cells, greater antibacterial activity resulted than with any of the treatments alone. This method of inactivation was developed by Ponce et al. (152) for the inhibition of *E. coli* and *L. innocua* in liquid whole egg. A method for preserving liquid egg including nisin addition followed by electroheating has been patented by Knipper (153).

V. POTENTIAL USE OF LACTICIN 3147 IN BIOPRESERVATION

When assessing lantibiotic members other than nisin for their potential as food preservatives, it is important to consider a number of characteristics. Ideally, they should be produced by a GRAS organism, have a history of safe food use, and have a broad spectrum of activity. Of the non-nisin lantibiotic bacteriocins discovered to date, only lacticin 481, lactocin S, carnocin U149, and lacticin 3147 are produced by food-grade bacteria. The only one of these that has a truly broad spectrum of activity is lacticin 3147 (154), which, like nisin, inhibits a wide range of gram-positive bacteria. This lactococcal bacteriocin is heat stable, particularly at low pH, and is active at physiological pH. Lacticin 3147 is composed of two peptides, both of which are required for biological activity (155). Both peptides contain lanthionine and dehydrated amino acids. The lacticin-encoding genetic determinants are located on a conjugative plasmid, pMRC01, which can be transferred between lactococcal cheese starter strains in a food-grade manner, using the bacteriocin itself for selection (154,156). As already outlined, there have been a number of problems related to nisin-producing

starter strains for cheese production, including their slow acid production and susceptibility to bacteriophage. In contrast, transconjugant starter strains that produce lacticin 3147 perform well as cheese starters for the manufacture of products such as Cheddar cheese (154,157) and cottage cheese (158). In addition, pMRC01 contains a phage-resistant region that renders transconjugant strains less susceptible to phage attack, which further distinguishes them from nisin-producing transconjugants (156,159).

A major application associated with lacticin 3147–producing starters is their ability to inhibit disadvantageous flora in fermented products. For example, Cheddar cheese made with such starters contains markedly less nonstarter lactic acid bacteria (NSLAB) levels in the ripened product. These NSLABs are present in the environment and are not directly added to the cheese vat. However, they gain entry during cheese making and proliferate in the cheese during ripening. The role of NSLABs in cheese quality and flavor is still unclear, but they undoubtedly contribute to the inconsistency of the final product in terms of flavor and quality. Defects in cheese associated with NSLAB include off-flavor development, formation of calcium lactate crystals, and slit formation. If a strain can be developed that can control these developing microflora, a more consistent end product with improved food safety qualities can be achieved. From a scientific point of view, it provides a system to determine the role of NSLABs in cheese flavor. Construction of these bacteriocin-producing strains involved the use of the genetic determinant of bacteriocin immunity as a food-grade selectable marker. Only strains containing pMRC01 are capable of growth on media containing lacticin 3147. Therefore, this mechanism forms the basis for a suitable food-grade selectable marker that is desirable when the end product is destined for consumption.

The control of the developing NSLAB population is also of benefit in the manufacture of reduced-fat Cheddar cheese. In a study carried out by Fenelon et al. (157), reduced-fat Cheddar cheese was manufactured using the lacticin 3147–producing strain described above and ripened at the elevated temperature of 12°C. The elevation of ripening temperature from 7 to 12°C results in accelerated ripen-

ing, which allows for a more cost-effective process. But consequently, more rapid proliferation of NSLAB occurs, with an associated increased risk of spoilage. This risk may be greatly decreased, however, in situations where lacticin 3147 starters are used to control the NSLAB population.

Lacticin 3147–producing cheese starters have also been assessed in the manufacture of cottage cheese where *L. monocytogenes* contamination poses a threat (158). In experiments where *L. monocytogenes*–containing cream was mixed with heat-treated curd made with a lacticin 3147 starter, *L. monocytogenes* levels were reduced by at least 3 log cycles within 5 days at 4°C, and the kill rate increased with higher storage temperatures. In the above examples, lacticin 3147–producing cultures were used as the starter; however, these strains may also be used as protective cultures on the surface of cheese products. For example, the use of such cultures applied as a spray on the surface of mold-ripened cheese can cause significant reductions in *L. monocytogenes* numbers (Ryan, Ross, and Hill, unpublished results, 1999).

As can be seen from these examples, lacticin 3147–producing strains have significant potential as protection cultures, particularly in the dairy industry. However, recent work has led to the development of a spray-dried fermentate from whey containing lacticin 3147 that may be added directly as an ingredient to food products. This research has demonstrated that lacticin 3147 activity survives the evaporation and drying process necessary for powder production. Interestingly, the bacteriocin-containing powder that resulted exhibited a greater killing effect at a neutral pH than at a pH of 5 on both *L. monocytogenes* and *S. aureus.* An example of the potential of this product is its use in providing an effective barrier against contamination with *Listeria* in infant formula. Results demonstrated that partial substitution of powder for formula killed 99.9% of *L. monocytogenes* (Fig. 2) in 3 hours. The effectiveness of lacticin 3147 at neutral pH may provide new opportunities for the preservation of many nonfermented food products using lantibiotics. In addition, the combined use of lacticin 3147 with nisin should provide very effective lantibiotic-based preservation against many of the gram-positive bacteria.
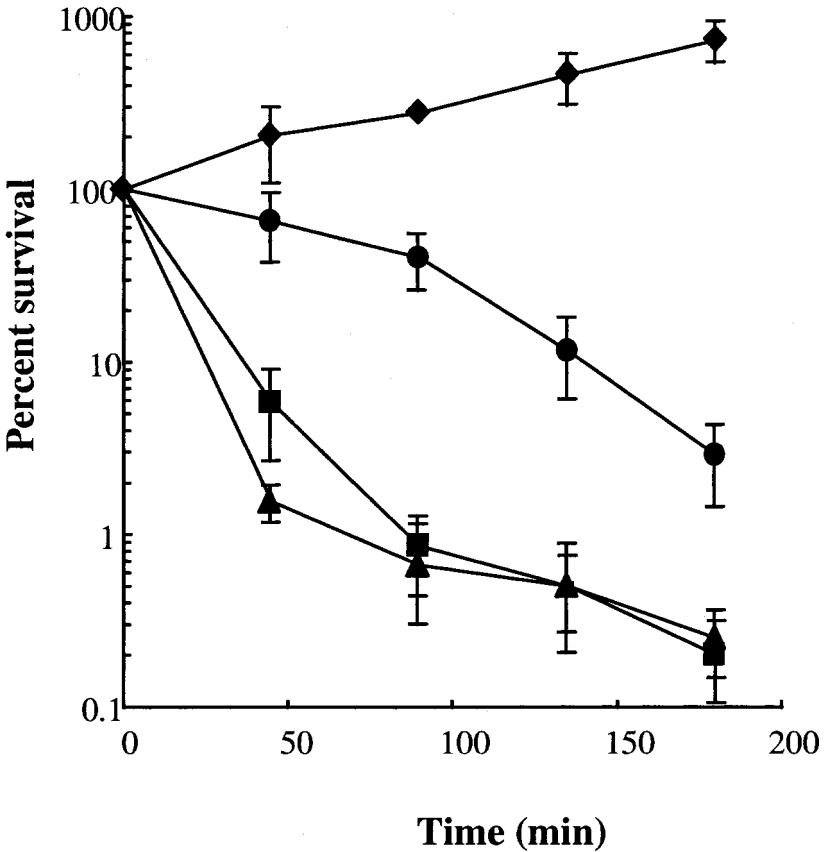
Figure 2 The two-component lacticin 3147 can be used to improve a variety of foods including those at neutral pH. This figure demonstrates the effect of a whey-based lacticin 3147 powder on the viability of *L. monocytogenes* Scott A when used as a component of infant milk formula. All samples contained an initial inoculum level of approximately 10^4 CFU/ml *L. monocytogenes.* The pathogen grew in the control formula, 15% infant milk powder (♦), but was killed where a whey-based lacticin 3147 powder was substituted for the formula at 5% lacticin powder, 10% infant milk powder (●); 10% lacticin powder, 5% infant milk powder (▲); and 15% lacticin 3147 powder (■). Error bars represent the standard deviation for duplicate experiments. (From Ref 159a.)

VI. BIOMEDICAL APPLICATIONS OF LANTIBIOTICS

A. Nisin and Related Lantibiotics

Lantibiotic peptides may provide valuable additions to the current range of antimicrobial compounds available for animal and human medicine. Such applications are especially important given the widespread emergence of antibiotic-resistant pathogens, such as penicillin-resistant pneumococci and methicillin-resistant staphylococci. The idea of using bacteriocins for such biomedical purposes is not a new one, nisin being recognized as having activity against tubercle bacilli as far back as 1928 (1). In addition, nisin was evaluated as an alternative to intramammary antibiotics for the treatment of bovine mastitis in the 1940s (3). While this work exhibited promising results, problems were encountered with irritancy that were possibly due to impurities and the particle size of the particular preparations used. It is well established that nisin is nontoxic when administered orally due to proteolytic breakdown in the gastrointestinal tract; however, some research is now focused on establishing its efficacy in inhibiting skin pathogens if applied topically. Valenta et al. (160) demonstrated a possible use for nisin as a preservative in topical formulations. In addition, nisin has been used in the formulation of a germicidal sanitizer for the teat skin surfaces of cows (161). Nevertheless, nisin must be evaluated for its potential side effects should it or any degradation product from it penetrate the skin. Even if immediate side effects are not observed, the formation of antibodies that could cause a severe allergic reaction may potentially occur. However, a study carried out by Bernkop-Schnurch et al. (162) demonstrated that the cumulative amount of nisin that permeated the skin during 6 hours of iontophoretic transport corresponds to only 0.015%.

In 1994, the National Institutes of Health issued a statement recognizing a link between peptic ulcer and the presence of the gram-negative organism *Helicobacter pylori.* Consequently, the disease is now treated with antibiotics. Nisin exhibits activity against *H. pylori* that may be enhanced by the presence of a chelator. Advantages to using nisin as a potential treatment for peptic ulcers (163) include its stability at acid pH and its resistance to the stomach protease pepsin.

