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Bacteriocins of Gram-Positive Bacteria

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INTRODUCTION

Historical Background

The frequency of scientific reports on the production by gram-positive bacteria of antibacterial molecules categorized as bacteriocins appears now to have entered exponential growth phase, and this review attempts to present the reader with an overview of certain aspects of contemporary research in this field. One traditionally sidestepped but ever-present issue is that of defining what constitutes a bacteriocin. Substances currently named bacteriocins comprise a rather ill-defined potpourri of proteinaceous molecules that typically first attracted the attentions of researchers because of their physiological capability of interfering with the growth on agar media of certain other, generally closely related, bacteria. Until relatively recently, most of the significant progress in bacteriocin research stemmed from investigations of the colicins, those prototype bacteriocins produced by various members of the family *Enterobacteriaceae*, and this resulted in considerable in-depth knowledge of the genetic basis, domain structure, mode of formation, and killing action of these molecules (161, 162). However, there now appears to be a remarkable renaissance of research activity centered upon the bacteriocin-like

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activities of gram-positive bacteria, particularly lactic acid bacteria. Many of these are food grade organisms that are already widely used in the food industry but now offer the further prospect of application to improve food preservation. Another contributing factor has been the burgeoning interest in possible applications of bacterial interference as a strategy for the prevention of certain infectious diseases. Several recent monographs and reviews have focused on characteristics of subcategories of the bacteriocin-like agents produced by gram-positive bacteria (24, 78, 105, 108, 120a, 169). The present review has as its particular emphasis some of the better-characterized, ribosomally synthesized, cationic, low-molecular-weight, heat-stable bacteriocins produced by gram-positive bacteria.

As was the case for so many seminal observations in microbiology, it was Pasteur who, together with Joubert, first systematically recorded an observation of antagonistic interactions between bacteria (156). In summarizing their findings that "common bacteria" (probably *Escherichia coli*) could interfere with the growth of coinoculated anthrax bacilli, either in urine (used as a culture medium) or in experimentally infected animals, they foreshadowed potential practical applications, stating "These facts perhaps justify the highest hopes for therapeutics." What followed over the ensuing three to four decades was an intensive search for bacteria of relatively low disease potential that could be used in replacement therapy (or bacteriotherapy) regimens to prevent the establishment of potential pathogens (50). Anthrax continued to be one of the most popular target diseases in these studies, and various antagonists of the in vivo or in vitro growth of *Bacillus anthracis* were reported, including both gram-positive (staphylococci, micrococci, and streptococci) and gram-negative (pseudomonads) species (reviewed in reference 51). It is not clear how many of these reported interactions can be attributed to antibiotic activities, since in most cases the observations were of a clinical rather than experimental nature and there is no information on the isolation and characterization of any inhibitory chemical substances. Nevertheless, on the basis of our present knowledge, it seems that many of the observed effects were probably due to broadly active inhibitory agents such as the production of pyocyanine by *Pseudomonas aeruginosa* or to the depletion of key nutrients or the accumulation of toxic levels of metabolic by-products in the coculture test systems.

Metchnikoff discussed the possibility of intestinal replacement therapy, claiming that the products of bacterial putrefaction contributed to the aging processes of the body (139). There followed a controversial period in which attempts were made to modify the microflora of the gut by implantation of lactic acid-producing bacteria. Another popular approach was that of introducing into the intestinal microflora "high-index" strains of *E. coli*; the index was introduced by Nissle as a measure of ability to kill *Salmonella typhi* in a coculture test system (152). The best strains were distributed commercially under the name Mutaflor, and their use was promoted for the treatment of constipation and dysentery and for typhoid carriers. However, there were persistent claims and counterclaims about the laboratory and clinical antagonistic effectiveness of these bacteria, and, once again, no characterization of an inhibitory agent that could account for the antagonism was reported. The first clear documentation of the nature of an antibiotic agent produced by *E. coli* was provided by Gratia, who demonstrated in 1925 that strain V (virulent in experimental infections) produced in liquid media a dialyzable and heat-stable substance (later referred to as colicin V) that inhibited in high dilution the growth of *E. coli* ϕ (70). There followed a period in which a whole series of colicins produced by *E. coli* and closely related members of the *Enterobacteriaceae* were discovered. Interestingly, it later became clear that the unusually small size and heat stability of colicin V set it apart from most of the subsequently isolated colicins, and it now more appropriately seems to fit the description of a microcin (48).