Table 1 Lantibiotics with Potential Applications

Lantibiotic	Type	Producing strain number	Peptide residue	Total
Nisin A	A	*Lactococcus lactis*	1	34
Lacticin 3147	A	*Lactococcus lactis*	2	30
				29
Gallidermin/ Epidermin	A	*Staphylococcus gallinarum/*	1	22
		Staphylococcus epidermidis	1	22
Mersacidin/ Actagardine	B	*Bacillus* subsp./	1	42
		Actinoplanes subsp.	1	45
Duramycin	B	*Streptomyces* subsp. and *Streptoverticillium* subsp.	1	19
Cinnamycin	B	*Streptomyces cinnamoneus*	1	19
Ancovenin	B	*Streptomyces* subsp.	1	19

Inhibitory activity of commercial interest	Proposed food applications	Potential biomedical applications
Gram-positive bacteria including pathogenic and food spoilage organisms	Food preservative in dairy, canning, fish meat/fish, alcoholic beverages, pasteurized egg products, bakery products, salads/ vegetables, animal feed	Bacterial mastitis Oral hygiene Treatment of MRSA and enterococcal infections Cosmetic deodorants and topical formulations
Gram-negative bacteria including *Helicobacter pyogenes*		Peptic ulcer treatment Treatment of enterocolitis Lung mucus clearing in, e.g., cystic fibrosis and asthma
Gram-positive bacteria including pathogenic and food spoilage organisms	Food preservative in dairy industry	Bacterial mastitis Treatment of MRSA and enterococcal infections Oral hygiene
Propionibacterium acnes, staphylococci, and streptococci	Not applicable	Acne, eczema, folliculitis, impetigo
Staphylococci including methicillin-resistant strains streptococci	Not applicable	Treatment of MRSA and streptococcal infections
Weak bacterial inhibition: Inhibitor of phospholipase A2	Not applicable	Inflammation
Inhibitor of phospholipase A2, angiotensin converting enzyme. (ACE), and herpes simplex virus	Not applicable	Inflammation Blood pressure regulation Viral infection treatment
Inhibitor of ACE	Not applicable	Blood pressure regulation

Additionally, it is broken down in the intestine, thereby reducing the effect on the intestinal microflora (5). A recent study has shown that nisin is inhibitory to *Clostridium difficile,* which is of particular importance due to its association with antibiotic-induced enterocolitis. The management of diarrhea caused by *C. difficile* infection is often complicated by relapses after apparently succcessful antibiotic therapy. Nisin may offer an alternative treatment for this condition, as it prevents the germination of clostridial spores and preliminary results suggest that the killing kinetics are similar to those of vancomycin (164). Lantibiotics may have a role in the prevention of periodontal disease, one of the major diseases responsible for tooth loss in the adult population. Early studies indicated a possible role for nisin in the control of this disease (165,166). More recently, the effect of a mouth rinse based on nisin was investigated (167) for its effectiveness against the development of plaque and gingivitis in beagle dogs. The nisin-treated groups showed an inhibition of gingivitis development; additionally, application of the nisin-based formulation did not appear to cause staining of the teeth. A number of patents have been filed in which nisin is incorporated as the active ingredient against tooth decay (168–170).

Research in recent years indicates that lantibiotics have considerable potential in the prevention of bovine mastitis. At present, antibiotics are used in the treatment and prevention of mastitis. Such routine use of antibiotics has obvious problems, such as the presence of antibiotic residues in the milk and the emergence of antibiotic-resistant strains. Bacteriocins may therefore offer an alternative to the prevention of this disease. Nisin inhibits a wide range of mastitis-causing pathogens (171). In studies conducted in vivo, efficient cure rates of 66%, 95%, and 100% for animals infected with *S. aureus, Streptococcus agalactiae* and *Str. uberis,* respectively, were observed. In addition, nisin has been evaluated in a germicidal sanitizer for teat skin surfaces (161) and has been shown to be effective. Notably, nisin is now the active ingredient in two commercial products that are used in the prevention of mastitis, Consept® (a teat dip) and Wipe-Out® (a teat wipe).

More recently, lacticin 3147 has been evaluated for its potential in the prevention of mastitis in cows. Like nisin, lacticin 3147 is effec-

tive in killing a wide range of mastitis pathogens (6). Moreover, the lantibiotic can be incorporated into a commercial teat seal product, where it exhibits excellent antimicrobial activity. In a further study, the formulation incorporating lacticin 3147 was tested in animal trials for efficacy, and only 6% of treated teats developed clinical mastitis or were shedding the challenge organism at the end of the experiment, compared to 61% of the control teats. In addition, the bacteriocin/teat seal formulation was well tolerated in the udder in that it did not cause a sustained increase in levels of somatic cell counts.

Nisin may also have potential as an animal feed additive. Feed-stuffs are fermented in the rumen of animals, and methane, heat, and ammonia are produced as metabolic by-products. As a result of subsequent metabolic reactions involving methane, the ratio of acetate to propionate increases, thus decreasing energy retention by the animal. At present, the ionophore monensin is used to inhibit methane production by dissipating the ion gradients of gram-positive bacteria. However, adverse side effects in both animals and humans has resulted in a search for alternative inhibitors of gram-positive ruminal bacteria. Due to its inhibitory spectrum and nontoxicity, nisin was evaluated for activity against ruminal bacteria in comparison to monensin in vitro. Results indicated that both nisin and monensin can inhibit ruminal methane, decrease the acetate to propionate ratio, and prevent amino acid deamination. Although more detailed in vivo investigation is required, these preliminary findings suggest that nisin may have potential as a feed additive (172).

Another lantibiotic with interesting biological activity is the Type A lantibiotic gallidermin, which is inhibitory to *Propionibacterium acne* (173), one of the causative agent in skin diseases such as acne. This condition is very often treated with antibiotics such as erythromycin, but given the emergence of antibiotic-resistant strains, alternative strategies such as the use of gallidermin and nisin are extremely attractive. The use of such lantibiotics in the preservation of cosmetics and deodorants has also been suggested (26). Gallidermin is stable at skin pH (5.4) and has quite a narrow spectrum of inhibition, thereby reducing possible side effects. In addition, the strains that produce gallidermin are isolated from human skin and are most probably normal flora of the skin, where they provide natural protection.

B. Type B Lantibiotics

The Type B lantibiotics cinnamycin, ancovenin, and the duramycins share considerable sequence homology, varying in only seven amino acids. Although their antimicrobial activity is restricted to quite a narrow spectrum (specific strains of *Bacillus* spp.), their novel activities against the functions of specific enzymes, such as phospholipase A2 and angiotensin converting enzyme (ACE), have resulted in renewed interest in them. Phospholipase A2 is involved in the immune system, while ACE is involved in the maintenance of circulatory blood pressure. Ancovenin inhibits ACE (168), the duramycins inhibit phospholipase A2 (174), while cinnamycin is inhibitory to both ACE and phospholipase A2 (175). In addition ancovenin inhibits herpes simplex virus 1 (176).

The remaining Type B lantibiotics, mersacidin and actagardine, have been shown to display considerable in vivo activity against *S. aureus* (177), including methicillin-resistant strains (MRSA) and streptococci. Effective treatment at present for MRSA and enterococcal infections is possible only with the glycopeptide antibiotics vancomycin and teicoplanin. The main concern is that *Enterococcus*-derived glycopeptide resistance, which is based on transposon-encoded mechanisms, will transfer to the MRSA, thereby rendering them untreatable by any antibiotics. Brotz et al. (8–10) have shown that these vancomycin-resistant strains remain sensitive to mersacidin, indicating that cross-resistance should not pose a problem. In vivo experiments carried out with mice show that concentrations of mersacidin similar to those of vancomycin are effective in treating infections with MRSA, thus indicating that this lantibiotic may have considerable therapeutic application. Additionally, it is not possible to digest mersacidin proteolytically, and antibodies to this lantibiotic are practically impossible to raise.

It has also been suggested that nisin may also have a role in the treatment of systemic disease caused by antibiotic-resistant streptococci. A recent report by Goldstein et al. (178) has shown that nisin exhibits excellent in vitro activity against clinical isolates of *Str. pneumoniae,* including penicillin-resistant strains. In subsequent mouse infection model studies, it was observed that nisin was 8 to 16

times more active than vancomycin against one of these clinical isolates. Additionally, low blood and tissue levels of nisin appear to be sufficient to treat mice infected with *Str. pneumoniae.* Further promising results were obtained by Severina et al. (179) regarding the activity of nisin against multidrug-resistant gram-positive pathogens. In this study, the efficacy of nisin was tested against 56 multidrug-resistant *Str. pneumoniae,* 33 *S. aureus,* and 29 vancomycin-resistant *Enterococcus faecium* and *E. faecalis* isolates. In the vast majority of cases, nisin caused a 3 to 4 log reduction in titer, although a concentration of up to 10 to 20 mg/l was required for the MRSA and enterococci (Fig.
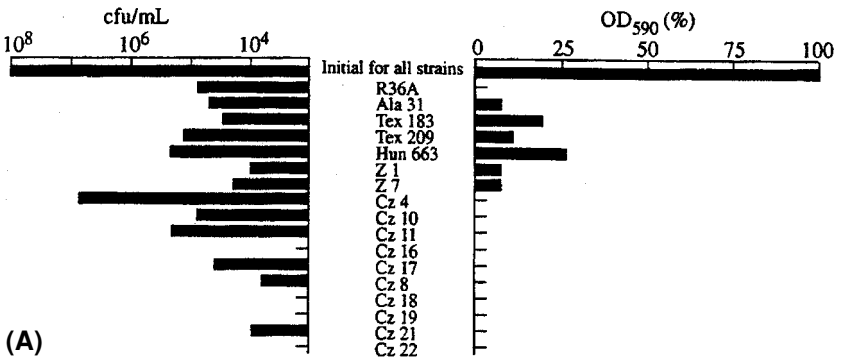
Figure 3 Antibacterial efficacy of nisin against multidrug-resistant gram-positive pathogens. Mid-log phase cultures were treated with nisin, and optical density (OD_{590}) was measured after incubation. Data were expressed as residual OD_{590}, with optical density at nisin addition taken as 100%. The titer of the cultures was also taken before and after nisin treatment, with the initial titer adjusted to 10^8 CFU/ml. (A) Bactericidal and bacteriolytic activity of nisin (1 mg/l) against a range of penicillin-resistant *Str. pneumoniae.* Treated cultures were incubated for 20 minutes at 30°C. (B) Activity of nisin (10 mg/l) against a range of methicillin-resistant, coagulase-negative staphylococci after a 3-hour incubation at 37°C. (C) Activity of nisin (20 mg/l) against a panel of vancomycin-resistant *E. faecium* and *E. faecalis* cultures after 4 hours at 37°C. The country of origin of the strains is indicated. The enterococcal strains were isolated in the New York Hospital (NY) and Memorial Sloan-Kettering Cancer Centre (MSK). (From Ref. 179.)
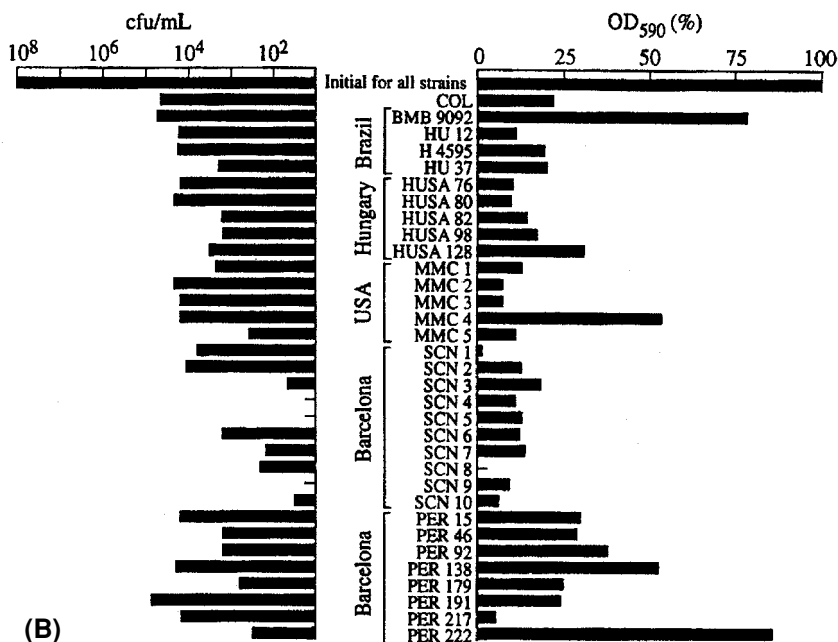
Figure 3 Continued

3). Of note, however, is the rapid appearance of stable nisin-resistant mutants following repeated exposure to the bacteriocin, thus indicating that as with the existing antibiotics, bacteria are also capable of adapting to the inhibitory effect of nisin. It is interesting that although lantibiotics appear to have considerable potential in clinical applications, many of the early studies have not been followed up. Hence, further research in this area should give greater insight into its mechanisms of action and thereby open up new areas of antimicrobial target research.

VII. FUTURE PROSPECTS

Considerable research to date has led to the isolation and development of a growing number of lantibiotic-type bacteriocins, but to date, only

(C)
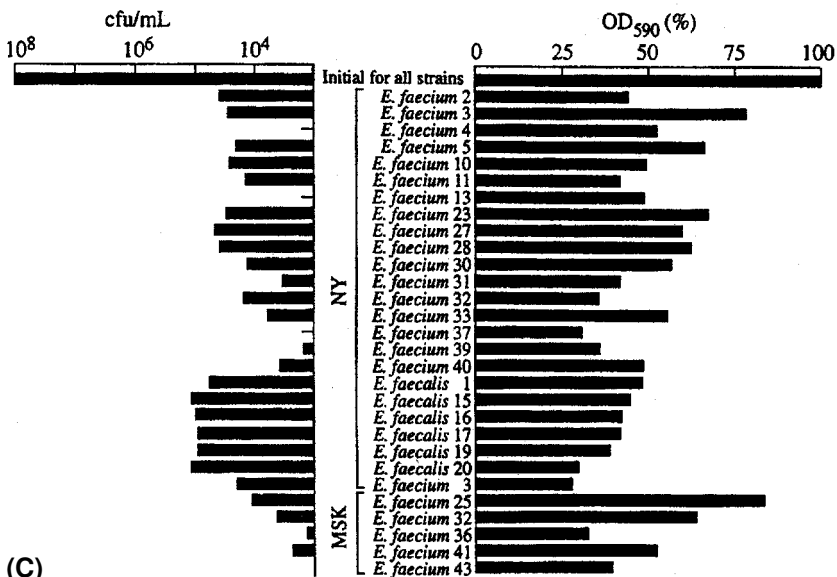
Figure 3 Continued

nisin has found commercial application. Consequently, there have been searches for natural variants of existing bacteriocins as well as for new bacteriocins. Such research should lead to the development of other useful lantibiotic peptides such as lacticin 3147, which may extend the applications of such peptides when used alone or in combination with other antimicrobials.

An alternative approach is the development of protein engineering strategies that can potentially alter the biological properties of bacteriocins and thereby extend their range of uses. Additionally, dehydrations and lanthionine formation modifications found in lantibiotics could be introduced into other proteins, where they may confer enhanced properties, such as increased thermal stability. Introduction of such properties into industrial enzymes may have a large economic impact. The use of recombinant genetics to prepare enzymes involved in the biosynthesis of lanthionine and/or dehydroamino acid–containing compounds has been patented by Dr. Karl Thomas GmbH (180).

Notably, more than 40 mutants of lantibiotics have been generated, but unfortunately, almost every mutation has resulted in decreased antimicrobial activity, with only a few exceptions, i.e., T2S nisin Z; M17Q/G18T nisin Z; and L6V gallidermin, which exhibit enhanced activity against some target strains (181).

Lactic acid bacteria and their by-products play a very important part in industrial food fermentations. Recent advances in controlled gene expressions have allowed regulated overexpression of desirable proteins produced by the LAB. One such technique harnesses the nisin regulatory machinery and is advantageous, as it is versatile, it is economically viable, and importantly, it has a safe history for use in food (182).

VIII.   CONCLUSIONS

The current trend in food processing toward the development of foods that are minimally processed, natural, and safe has been fueled by consumer concerns over the safety of existing chemical preservatives. Moreover, the growing incidence and emergence of food pathogens has led to the concurrent need for effective alternatives to chemical preservatives. Fermented foods are perceived as natural and healthy; hence inhibitory by-products of fermentation processes are attractive alternatives for use in biopreservation. The safe use of nisin, one such by-product, paves the way for the use of other bacteriocins/lantibiotics as food preservatives. Nisin is the only lantibiotic that is commercially available, and its use has been demonstrated in foods such as low-fat dairy products, where its addition increases shelf life by up to 6 weeks (Aplin and Barrett, Applied Microbiology, Inc.). Apart from their food uses, lantibiotics may also provide valuable additions to our antimicrobial arsenal to combat infections caused by biomedical pathogens that have become resistant to commonly used antibiotics. For such applications, the gradual emergence of lantibiotic-resistant pathogens may also be problematic. Given the intensive research effort directed at the discovery and characterization of novel lantibiotics, it is expected that concomitant applications will emerge for these unusual peptides (183). However, their potential will be realized only if limitations such as

those imposed by their properties can be overcome. In response to this, protein engineering strategies have been developed that can potentially alter the biological properties of lantibiotics, thereby extending their range of uses. In this respect, the search for new peptides should be encouraged, while fundamental research into existing ones could undoubtedly lead to a new generation of peptides engineered toward particular food and biomedical applications.

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8
Amphibian Antimicrobial Peptides

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I. INTRODUCTION

This review updates an earlier summary of pharmaceutical development published in 1994 (1). An excellent general review specifically covering amphibian peptides has been published recently (2), and thus I wish here to highlight areas that have not been extensively addressed elsewhere. A summary of our understanding of the mechanism of action of linear antimicrobial peptides will be presented from the perspective of my analysis of current data. I will briefly review the clinical experience with pexiganan, the first antimicrobial of animal origin to complete Phase III human trials. In addition, several applications of antimicrobial peptides undertaken by Magainin Pharmaceuticals, Inc., in other therapeutic areas will be discussed to provide the reader with some of the strategies and insights that we have gathered over the past 5 years since the prior review in our efforts to identify therapeutic opportunities for these peptides.

II. NATURALLY OCCURRING ANTIMICROBIAL PEPTIDES FROM AMPHIBIANS

Over the past 10 years antimicrobial peptides have been discovered in many species of amphibian, generally from skin. As noted previously,

this family exhibits remarkable diversity in antimicrobial peptide sequence, with no two species bearing the same peptide sequences. Indeed, this diversity is so extensive that relatedness of antimicrobial peptide sequences has been used as a taxonomic tool to classify species within the *Rana* genus (3,4) and *Littoria* families (5). Two groups of peptides have been identified based on structural similarities: linear cationic amphipathic peptides and cationic peptides with a single disulfide linked loop at the carboxyl end.

Of the linear amphipathic class, magainin, isolated from *Xenopus laevis,* represents the prototype (6). The class includes peptides from *Bombina* species (7,8), such as the bombinins; from the *Phyllomedusa* genus (9), such as dermaseptins (10–12), adrenoregulin (13,14), and phylloxin (15); from the Australian genus *Littoria,* such as caerins (5), frenatins (16), maculatins (17), citropins (18), and aureins (19); and from the African running frog, *Kassina sengalensis,* kassinateurin (20).

A curious modification noted in several linear amphibian antimicrobial peptides is the presence of D-amino acids in certain peptides (21). Thus bombinin H7 bears a D-leucine in the second position, while bombinins H3, H4, and H5 bear D-allo-isoleucine in that position (22). It appears that this modification in the antimicrobial peptides affects the proteolytic stability of the molecule rather than its antibiotic spectrum. As in the case of the opioid peptide dermorphin, where the phenomenon of a D-substituted amino was first discovered, the modification is likely to occur posttranslationally (23).

The other large class of antimicrobial peptide (with a single disulfide loop) is represented by the prototype, brevinin I, isolated from the Japanese frog, *Rana brevipoda* (24). This class, exclusively isolated from species of the *Rana* genus, is characterized by an amino terminal sequence of varying length, followed by a cyclic motif at the C-terminal end formed by a disulfide link between an internal and a carboxyterminal cysteine, comprising six residues [brevinin (25,26), esculentin (27), gaegurins (28), ranatuerin (29), rugosin (30), ranatuerin 2 (31)], seven residues [ranatuerin 1T (32,33)], or eight residues [tigerinins (34)]. Although the *Rana*-derived peptides are characterized by sequence diversity, they share sufficient similarity to be grouped into classes most closely similar to an initially discovered

molecule. They differ in overall size from the very short brevinin 1Ta molecule (15 amino acids) (28) to palustrin 3A (48 amino acids) (33). A very useful, up-to-date online database of all reported antimicrobial peptides, including amphibian-derived molecules, as well as an extensive bibliography of reviews and original papers on antimicrobial peptides, should be accessed for specific sequence data (35).

Many of the amphibian peptides exhibit broad-spectrum antimicrobial activity against bacteria, fungi, and protozoa (36) and are non-hemolytic at effective antimicrobial concentrations. However, certain peptides are both antimicrobial and hemolytic (37), and a few appear to be highly specific for certain species of bacteria, such as the short linear peptide temporin from *Rana gyrlio* (38), its synthetic analogs (39), or the linear peptide caerin 1.1 from White's tree frog, *Littoria caerulea* (40) that exhibit relative specificity for gram-positive bacteria, including *Staphylococcus aureus.* As noted previously for magainin (6,41,42), peptides of this class can kill protozoa including the malaria parasite. In a recent report, dermaseptin was shown to kill *Plasmodium falciparum* in its intraerythrocyte stage in the absence of concurrent erythrocyte lysis, suggesting that the peptide was by some mechanism transported across the red cell membrane prior to interacting with the parasite (43).

In general, amphibian peptides are created from a precursor bearing a secretory leader sequence followed by an acidic spacer of variable length, subsequently followed by the peptide. Processing of the polyprotein precursor is effected by a series of endo- and exopeptidases (2,44). Within the antimicrobial peptides from a single species, and even between certain classes of different peptides from diverse species, significant conservation of amino acid sequence can be recognized in the prepro region of the precursor molecules; the existence of a conserved signal sequence suggests that while the antimicrobial peptides have been given the opportunity to mutate, constraints exist on the design of the sequences involved in synthesis, secretion, or intracellular trafficking of this class of membrane-disruptive peptide (2,44–46). Analysis of the genes of both bombinin and dermaseptins reveals that the signal sequence lies within its own first exon, separated from the second or third exons, which harbor the peptide coding sequences, further highlighting the independent functional property of this

segment of the anitimicrobial peptide precursor. With respect to the biological controls modulating expression of the amphibian antimicrobial genes, examination of the 5′ DNA sequences of genes from both *Bombina* spp. and *Phyllomedusa bicolor* revealed nuclear factor κB (NF-κB) and nuclear factor interleukin 6 (NF-IL6) consensus sequences, features shared with many of the genes that participate in the network of mammalian immunity (47–49).