According to Florey et al. (51), perhaps the first description of bacteriocin-like antagonism between gram-positive bacteria was that of Babes, who in 1885 observed "le staphylococcus empêche surtout le staphylococcus" during growth on solid medium. A series of clinical observations further suggested that staphylococci were also inhibitory to *Corynebacterium diphtheriae*, and there followed extensive use of staphylococcal nasal and throat sprays for the treatment of diphtheria infection and carriage.

In recognition of the discovery that antibiotic substances of the colicin type may also be produced by noncoliform bacteria, the more general term "bacteriocin" was coined by Jacob et al. in 1953 (98). Bacteriocins were specifically defined as protein antibiotics of the colicin type, i.e., molecules characterized by lethal biosynthesis, predominant intraspecies killing activity, and adsorption to specific receptors on the surface of bacteriocin-sensitive cells. Further distinguishing features of the colicins included their relatively high molecular weights and the plasmid association of their genetic determinants.

As a result initially of the influence and efforts of Fredericq (53–55), knowledge of the colicins advanced at a great rate and more than 20 different types were identified on the basis of their actions against a set of specific colicin-resistant (generally receptor-deficient) mutants. Fredericq (52) also observed that certain strains of staphylococci produced substances, named by him "staphylococcines," that were inhibitory to the growth of other staphylococci and some other gram-positive bacteria but not gram-negative bacteria. However, attempts to categorize the staphylococcins in a manner similar to the receptor-based colicin classification scheme were not successful.

In 1976, a review of the bacteriocins of gram-positive bacteria opened with the remark that most of the definitive investigations in the field of bacteriocins had centered on those of gram-negative bacteria but predicted an increase in research emphasis on bacteriocins of gram-positive bacteria (211). Indeed, the majority of current reports of bacteriocin-like activities relate to those produced by gram-positive bacteria. It seems that much of the renewed interest in these substances is a direct response to the perceived potential practical applications of these agents either to preservation of foods or to the prevention and treatment of bacterial infections.

Food preservation. Many lactic acid bacteria have important roles in the production of fermented foods, and some of these bacteria have been shown to be capable of inhibiting the growth of a wide variety of food spoilage organisms (169, 207). The classic example of a commercially successful naturally produced inhibitory agent is nisin. Known since 1928 (178) to be produced by some *Lactococcus lactis* isolates and structurally characterized in 1971 as a lanthionine-containing peptide (73), nisin and nisin-producing strains have had a long history of application in food preservation, especially of dairy products (89, 90, 140). Recognition that nisin may be produced by *L. lactis* strains while they are naturally associated with certain foods during processing and that it has no apparent adverse effects when ingested has led the U.S. Food and Drug Administration to accord GRAS (generally recognized as safe) status to nisin (49a). With the advances that have occurred in protein purification and genetic technology, some molecular details of nisin formation have now been revealed, showing that it is formed by posttranslational processing of a prepeptide molecule (24, 42, 108, 117). Access to processing systems such as

this now offers molecular engineers the prospect of relatively facile bioproduction of novel protein structures that are not confined in their amino acid content to the 20 amino acids directly specified by the genetic code. Since techniques are now available for the site-directed mutagenesis of bacteriocin structural genes, the possibility of constructing new families of designer peptides with enhanced antimicrobial activity or improved stability and specificity characteristics has now become a reality (76, 78, 134). Peptide structure-function studies will help to reveal the molecular basis of the specificity of bacteriocin targeting and mode of action. Once the various ethical and regulatory hurdles are crossed, the path should be clear for commercial applications of starter cultures that produce a wide range of antibacterial peptides, and this in turn should bring about a significant reduction in the usage of potentially toxic nitrites in food preservation and should improve the safety of fermented foods.