In frogs, these peptides are almost universally synthesized and stored within highly specialized neuroendocrine structures called granular glands, innervated structures that release their contents onto the surface of the skin following injury or adrenergic stimulation (2,44). Complex multinucleated cells related ultrastructurally to the granular glands of the skin and capable of synthesizing and secreting the known skin antimicrobial peptides have been identified in the intestinal tract of *Xenopus* (50,51), and peptides have been isolated from the gastric tissue of *Rana esculenta* (52). Thus, it is believed that the antimicrobial system provides protection to both the inside and outside milieu of the frog defended by epithelial barriers. Of particular interest to those studying the physiological role of this system in the setting of host defense are recent studies that deal with the regulation of antimicrobial peptide synthesis. For example, recent work with *R. esculenta* has suggested that expression of the genes encoding antimicrobial peptides is under the control of NF-kB, a transcription factor widely implicated in vertebrate immunity (48). Application of topical glucocortocoids to the skin of the frog impairs de novo synthesis of antimicrobial peptides following pharmacological discharge, via electrical stimulation of the skin, of the contents of the granular glands, in part through inhibitory effects exerted through the NF-kB circuit (53). Studies dealing with the nature of the stimuli that naturally promote synthesis of antimicrobial peptides have been reported. Frogs that have been pharmacologically depleted of skin antimicrobial peptides will not reaccumulate these peptides unless the animals are exposed to bacteria in their environment (54). Freeze-tolerant wood frogs, such as *Rana salvatica* collected in the cold and maintained at 5°C, exhibited no peptides in skin; maintaining the animals at 30°C led to an increase in peptides by 3 weeks, likely a response to ambient bateria or physiolog-

ical stimuli under thermal control (55). These studies highlight certain attractive features of the use of the frog to study the physiology of the antimicrobial defense system in a vertebrate species (56).

Not all amphibian peptides, however, appear to be produced from specialized precursors and stored within and secreted from specialized epithelial structures. Recently, an exception has been discovered in the Asian toad, *Bufo bufo* (57). The stomach of this animal yielded a potent antimicrobial peptide called buforin I and a shorter fragment, representing amino acids 16–36 of buforin, called buforin II. Surprisingly, buforin is a fragment of histone 2A. It is generated in the gastric mucosa through the action of an isoform of pepsin. It appears that histones are synthesized in excess of the needs required for DNA packaging in the gastric mucosal cell, and they accumulate within cytoplasmic secretory granules of the gastric epithelial cell. During digestion, secretion of pepsin results in conversion of histone 2A to buforin I, which remains adherent to the mucous biofilm coating the stomach surface, thus providing the stomach with a protective antimicrobial coat. A comparable situation has been shown to occur in human gastric mucosa. The role of this system in human gastric immunity and its relationship to infections such as those caused by *Helicobacter pylori* remain to be determined. One might speculate, for example, that a defect in pepsin secretion could impair gastric mucosal defense by preventing histone 2A conversion to buforin, which, if true, would suggest radical new treatments for gastric ulcers.

III. MECHANISM OF ACTION

The basic mechanism has been studied in many laboratories using many diverse biophysical approaches over the past decade (for recent reviews of mechanism see refs. 58–61). The following picture emerges: Linear antimicrobial peptides generally exhibit little secondary structure in water. Because of their net cationic charge, these peptides bind electrostatically to membranes that contain accessible negatively charged phospholipid head groups. Upon interaction with the membrane, the linear peptides assume an α-helical secondary

structure. Within the membrane they lie parallel to the surface, with the positively charged hydrophilic surface of the helix face in contact with the aqueous boundary and the hydrophobic face buried within the lipid phase (62,63). The depth at which the peptide resides is generally not sufficient to disrupt the packing of the fatty acids of the phospholipids. Because of their considerable mass and size the peptide helices, however, significantly displace the normal packing of the phospholipid lipid head groups. The insertion of peptides thus leads to an expansion of the surface area of the lipid film into which they have inserted. In the setting of a bilayer, peptide insertion into one leaflet results in imposition of a positive curvature, caused by the expansion of the outer leaflet relative to the inner leaflet of the bilayer. As peptides continue to enter the membrane from one leaflet, the curvature increases. To relieve this growing strain, the membrane then reorganizes its components. Precisely how the strain is relieved remains a source of discussion and controversy. In one model (64), peptides bend like flexible logs falling over a circular waterfall. One imagines that a transient "torus"-like hole is formed in the membrane as several peptides are swept down in concert. Once they pass through the bilayer they remain attached to the lower face of the bilayer, oriented on the inner leaflet as they had been on the outer. As these peptides course through the membrane, they drag lipids to which they are electrostatically associated. Lipids might be organized in certain ordered arrays in the vicinity of the peptides (65–67). This process results in the formation of transient imperfections in the membrane associated with alterations in permeability. A second model (68), established using low-angle X-ray scattering techniques, suggests that at sufficiently high peptide to lipid ratios highly discrete channels form, not simply transmembrane disruptions. Evidence supporting the existence of stable transmembrane arrays of magainin 2 at high peptide to lipid compositional ratios using photo-labeled lipids has also been reported (69). It is suggested that these transmembrane structures underlie the killing mechanism of the peptide (68). However, unlike alamethicin, which forms physical barrel/stave channels through peptide–peptide interactions, the channels formed by magainin are stabilized toroidal holes in the bilayer, a structure created between lipid and peptide, with four to seven peptides sta-

bilizing each hole. Using neutron scattering, and D_2O to fill the gap, the magainin-stabilized hole has been visualized (70). A third model suggests that linear antimicrobial peptides behave like detergents (71). When their concentration in a membrane becomes high enough, they simply pinch off membrane, forming micelle-like structures. As fragments of membrane pinch off, the permeability properties of the membrane are disrupted and, in the case of a microbe, large numbers of antimicrobial peptide can now penetrate the cell unhindered by the membrane, killing the cell.

What is curious about these models is that the formation of a physically distinct alamethacin-like peptide channel is no longer considered to be central to the mechanism of action of linear amphipathic antimicrobial peptides. How a membrane responds to peptide contamination will depend on the forces that maintain the structural integrity of that membrane. This will be dependent in part not only on the precise structure of the peptide but also on the structure of the lipids that comprise the membrane, independent of the charge of their headgroups (72). Specificity for microbes over vertebrate cells is generally regarded as being based on the electrostatic interaction of the peptide with negatively charged headgroups, which are a feature of the composition of the outer leaflet of the most accessible membranes of many species of microbe (73). Thus, while magainin, for example, can interact with mammalian lipid membranes composed of neutral phospholipids, the dynamics or energetics of that interaction must not be sufficiently favorable to overcome membrane stability (74). The balance between hydrophilic and hydrophobic groups, the extent of the faces that each occupies on an α helix, generally correlate with specificity (75–77). Peptides that are excessively hydrophobic (relative to magainin) are driven into the membrane through hydrophobic interactions and are less dependent on the presence or absence of bacterialike lipid headgroups. In addition, peptides that aggregate to form complexes that in solution create hydrophobic faces prior to interaction of the peptide with the membrane exhibit reduced specificity (78). Membranes, which contain cholesterol, are less receptive to the action of peptides like magainin (79), perhaps because the cholesterol molecule itself interacts with the peptides, hindering their normal behavior

in the bilayer (80). Computer-based molecular simulation of antimicrobial peptide–lipid interactions, utilizing several of the experimental variables describe above to model the behavior of both the peptide and lipid components, has been reported (81,82).

A recent paper has suggested that the presence of a proline motif in the sequence of buforin II introduces a surprising property to this peptide when studied in microbial cells. Like magainin, buforin II binds to a bilayered membrane through electrostatic interactions and translocates to the inner face; however, as a consequence of the presence of proline within the sequence, the peptide, once translocated, diffuses from the inner leaflet of the membrane and enters the cytoplasm of the bacterial cell (83). Removal of the proline residue causes the peptide to remain associated with the cellular membrane and severely reduces antimicrobial activity. Similarly, removal of a naturally occurring proline in the center of the α-helical peptide gaegurin is associated with dramatic loss of activity (84). This striking and unanticipated influence of a proline residue in a specific position within these peptide sequences suggests that simple sequence modifications can profoundly influence peptide–membrane dynamics in the setting of microbial cells.

How do antimicrobial peptides actually kill microbes? Many investigators have addressed the mechanism of killing, but no consensus has been reached.

Do the channels created by these peptides in normally energized bacterial membranes result in fatal depolarization (85,86)?

Do the peptides actually organize to form physical holes responsible for leakiness of larger molecules (68)?

Does minor damage to the membranes of microbes lead to activation of other death-inducing processes such as induction of cell wall–degrading hydrolases (87)?

Do the peptides, translocating between outer and inner leaflets of a bilayer, scramble the lipids between the leaflets of the bilayer, resulting in disturbance of membrane functions?

Do some peptides kill their targets by gaining access to the cellular interior, where they inactivate critical intracellular targets, as suggested by the example of buforin II?

Does a cell die when damage imposed cannot be repaired fast enough (86)?

IV. PEPTIDE SYNERGY

The most abundant antimicrobial peptides present in *Xenopus* skin are magainins 1 and 2 and PGLa (a peptide beginning with glycine and terminating in leucine amide). These peptides, while individually antibiotic, exhibit a striking functional synergy when assayed together against microbial targets (44,88) amounting to a 10-fold or greater activity at optimum ratios of the two peptides. A recent study explores the underlying mechanism of synergy (89). Like magainin, PGLa exhibits little secondary structure in solution and, high affinity for anionic lipids, and interacts initially with a lipid to form α-helical structures lying parallel to the membrane surface (62) leading to specificity. PGLa also forms transient physical imperfections that alter the permeability properties of model bilayers and through which both peptides and lipid can relocate to the opposing face of the bilayer. These pores form more rapidly than those seen with magainin, but are shorter-lived. At a 1:1 molar ratio, magainin and PGLa exhibit maximal membrane-disruptive activity, suggesting that a heteromolecular pore of precise stoichiometry is created. In addition, substitutions in the magainin peptide can be created that interfere with synergy but not with individual activity. No evidence of peptide–peptide interaction can be demonstrated when the peptides are studied as a mixture in water or together in membrane vesicles. Upon mixing, the heteromolecular pores formed appear to share the best properties of both peptides, namely, rapid formation and long lifetimes. The precise molecular structure of the pore, with respect to lipid/peptide organization, is not yet reported.

Synergy has also been reported in the peptide secretions of *Phyllomedusa* (90) and *Bombina* (2), and although the molecular details underlying the synergistic interactions remain to be elucidated, they likely reflect a picture similar in overall design to the *Xenopus* story.

The discovery of peptide synergy is important for several reasons.

Since it is possible to alter the primary sequences of both the magainin and the PGLa molecule in ways that eliminate synergistic interactions without reducing the antimicrobial properties of either peptide when studied independently, these two peptides have likely evolved in nature as part of a codependent system. In other words, synergy between these molecules likely provides the frog with certain benefits not achievable through additive interactions. What might these benefits be? Clearly, synergistic interactions provide the animal with a means of creating a membrane-disruptive weapon that can be regulated more precisely than is possible with a one-component system. For example, a synergistic mixture creates an activity that exhibits far greater concentration dependence than an additive mixture, a strategy useful in providing the skin with high local activity but permitting dissipation of activity as the molecules diffuse from the site of secretion. Of importance to those involved in drug development, however, is the realization that certain peptides might exhibit properties in vivo, either favorable or not, which result from unanticipated synergies with endogenous molecules.

V. RESISTANCE

Addressing the issue of resistance is essential to the development of a commercially viable antibiotic. We recognize several types of resistance to antimicrobial peptides. These include both resistance intrinsic to a species and resistance that is acquired by a previously sensitive species. It is also important to remember that results of studies involving resistance conducted on specific peptides might or might not apply to other antimicrobial peptides. Since it remains unclear why peptides exhibit certain antimicrobial spectra (i.e., species susceptibility and potency) or why subtle changes in sequence can profoundly influence the antimicrobial spectrum of a peptide, we still have only a rudimentary understanding of those parameters that influence antimicrobial potency.

A. Pexiganan

Pexiganan has been extensively evaluated against thousands of clinical isolates as a consequence of its development as a therapeutic agent for

the treatment of infected diabetic foot ulcers. The surveys of activity against bacterial species demonstrated a spectrum of sensitivities ranging from very sensitive species [minimum inhibitory concentration (MIC) about 1–5 µg/ml] to those resistant (MIC > 256 µg/ml). Included in the sensitive species are gram-negative and gram-positive aerobes and anaerobes. Indeed, more than 90% of all clinical isolates, encompassing over 200 bacterial species, exhibited MICs less than 64 µg/ml (91,92). Strikingly, both gram-positive and gram-negative anaerobes were among the most sensitive (93). The minimal bactericidal concentration of pexiganan determined against over 200 strains of aerobic bacteria differs by no more than twofold from the MIC, consistent with the microbicidal mode of action of pexiganan. Species with intermediate MICs included common commensals such as *Lactobacillus* spp. This feature of the spectrum clearly is of therapeutic value in that pexiganan would be expected to spare certain bacterial species considered to be beneficial colonizers. Other naturally occurring resistant strains include those of *Serratia, Proteus,* and *Aeromonas.* In fact, all gram-negative species resistant to polymyxin were invariably found to be resistant to pexiganan, likely a result of the "nonreceptive" lipid composition of the outer membrane of these bacteria. In an infection, however, we found evidence that resistance in vitro was inadequate to predict activity in humans due to probable synergy between pexiganan with other proteins such as lysozyme, lactoferrin, and complement (J. Ge and M. Zasloff, unpublished); indeed, application of 1% pexiganan led to eradication from open diabetic ulcers of organisms such as relatively resistant *Enterococcus faecalis.* A second class of intrinsically resistant species includes those species that secrete proteolytic enzymes, such as *Porphyhromonas gingivalis* (94,95). Distinguishing resistance on this basis can be accomplished readily by utilizing the protease-resistant all D-form of an active peptide (96). Species such as those of *Serratia* are resistant regardless of the epitope, while *P. gingivalis* is sensitive to certain peptides composed of all D-amino acids but not the L isomers (M. Zasloff and G. Bedi, unpublished).

Preclinical studies have failed to elicit resistance to pexiganan following repeated passage studies of strains including those of *Staphylococcus aureus, Staphylococcus epidermidis, Enterobacter cloacae,*

Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumanii, and *Stenotrophomonas maltophila.* In the clinical trials, strains isolated from wounds prior to therapy with pexiganan exhibited no increase in antibiotic resistance to pexiganan after 28 days of twice daily application. Additionally, efforts to induce resistance in strains of *Escherichia coli* and *S. aureus* through the use of chemical mutagens have failed (94). Furthermore, no evidence has been reported documenting transfer of a resistance phenotype through either plasmid or chromosomal transfer for pexiganan or any molecule closely related to magainin. No cross-resistance was observed between pexiganan and the following classes of antibiotics: penicillins, cephalosporins, carbapenems, quinolones, macrolides, lincosamides, sulfonamides, nitroimidazoles, polymyxin B, and polymyxin E. Pexiganan was shown to be active against pathogens that were resistant to these antibiotics.

B. Magainin

The natural peptide magainin, which is considerably less potent than pexiganan, has been studied by investigators interested in exploring mechanisms of resistance in certain gram-negative enterobacteria, principally *Salmonella.*

An outer membrane surrounds the gram-negative organism, and peptides such as magainin interact initially with cation binding sites on the lipopolysaccharide (LPS) of the outer envelope (97–99), normally occupied by Mg^{2+} and Ca^{2+}. Binding results in displacement of the metal ions and subsequent disruption of the integrity of the barrier. The peptides then gain access to the inner cytoplasmic membrane (97,99). Hence, in discussions of resistance against gram-negative organisms, the subject of most of the rigorous investigations, areas of focus have been on the availability of initial peptide binding sites on the outer membrane, as well as on events that affect their ability to permeabilize the cytoplasmic membrane.

The PhoPQ regulon (100–102), involves a two-component system that includes a sensor (PhoQ) and an intracellular effector (PhoP). The periplasmic domain of PhoQ senses Mg^{2+}. Strains defective in the PhoPQ system are most sensitive to killing by magainin. In the presence of Mg^{2+}, the PhoPQ operon is shut off, rendering the cells more

sensitive to the action of magainin. Expression of this regulon by a microbe is believed to vary during the course of an infection. In the extracellular space, where magnesium ion concentrations are high, the system should be off, creating a more sensitive species. Inside the phagolysozome, where Mg^{2+} levels are believed to be low, the system is turned on, decreasing sensitivity toward antimicrobial peptides. The PhoPQ protects the bacterial cell from low Mg^{2+} states. It serves to facilitate magnesium uptake. But the genetic system also profoundly alters the cation binding sites on the LPS, reducing the dependence of the outer membrane on Mg^{2+} to maintain integrity. The PhoP/PhoQ regulon influences the structure of the outer membrane through modulation of the Pmr A regulon (103), which itself controls a bank of genes that mediate modifications of the outer membrane with basic moieties, ethanolamine and 4-aminoarabinose. Hence, as the Mg^{2+} concentration surrounding a bacterium falls, or as external pH falls, the outer membrane must adapt chemically to retain its integrity, which it does by incorporating organic cationic groups for metal ions, reducing accessibility of magainin-like peptides. Whether this would affect peptides with a denser cationic charge, such as pexiganan, remains to be determined. Recently, parallel studies on the role of the PhoPQ operon in the resistance of *Ps. aeruginosa* to cationic antimicrobial peptides have been reported (104).

A series of genes has been identified that, when knocked out, increase the sensitivity of *Salmonella typhimurium* toward antimicrobial peptides such as magainin (105). These include *Sap* ABCDF, *Sap* G, and *Sap* J. *Sap* ABCDF encodes an ABC-type transporter, *Sap* G an NAD binding protein, and a membrane protein that interacts with *Sap* J. *Sap* G, *Sap* J, and the *Sap* ABCDF transporter are components of a low-affinity K^+ uptake system in *E. coli,* suggesting that failure to manage potassium homeostasis after an antimicrobial peptide insult can alter the sensitivity of the target.

VI. STRUCTURE–ACTIVITY STUDIES

An extensive review of the relationship between peptide sequence and antimicrobial activity for a wide range of peptides, both natural and

synthetic in design, was presented in an earlier review (106). The basic message that has emerged over the past 15 years is that an optimally designed antimicrobial peptide has a sequence comprising hydrophilic, hydrophobic, and cationic amino acids, that can organize into a "tuned" cationic amphiphilic molecule. We still cannot predict, a priori, how to construct such a molecule from first principles. Recent studies confirm this deficiency in our understanding, as highlighted in a very recent study on sequence relationships affecting the activity of dermaseptin analogs (107). The effects of a helix-breaking motif in the center of an α-helical peptide are well recognized (108,109). The selective introduction of D-amino acids into the N or C terminus is associated with enhanced proteolytic stability (110). Synthetic peptides composed of all D-amino acids (111,112) retained full antibiotic potency while exhibiting expected resistance to enzymatic proteolysis, and showed activity in models of cancer and viral infection. (113–115). Systematic study of D-lysine and D-leucine substitutions into short amphiphilic peptides demonstrated that sequences can be generated through these modifications, with various degrees of selectivity and antimicrobial potency (116). The principal issues, beyond efficacy and toxicity, that will determine whether D-amino acid–containing peptides can be commercially developed will focus on the cost of synthesis, since such molecules cannot presently be prepared by recombinant methodologies.

Recently, antimicrobial peptides comprised of β-amino acids have been constructed (117,118). Like α-amino acids, they can be assembled into sequences that adopt α-helical secondary structures. Unlike peptides derived from natural amino acids, these peptides are resistant to the action of proteolytic enzymes (119). As such, they clearly have application in settings where proteases limit the pharmacological lifetime of a peptide, such as systemic therapeutics or those to be delivered orally for the purpose of treating gastrointestinal infections. β-Peptides composed of β-leucine and β-lysine adopt an α-helical amphipathic conformation but do not display selectivity for microbial cells over human red blood cells (118). However, very recently, a peptide composed of 17 β-amino acids was described (119). Monomers consist solely of (R,S,)-trans-4-aminopyrrolidone carboxylic acid, and (R,R)-trans-2-amino cyclopentanecarboxylic acid.

This molecule has been composed in such a fashion that it can organize into a positively charged amphiphilic helix; it has broad-spectrum antibacterial activity; and it is not hemolytic.

An attempt to create a true peptidomimetic of magainin has been reported recently (120). Using the indane ring as a scaffold, moieties have been arranged around the ring system to recreate the hydrophobic:hydrophilic cross section of an amphipathic helix, resembling either lysine-phenylalanine or phenylalanine-lysine. Although these molecules exhibit antimicrobial activity, it is not clear if the mechanism resembles that of alkyl amines or, as suggested by the investigators, antimicrobial peptides, mimicked through the transient formation of stacked monomeric aggregates reminiscent of an amphiphilic helix.

VII.   RECOMBINANT SYNTHESIS

Assuming that an antimicrobial peptide exhibits an MIC in vitro of 1–10 μg/ml for targeted organisms, we can crudely estimate that a daily systemically administered dose would amount to about 1 g, roughly equivalent to the amounts of conventional antibiotics required. Successful commercial development of a linear antimicrobial peptide, then, would necessitate development of a cost-effective means of production for the new agent to remain competitive with existing agents. A solution phase chemical synthesis of pexiganan (see below) developed by Magainin Pharmaceuticals in collaboration with the chemical division of Abbott Pharmaceuticals resulted in the production of pharmaceutical-grade peptide at a cost of about $200 per gram. I and my colleagues were convinced that a significant further reduction in the cost of manufacture of peptides of this type could be achieved only through recombinant microbial production, and significant progress in this area was made (unpublished results). Recombinant production has lagged behind chemical synthesis in the commercial development of peptides, in large part due to the successful scale-up of peptide synthesis by chemical methods and the general comfort of both the Food and Drug Administration (FDA) and the pharmaceutical industry in dealing with regulatory issues surrounding chemically synthesized peptides. However, the potential for cost re-

duction makes recombinant production attractive in a setting where dosing requires large amounts of material. Several recently published reports demonstrate the feasibility of commercially producing antimicrobial peptides in bacteria through genetic engineering. Pexiganan's unamidated precursor, MSI-344, a 21 amino acid linear peptide, has been effectively synthesized as a fusion partner with a truncated portion of PurF. The cellular product forms insoluble inclusion bodies that sequester the polycationic peptide, thereby effectively reducing toxicity to the bacterial cell. This permits recovery of the product, representing over 30% of total cellular protein, through simple centrifugation and water washing (121). A similar strategy has been reported for the synthesis of ^{15}N-enriched fragments of lactoferrin, using a ketosteroid-isomerase fusion partner (122), and for the amphibian peptide esculentin (123). The active peptide is cleaved from its leader chemically and readily purified by low-pressure chromatographic procedures based on its high cationic charge density and low molecular weight. Almost certainly, future commercialization of products such as these will be based on recombinant material.