Bacterial interference. Peptide antibiotics such as bacitracin and gramicidin that are synthesized in bacteria by multienzyme complexes or sequential enzyme reactions (120) have not yet achieved widespread application in the treatment of infectious diseases. However, recent studies of mersacidin and epidermin, ribosomally synthesized peptides of the lantibiotic class, have suggested that they may be at least as effective as some currently used therapeutic agents for the treatment of staphylococcal infections in mice (131) and acne in humans (222), respectively. Ever since the days of Pasteur, it seems that there has been a small subgroup of microbiologists who have stubbornly promoted bacteriotherapy and microbial interference for the treatment and prevention of infectious diseases. The discovery and dramatic success of penicillin, signaling the advent of the "antibiotic era," for a time quelled most interest in the possible applications of antagonistic microorganisms to protect the human or animal host against infection. However, one notable exception was the (generally) successful application of the relatively avirulent 502A strain of *Staphylococcus aureus* in the prevention of serious staphylococcal diseases in neonates and in the treatment of furunculosis (8). In recent years, there has been increased concern that because of the widespread overprescribing of antibiotics and consequent increased development of antibiotic resistance, the pharmaceutical industry may no longer be able to develop effective novel antibiotics sufficiently quickly. This concern is now being translated into a resurgence of interest in the implantation into the indigenous microflora of bacteriocin-producing bacterial strains of apparent low virulence that are potentially capable of interfering with colonization and infection by more pathogenic species.

General Nature of Bacteriocins

What's in a name? In the broadest sense, an antibiotic can be considered to be a chemical produced by one organism that is harmful to the growth of some other organism(s) (38). However, in practical terms, the antibiotics are generally considered to be secondary metabolites that, on the basis of laboratory observations, are growth inhibitory when present in relatively small concentrations, thereby excluding inhibition caused by metabolic by-products like ammonia, organic acids, and hydrogen peroxide. It seems likely that most if not all bacteria are capable of producing a heterogeneous array of molecules in the course of their growth in vitro (and presumably also in their natural habitats) that may be inhibitory either to themselves or to other bacteria (211). These molecules include the following: (i) toxins (many substances traditionally thought of as bacterial toxins because of the action against eukaryotic cells can also be shown to have antibacterial activity [159]; indeed, some bacterial toxins [e.g., diphtheria, tetanus, and cholera toxins] resemble the colicins in having domain structures that convey binding and toxic activity characteristics upon the molecules [155]—furthermore, some partially purified bacteriocins from *E. coli* [49] and *Streptococcus pyogenes* [212] have also been reported to be toxic for eukaryotic cells); (ii) bacteriolytic enzymes such as lysostaphin (192), phospholipase A (231), and hemolysins (64); (iii) bacteriophages and defective bacteriophages (28); (iv) by-products of primary metabolic pathways such as organic acids, ammonia, and hydrogen peroxide, and various other secondary metabolites (idiolytes) produced by bacteria that have demonstrable antibacterial activity (137); (v) antibiotic substances like gramicidin, valinomycin, and bacitracin that are synthesized by multienzyme complexes (120) (the biosynthesis of these, in contrast to that of bacteriocin-like agents, is not directly blocked by inhibitors of ribosomal protein synthesis); and (vi) bacteriocins and bacteriocin-like molecules that are directly produced as ribosomally synthesized polypeptides or precursor polypeptides.

Detection of inhibitory molecules in the laboratory is dependent on the creation or simulation of environmental conditions (pH, nutrients, temperature, etc.) that will facilitate their effective interaction with a susceptible organism, plus the application of a sensitive method of detecting how these cells have responded to the inhibitory agent. Most reports of interbacterial antibiosis have typically resulted from studies involving cross-testing different combinations of bacterial strains on agar media in either deferred (delayed) or simultaneous (direct) antagonism methods (211). Various attempts are then made to recover and purify the inhibitory agent(s) either from liquid or agar-based cultures of the producer strain. Simple methods are available to enable non-bacteriocin-related inhibitory effects to be identified, and these should be applied before antagonistic effects are attributed to the action of bacteriocin-like agents. Unfortunately, the name "bacteriocin" has sometimes been rather prematurely applied following preliminary "sightings" of inhibitory interactions. Examples of inadequate data include (i) predictions of protein nature based only on inactivation of the bactericidal activity by proteolytic enzymes, (ii) estimates of molecular weight only from gel chromatography data, and (iii) reports of inhibitory spectra that are based not upon the use of the purified inhibitory product but on the results of determining the total inhibitory activity of the producer strain when grown on agar media with the use as detector strains of only a few (sometimes poorly characterized) representative isolates of a rather small number of other bacterial species.