VIII. DEVELOPMENT OF THERAPEUTIC AGENTS

A. Pexiganan: Topical Antibiotic for the Treatment of Infected Diabetic Foot Ulcers

Pexiganan was the first antimicrobial peptide of animal origin to be developed as a human therapeutic agent and the first to complete Phase III human clinical trials. It was developed to treat infected foot ulcers, a condition that commonly occurs in older people with diabetes. The current therapy includes the use of broad-spectrum oral antibiotics and chronic local wound care, involving debridement of nonviable tissue and daily replacement of wound surface dressings. Pexiganan was intended for use in those individuals in whom the extent of infection was not severe, providing both patient and physician a topically applied alternative to a systemic agent. In contrast to systemic agents, which have several associated toxicities, and which profoundly disturb normal commensal microbial populations, topical pexiganan would be expected to have few if any deleterious side effects.

Pexiganan (MSI-78): GIGKFLKKAKKFGKAFVKILKK-NH2
Magainin 2: GIGKFLHSAKKFGKAFVGEIMNS-OH

Pexiganan is structurally similar to magainin 2. Through extensive sequence modification, a molecule was created by W. L. Maloy and U. P. Kari with an activity profile and a potency "tuned" to the spectrum of human dermal infections (91). The methionine was removed to eliminate the likelihood of oxidation, resulting in a molecule of greater chemical stability. The molecule was synthesized commercially by solution phase chemistry:

Step 1. GIG (segment A) + KFLKKAKKFG (segment B)
↓
GIGKFLKKAKKFG (segment AB)

Step 2. Segment AB + KAFVKILKK-*O*-methylester (segment C)
↓
GIGKFLKKAKKFGKAFVKILKK-*O*-methylester
↓ ammonolysis
pexiganan

The efficacy of pexiganan (1% cream) for the topical treatment of infected diabetic ulcers was established in two Phase III multicenter, double-blind studies. A total of 835 adult outpatients were divided randomly into two groups receiving pexiganan cream 1% or ofloxacin, 400 mg orally twice a day. Treatment lasted for 14 to 28 days unless the investigator directed earlier discontinuation. Both groups received appropriate local wound care. The overall clinical response to the treatment, assessed at the end of treatment or at follow-up (2 weeks after treatment ended), was defined as either cured or improved (Table 1).

Analysis of wound-healing parameters, which involved evaluation of signs distinct from those associated with infection, included wound closure with respect to area and depth. The time course for wound healing appeared very similar for the pexiganan and ofloxacin groups, leading to the conclusion that topical administration of pexiganan did hot impede wound healing in this clinical setting. Microbiological outcomes in this trial were also monitored. The infected diabetic ulcer is regarded as a polymicrobial infection. Indeed, at least

Table 1 Pexiganan (1%) Phase III Clinical Trial Results as Topical Therapy for the Treatment of Infected Diabetic Foot Ulcers: Overall Clinical Outcome (Cure or Improved)

Evaluation point	Pexiganan	%	Ofloxacin	%	95% CI
End of therapy	363/418	87%	377/417	90%	–7.87, 0.74
Follow-up	320/406	79%	338/403	84%	–10.41, 0.31

139 different bacterial species were cultured from this patient population, and on average, patients had at least 2 pathogenic species cultured from each ulcer. Organisms included gram-negative and gram-positive anaerobes and aerobes. Although both treatments resulted in reduction in the fraction of patients with recoverable bacteria at the end of treatment, complete eradication was not always observed despite clinical improvement. This suggests that total sterilization of an infected foot ulcer is not necessary for clinical improvement. For example, in the pexiganan group, 50% of those patients from whom *S. aureus* was cultured at the start were found free of the organism at the end of therapy, compared with 60% in the ofloxacin group. Since the skin is not a sterile organ, wound colonization with skin flora might have contributed to the relatively low rates of eradication. This result highlights the difficulty of distinguishing pathogen from colonizer in polymicrobial skin infections such as diabetic foot ulcers.

No adverse events assignable to the pexiganan treatment group were reported. The absence of allergic reactions at the treatment site, both acute and delayed, was notable.

The results suggested that pexiganan applied locally to an infected diabetic foot ulcer could achieve therapeutic resolution equivalent to that observed with a systemically administered broad-spectrum fluoroquinolone, without the toxicities associated with the systemic agent.

A New Drug Application documenting the pexiganan development program was submitted to the FDA, with the result that a ma-

jority of the members of an advisory panel convened by the anti-infective branch of the FDA agreed that pexiganan was clearly well tolerated and worked about as well as an oral fluoroquinolone. Medical and ethical questions remain as to the desirability of a placebo-controlled trial, which was requested by the panel, and development of pexiganan is on hold until these regulatory issues have been resolved.

B. Systemic Anti-Infectives

The naturally occurring antimicrobial peptide magainin 2, although active in vitro against organisms such as *E. coli,* was disappointing when evaluated as a systemic agent in mouse models of infection (124). Magainin 2, administered by the intravenous route, was effective in protecting mice inoculated via the intraperitoneal route with lethal doses of *E. coli;* however, the doses required approached 200 mg/kg, very close to the median lethal dose (LD_{50}) of the peptide. Both the low therapeutic index and the large amount of peptide required for efficacy eliminated this molecule from consideration as a drug candidate. Drs. W. L. Maloy and U. P. Kari initiated an extensive program to evaluate the effects of sequence variations on pharmacological activity. After creating several thousand molecules, however, the Magainin team found that few peptides active in vitro, exhibited favorable properties in vivo. The precise reason underlying the poor efficacy of most of the linear amphiphilic peptides is still not understood. The obvious concerns relating to chemical lability of the peptides were discounted when protease-resistant analogs synthesized from all D-amino acids proved equally ineffective, differing neither in efficacy nor in LD_{50} from the analogs composed of the corresponding all L-amino acids. One solution to the problem was achieved when a design motif from polymyxin, a short amphipathic peptide, itself without antimicrobial activity, was linked to a fatty acid (W. L. Maloy and U. P. Kari, unpublished).The basic logic was to create a molecule that could anchor to the bilayer by virtue of its lipid moiety, whereupon the peptide would organize into an amphiphilic helix.

1. MSI-843: A Distant Relative of a Linear Antimicrobial Peptide

MSI-843 is a 10 amino acid peptide with an octanoyl group attached to the amino terminus of the peptide:

2. MSI-843: $CH_3(CH_2)_6CO$-Orn-Orn-Leu-Leu-Orn-Orn-Leu-Orn-Orn-Leu-NH_2

As a consequence of the presence of ornithine moieties in place of lysine, the peptide is resistant to the action of proteases such as elastase or cathepsin G. Thus, incubation of MSI-843 with either human neutrophil elastase or human neutrophil cathepsin G, each at a concentration of 200 µg/ml, failed to degrade or cleave MSI-843. Since these protease concentrations are comparable to those observed in the sputum of individuals with cystic fibrosis, MSI-843 was a candidate for delivery as a systemic or aerosolized agent to individuals with that condition.

The MIC of MSI-843 against several standard bacterial strains was determined via the micro broth dilution assays according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (Table 2).

In addition, 45 strains of *Ps. aeruginosa* isolated from the sputum of individuals with cystic fibrosis patients were assayed. These strains were resistant to one or more of the following antibiotics: carbenicillin, ticaricillin, piperacillin, gentamycin, tobramycin, cefoperazone, imipenem, and ciprofloxacin. The MIC_{50} and MIC_{90} (the minimal inhibitory concentration that inhibits the growth of 50% and

Table 2 Antibiotic Spectrum of MSI-843

Organism	MIC (µg/ml)
E. coli ATCC 25922	16
S. aureus ATCC29213	16
Ps. aeruginosa ATCC27853	2
C. albicans ATCC 14053	32

90%, respectively, of the strains) and the MIC range of these 45 strains were determined (Table 3).

To assess whether on-therapy resistance might develop to MSI-843, an in vitro passage study was conducted. Seven clinical isolates of *Ps. aeruginosa* (obtained from Dr. Lisa Saimon, Columbia University) were passaged up to 14 times on agarose plates containing 50% of the MIC of MSI-843. The MICs were determined prior to passages and after the 4th, 7th, and 14th passages (Table 4). No significant increase in MIC was observed in these studies.

A study was carried out to determine whether MSI-843 could complex LPS, since this is a feature of many of the naturally occurring linear antimicrobial peptides (97) and is a theoretically beneficial characteristic of a human therapeutic agent. A competitive binding assay was used in which a constant amount of LPS (*E. coli* serotype 0111:B4) was bound to a constant amount of carbocyanine dye (1-ethyl-2-(3-[1-ethylnapthol (1,2-*d*)-thiazolin-2-ylidene]-2-methylpropenyl) naptho-

Table 3 Antibiotic Activity of MSI-843 *Ps. aeruginosa* Strains from CF Sputum

Agent	No. of strains	MIC_{50}	MIC_{90}	Range
MSI-843	45	4 µg/ml	8 µg/ml	2–8 µg/ml

All strains were obtained from CF centers and were resistant to multiple antibiotics.

Table 4 Attempt to Select MSI-843–Resistant Mutants by Repeated Passage

Ps. aeruginosa (ATCC #)	Initial MIC (µg/ml)	MIC following 4th passage	MIC following 7th passage	MIC following 14th passage
33599	2	2	2–4	
33718	2	2–4	2–4	
74E	4	8	8	
30398	4	4–8	4	
29820	4	16	16	8–16
33718-1	2	2	2–4	
29027	2	2–4	4	

(1,2-*d*)-thiazolium bromide) and then displaced with various amounts of MSI-843. MSI-843 at a concentration of 2 μg/ml displaced 100% of LPS. For comparison, polymyxin B, a known LPS-binding peptide, exhibited similar potency in this assay.

In CD-1 mice the LD_{50} of MSI-843 administered intravenously was 24.5 mg/kg, and when administered intraperitoneally it was between 25 and 50 mg/kg.

MSI-843 protected mice against live *E. coli*–induced lethality. Efficacy was observed with all doses of MSI-843 ($p < 0.05$); protection was provided in a dose-dependent manner. Survival of 100% was observed in animals receiving 20 mg/kg. The median effective dose (ED_{50}) was 5 mg/kg, total dose (Fig. 1).

MSI-843 protected mice against endotoxin-induced lethality, with efficacy seen at all doses greater than 5 mg/kg (Fig. 2).

MSI-843 protected mice against live *Ps. aeruginosa*–induced lethalityin a dose-dependent manner (Fig. 3).

These studies demonstrate that it is possible to modify linear peptides by shortening the peptide and adding a fatty acid to the molecule. MSI-843 is a prototype of a second-generation antimicrobial peptide, and reveals certain design insights that could be exploited further in the development of systemically administered therapeutic molecules.

3. MSI-1324: Reversible Modification of the Cationic Charges

Although the basis of the toxicity underlying linear cationic amphipathic antimicrobial peptides remains unexplained, it can be readily observed experimentally to be related to the density of cationic charges on the peptide. One successful strategy used to reduce the toxicity of polymyxin was the introduction of methanesulfonate groups onto the basic residues (125). Although the precise effects of this modification on polymyxin's pharmacodynamic profile remains unclear, the methanesulfonate groups are believed to undergo reversible hydrolysis in vivo. This probably occurs gradually, thus permitting the "masked" peptide to distribute throughout the body and only gradually convert to the cationic species. The activity of colistin in vitro also
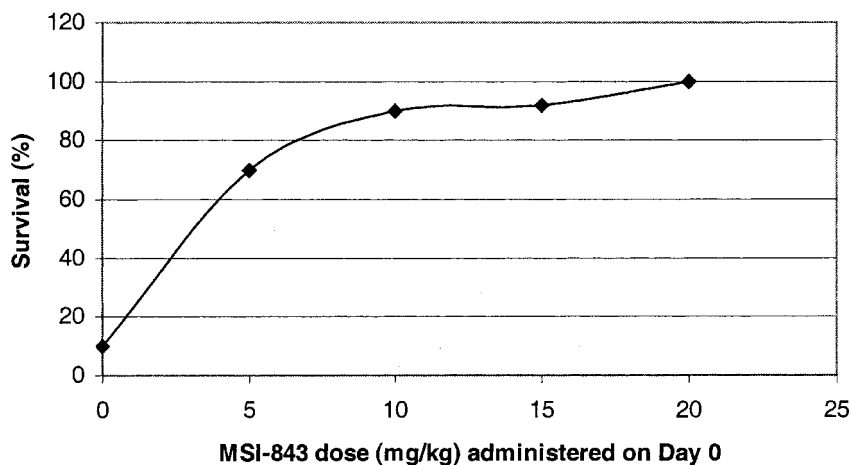
Figure 1 CD-1 mice were inoculated intraperitoneally with 9.5×10^6 colony-forming units per kilogram (CFU/kg) of *E. coli* 21915-1. At 1 and 5 hours postinoculation, MSI-843 was administered intravenously at a concentration of 0, 5, 10, 15, or 20 mg/kg in total, divided into two equal doses (10 mice per dose). Efficacy was observed at all doses ($p < 0.05$), and the protection was offered in a dose-dependent manner. Survival of 100% was observed in animals receiving 20 mg/kg. Three additional replicate experiments were conducted, with similar results.
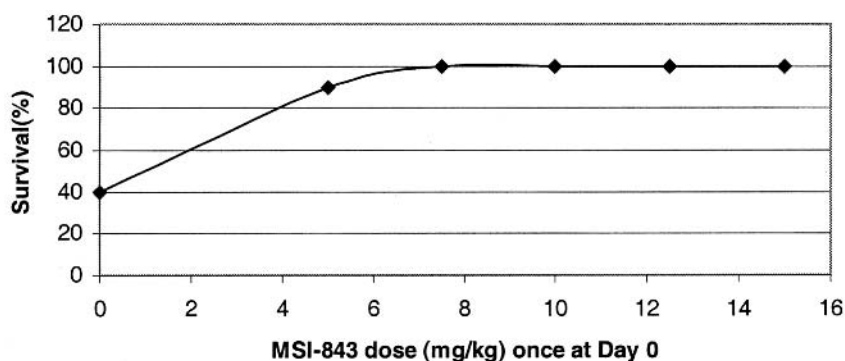
Figure 2 C57BL/6J mice were treated with MSI-843 intravenously at a concentration of 0, 5, 7.5, 10, 12.5, or 15 mg/kg (10 mice per dose) 2 minutes prior to an intraperitoneal dose of endotoxin (0.1 mg per mouse, *E. coli* LPS 0111:B4) and galactosamine (8 mg per mouse).
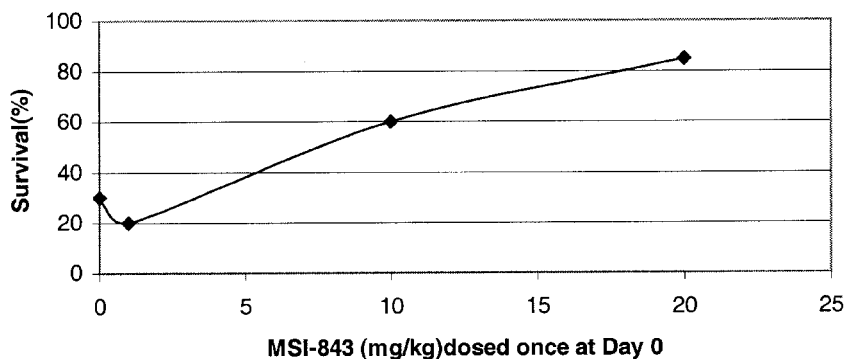
Figure 3   CD-1 mice were inoculated intraperitoneally with 10^8 CFU/kg of *Ps aeruginosa* 27853. At 1 and 5 hours postinoculation MSI-843 was administered intravenously at a concentration of 0, 1, 10, or 20 mg/kg in two divided doses (10 mice per dose).

suggests that chemical conversion might occur upon interaction between peptides and the microbe. These issues have not been resolved. However, we have successfully introduced a methanesulfonate group into the MSI-843 molecule, reducing its acute toxicity while retaining its therapeutic efficacy. This molecule was designed to be used for inhalation purposes in the treatment of cystic fibrosis.

When assayed under standard NCCLS guidelines via the micro broth dilution assay, MSI-1324 exhibited potent antimicrobial activity (Table 5). In addition, strains of *Ps. aeruginosa* isolated from cystic fibrosis (CF) patients were tested. All strains were multiply resistant to several antibiotics (Table 6). Furthermore, in vitro, the antimicrobial activity of MSI-1324, like that of MSI-843, was not significantly affected by the NaCl concentration across a physiological range (Table 7). Because of the elevated concentrations of NaCl in the surface fluid of the airway of the individual with CF, a result of the basic genetic defect in the cystic fibrosis transmembrane regulator (CFTR), endogenous antimicrobial peptide defenses fail to control microbial flora adequately in the airway (126,127). Hence, any

Table 5 Antibiotic Profile of MSI-1324

Organism	MIC (μg/ml)
E. coli ATCC 25922	8
S. aureus ATCC 29213	16
Ps. aeruginosa ATCC 27853	4
C. albicans ATCC 90028	64

Table 6 Activity of MSI-1324 and MSI-843 Versus *Ps. aeruginosa* Strains Isolated from CF Patients (MIC in μg/ml)

Compound	LS-6	LS-31	LS-40	LS-43	ATCC 27853
MSI-1324	8	16	4	2	4
MSI-843	8	16	4	2	4

Strains obtained from Dr. Lisa Saimon, Columbia University.

Table 7 Effect of NaCl Concentration on the Activity of MSI-1324 and MSI-843 Versus *Ps. aeruginosa* (MIC in μg/ml)

	NaCl concentration (mM)			
Compound	50	100	150	300
MSI-843	2	2	2	2
MSI-1324	2	2	2	2

ATCC strain 27853 was used.

antimicrobial to be considered for use in treating the chronic bronchial infections in the CF airway must exhibit activity through a range of NaCl concentrations expected to be present in the fluids of the airway of these patients.

In vivo toxicity of MSI-1324 was dramatically reduced compared with that of MSI-843. The LD_{50} of MSI-1324 was 400 mg/kg, administered as a bolus intravenously to mice. By comparison, the

LD$_{50}$ of MSI-843 was 20 mg/kg. Furthermore, MSI-1324 was well tolerated when administered by nebularization to guinea pigs. Indeed, following a 2-hour exposure of Hartley strain guinea pigs to a 30 mg/ml solution of either MSI-1324 or MSI-843, lung histology of the MSI-1324–treated animals was normal, while that of the MSI-843–treated animals was characterized by marked congestion, a grossly mottled appearance, and scattered microhemorrhages.

MSI-1324 was effective when administered systemically (intravenously) to mice previously inoculated with *E. coli* via the peritoneal route, following the identical protocol described above for evaluation of MSI-843 (Fig. 4).

In conclusion, modification of an antimicrobial peptide by methanesulfonate addition to the basic amino groups of the molecule can profoundly reduce acute toxicity while preserving both in vitro and in vivo activity. It is likely that other modifications can be devised that achieve comparable effects.
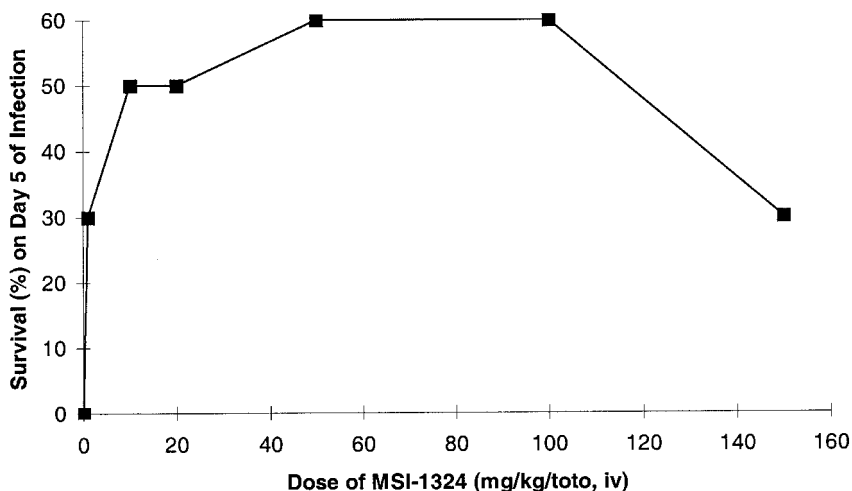
Figure 4 Effect of MSI-1324 dose on survival (day 5) of mice infected with *E. coli.*

C. Agents for the Prevention of Sexually Transmitted Diseases

The broad antimicrobial spectrum of amphibian peptides has encouraged their development as agents to limit the spread of sexually transmitted diseases (STD). To explore this therapeutic application, potential compounds have been evaluated against several of the major STD pathogens: herpes simplex virus (HSV), human immunodeficiency virus (HIV), *Neisseria,* and *Chlamydia.* The basic therapeutic strategy is to utilize these broad-spectrum agents as a "chemical condom," rather than as a therapeutic to treat an incurrent infection. In addition to the efficacy of the agent and the cost, toxicity to the female genitourinary tract is a critical issue. Limited studies in several animal models have yielded encouraging results regarding both safety and efficacy for several peptides, but as yet there is no reported human clinical experience.

The activity of magainin against viral pathogens as a microbicide has been explored. In a recent publication, the activity of numerous magainin analogs has been evaluated against HSV-1 in vitro by means of a plaque reduction assay (115). The peptides studied included protease-resistant molecules composed of all D-amino acids, peptides with *N*-octanoyl modifications, and synergistic combinations of natural magainin and PGLa (Table 8 for sequences). Studies were conducted by pretreating either the virus or the target cell (in this work a Vero cell) to determine whether the agent was virucidal or itself capable of rendering the target cell resistant. The study clearly demonstrated potent virucidal activity at concentrations ranging from 12.5 µg/ml to 50 µg/ml. Since pretreatment of cells was ineffective, the authors concluded that the inhibitory activity was due to disruption of the viral envelope.

Studies have been conducted against HIV in collaboration with the Contraceptive Branch of the National Institute of Child Health and Human Development (Bethesda, Maryland). In these studies, inocula of HIV (strain IIIB) were treated in cell-free media with a given peptide for 1 hour and introduced onto SupT cells in tissue culture. Infection was monitored 48 hours later by enumeration of syncytia or through measurement of viral reverse transcriptase. As seen in Table 9, peptides such as MSI-63 exert potent virucidal activity, comparable to that of the detergent Nonoxynol 9 (N9).

Table 8 Antimicrobial Peptides Evaluated as Microbicide Candidates for the Prevention of STDs

MSI-420	KKLLKKLKKLLKKL
MSI-94	GIGKFLKKAKKFGKAFVKIMKK-NH2
MSI-591	Oct-LKKLLKKLKKL-NH2
MSI-93	GIGKFLKSAKKFGKAFVKIMNS-NH2
MSI-63	GFASFLGKALKAALKIGANLLGGTPQQ-OH

Table 9 HIV Antiviral Activity (Cell-Free Assay)

Compound	HIV infectivity—log reduction			
	0.1%	0.05%	0.01%	0.005%
MSI-94	3.5	2.2	<0.5	<0.5
MSI-420	1.7	1.8	1.7	ND
MSI-591	1.7	1.8	<0.5	<0.5
MSI-63	ND	ND	>4	>3
Nonoxynol 9	>4	>5	4.5	2.5

Numerous peptides have been assayed against *Neisseria* and *Chlamydia,* two pathogens associated with STD. In the example shown in Table 10, the concentration of peptide observed to achieve complete sterilization is listed. Of note are the potency of the peptide and the rapidity of killing.

Chlamydia trachomatis is recognized as the most common sexually transmitted bacterial pathogen in the United States, with over 4 million cases occurring annually. Antimicrobial peptides related to the amphibian family have been shown to exhibit potent activity against this important pathogen (Table 11). In these experiments, susceptibility studies were performed with *C. trachomatis* serovar E. The peptides themselves exhibited no cytopathic effects on McCoy cells in uninfected controls, as determined by microscopic examination and trypan blue exclusion. The data presented in Table 11 demonstrate that these peptides exert potent activity against an important STD pathogen during its infections stage, and hence establish the rationale for their use as topical microbicides directed against this organism.

Table 10 Activity of Various Antimicrobial Peptides Versus *N. gonorrhoeae* In Vitro: Lowest Killing Concentration of Peptide (µg/ml) After Timed Exposure

Compound	15 minutes	30 minutes	60 minutes
MSI-63	15.6	7.8	7.8
MSI-843	62.5	62.5	62.5
MSI-148	62.5	31.2	31.2

A strain of *N. gonorrhoeae* (F62) that causes uncomplicated gonococcal infections was grown in gonococcal broth, diluted to 2×10^4 cells per milliliter, and exposed to peptides for the stated times at various concentrations. Aliquots were removed, diluted, and plated on gonococcal agar plates (GCA plates, DIFCO). Viable bacteria were counted the next day.

D. Contraceptive Agents

The spermicidal activity of magainins has been noted (128). Recent studies have demonstrated the efficacy of magainin A (129) as a contraceptive in the rat (130) and the rabbit (131). The investigators demonstrated that this compound was spermicidal against rat, human, and rabbit spermatozoa, although it required a higher concentration when assayed in saline as opposed to semen. When instilled as a 1 mg/ml saline solution into the vagina of the rabbit, magainin A afforded complete contraceptive control (131). The persistence of efficacy could be maintained for up to 24 hours following initial application. Subsequent histological examination of the vaginal epithelium demonstrated that the peptide was well tolerated, with no evidence of erythema, erosion, or inflammation noted. Introducing cyclodextrin into the contraceptive cocktail, to sequester cholesterol from the membranes of the sperm, potentiated the effects of magainin (132). The spermicidal activities of several of the peptides being considered for development as STD agents are shown in Table 12. While MSI-63 is quite potent, the other peptides with clinically acceptable antimicrobial potency are considerably less active. Thus, it is possible,

Table 11 Activity of Various Antimicrobial Peptides *C. trachomatis* In Vitro: Percent Inhibition at Two Drug Concentrations

Compound	1 µg/ml	256 µg/ml
MSI-63	40	100
MSI-843	35	85
MSI-148	100	100

Susceptibility studies were performed with *C. trachomatis* serovar E. Peptides were added to a volume of Bartels medium (Baxter Diagnostics) containing 10,000 elementary bodies per milliliter. Samples were incubated at 35°C for 60 minutes. An inoculum of the suspension was applied to prepared McCoy cell cultures (10^4 McCoy cells per tube), and the cultures were centrifuged at $600\times g$ for 1 hour at 35°C. The supernatant was removed, and the cells were overlaid with fresh Bartels medium free of antibacterial antibiotics, containing 10% fetal bovine serum, 1% glucose, and 1 µg/ml cycloheximide. Following 48 hours of incubation at 35°C, the cells were washed, fixed, and stained for inclusions using commercial fluorescein-labeled monoclonal antibody. Percent inhibition is defined as the number of inclusions observed in the pretreated sample relative to the untreated control.

in principle, to create an STD that either offers contraceptive benefit or contains solely anti-infective properties.

E. Potentiation of Clinically Useful Antibiotics

Antimicrobial peptides can enhance the potency of existing antibiotics. This probably occurs as a consequence of the damage caused by these peptides to the outer and cytoplasmic membranes, along with induction of hydrolases that degrade the proteoglycan cell wall,

Table 12 Spermicidal
Activity of Antimicrobial
Compound

Compound	MEC (mg/ml)
MSI-63	0.075
MSI-94	0.714
MSI-420	No activity
MSI-591	0.625
Nonoxynol 9	0.085

The basic spermicidal assay involved exposure of human semen to a solution of the peptide for 20 seconds, followed by direct examination of spermatozoon motility for up to 3 minutes. The minimum concentration of peptide that immobilizes sperm in <20 seconds (MEC) is noted.

facilitating access of antibiotics to the bacterial cell (99). This phenomenon was noted in studies of the action of the cationic peptide component of polymyxin, which itself was not antibiotic but which enhanced the activity of numerous conventional agents as well as components of complement (133). This principle was first applied by Darveau et al., who demonstrated synergy in vivo between magainin 2 and a β-lactam antibiotic in a murine model of an *E. coli* infection (124). Numerous studies by Scalise and associates have confirmed the utility of this approach in vitro for both gram-negative and gram-positive bacterial species (134–138). In a recent study, the antimicrobial peptide ranalexin was shown to synergize with clarithromycin, rifampin, and vancomycin versus methicillin-resistant *S. aureus* (134). Peptides such as buforin II, ranalexin, and magainin 2 exhibited activity against clinical multidrug-resistant isolates including *Acinetobacter baumanii, Stenotrophomonas maltophila, Ps. aeruginosa,* and *Rhodococcus equi,* and synergistic activity was observed in several combinations of peptides and conventional antibiotics against these strains (135–138).

F. Antimicrobial Polymeric Materials

Microbial colonization and growth on the surfaces of synthetic polymeric materials is a problem that complicates the use of medical devices such as intravenous catheters. One novel solution has been suggested by the successful demonstration that magainin peptides, covalently bound to insoluble polymeric beads, retain antimicrobial activity (139). In these studies, peptides were synthesized directly on the bead using an approach identical to that of the classical Merrifield solid-phase synthesis. The peptide density achieved ranged between 1 and 3.4 mg/g of polymer. Despite immobilization, polymer-bound magainin 2 exhibited bactericidal activity against *S. aureus, E. coli, K. pneumoniae,* and *Candida albicans;* curiously, activity against *Ps. aeruginosa* exhibited by the free peptide was lost in the insoluble form. Kinetics of bactericidal action were rapid, with a 5 log reduction in viable *E. coli* cells observed in about 30 minutes of exposure. The investigators comment that peptides have also been effectively incorporated into silicon-based plastics. In addition to providing new insights into the creation of polymeric materials with antimicrobial surface properties, these studies argue that internalization of certain antimicrobial peptides is not required for target cell killing.

Antimicrobial peptides have demonstrated efficacy in a rat skin graft model in which they were soaked into a Dacron graft permeated with collagen. In this study, ranalexin and buforin II proved as effective as or superior to rifampin, teicoplanin, and vancomycin in preventing experimental infection of the grafts following inoculation with methicillin-resistant S. *epidermidis* (140,141).

IX. WHERE DO WE STAND REGARDING COMMERCIALIZATION?

There is little doubt that antimicrobial peptides of vertebrate origin, such as pexiganan, will be used initially as topically applied anti-infectives. The efficacy of pexiganan in treating an existing polymicrobial infection, along with its safety, broad antimicrobial spectrum, bacteri-

cidal mechanism, and favorable resistance profile, augur well for the therapeutic possibilities of the class. Related peptides under development by Micrologix, Inc., are being used topically to control infection at skin sites of intravenous catheter insertion. In addition, Intrabiotics, Inc., is developing a peptide for the local treatment of oral mucositis, a polymicrobial infection that frequently occurs in individuals immuno-compromised by treatment with chemotherapeutic agents. As these products enter the market, the costs associated with peptide synthesis will certainly fall as a consequence of the introduction of newer technological improvements. Assuming that sufficient cost reductions can be achieved, the use of these peptides in relatively low-priced applications such as microbicides is likely to follow.

More uncertain is whether antimicrobial peptides of vertebrate origin will enter the armamentarium of systemic anti-infective agents. Our conventional antibiotics are truly "wonder drugs." They have margins of safety and degrees of efficacy that represent extraordinary challenges to those attempting to create new classes of therapeutics. Furthermore, because these conventional antibiotics, as natural unmodified substances, frequently work so effectively without additional chemical modification, very little effort has been invested in determining why they are so effective. Antibiotics such as penicillin, streptomycin, and erythromycin have distributive properties in the body that permit the agent to achieve therapeutic levels in all sites in the body where bacteria must be accessed in order to treat an infected human effectively. In general, we prefer to improve an already effective agent, generally through manipulation of changes that impact on bacterial resistance, metabolism, or stability in vivo; rarely can we successfully reengineer a systemic therapeutic from a molecule active in the test tube that exhibits no activity in vivo. In my opinion, the future of the commercial development of vertebrate antimicrobial peptides awaits the discovery of prototypic peptides with reasonable characteristics of therapeutic index and potency, upon which we can base further optimization. It is likely that the public appreciation of the growing crisis resulting from the increasing prevalence of pathogens resistant to conventional antibiotics will fuel these efforts with increasing energy over the coming years.

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