As details of the structures and functions of the bacteriocin-like substances produced by gram-positive bacteria accumulate, it is becoming evident that these substances comprise a great variety of agents and differ considerably from the classical colicin-based model of what constitutes a bacteriocin. This can be illustrated by reference to the typical defining characteristics of the colicins: (i) a narrow spectrum of inhibitory activity centered about the homologous species (211); (ii) the presence of an essential, biologically active protein moiety; (iii) a bactericidal mode of action; (iv) attachment to specific cell receptors; (v) plasmid-borne genetic determinants of bacteriocin production and of host cell bacteriocin immunity; and (vi) induced (SOS) release of the bacteriocin from the producer cell associated with death of that cell.

Inhibitory spectrum. Although some bacteriocin-like substances produced by gram-positive bacteria (especially some of those produced by lactococci and lactobacilli) do appear to have relatively narrow inhibitory spectra, most are much more broadly active than the colicins. In general, they tend to be

active against a wide range of gram-positive bacteria, and some have also been reported to inhibit gram-negative species (26, 109, 206). The degree of activity of bacteriocin-like agents against sensitive bacteria and, indeed, the range of apparently sensitive species can sometimes be substantially increased by testing either at particular pH values (241) or in the presence of chemical agents that weaken cell wall integrity (109, 168, 206).

Many of the bacteriocin-like agents produced by gram-positive bacteria kill species other than those that are likely to have the same ecological niche. Some relatively resistant strains can usually be detected even within a generally sensitive species, and various typing schemes have been devised which are based on either determination of the inhibitory activity of the test strains against standard sets of indicator bacteria or evaluation of the sensitivity profiles of the test strains when exposed to sets of bacterial strains known to produce different bacteriocin-like agents (128, 210, 211, 215, 233). Interpretation of spectra of inhibitory activity in terms of specific bacteriocin activities can sometimes be difficult if the producer strains release more than one bacteriocin-like agent (225, 227) or if the inhibitory activities of other metabolic products such as acids and hydrogen peroxide are not eliminated (211).

Although the specific "immunity" (or producer strain self-protection) of gram-positive bacteriocin-producing cells to their homologous bacteriocin is less strong than that found in colicin-producing bacteria, genes encoding membrane-associated molecules that confer a degree of specific protection upon the producer strain have been found in some gram-positive bacteria (117, 124, 150). In the cases of Pep5 (174) and nisin (166), it has been found that the presence of the bacteriocin structural gene as well as the immunity gene is required for expression of immunity. Immunity to lactococcin A has been shown to function at the membrane level via a mechanism that presumably blocks access to a putative receptor molecule, prevents its insertion, or inactivates the bacteriocin (150, 228).

Protein nature. Although by definition all bacteriocins have a protein or peptide component that is essential for their bactericidal function, some have been reported to consist of combinations of different proteins (7, 151, 226) or are composites of proteins together with lipid or carbohydrate moieties (101, 129, 198, 223, 224). Improved protein purification protocols have shown that some bacteriocins previously considered to be high-molecular-weight protein aggregates may be small peptides that, because of their highly hydrophobic nature, had previously copurified with some other cellular components (183). Although some gram-positive bacteria have been shown to form relatively high-molecular-weight, heat-labile bacteriocin-like substances (102, 103, 219, 220, 230, 236), most of those described to date have been small, heat-stable cationic peptides that are structurally quite unlike the colicins. The colicins are generally high-molecular-mass (29- to 90-kDa) proteins that contain characteristic domains specifying either attachment specificity, translocation, or killing activity (162). Similar domain constructs have been found in some of the pyocins produced by *P. aeruginosa* (189a). In contrast, the bacteriocins produced by gram-positive bacteria appear to be formed initially as prepeptides, which are subsequently separated from a leader peptide to form the biologically active molecule. In some cases, such as the lantibiotics (108), posttranslational modifications are introduced into the propeptide region of the precursor molecule prior to cleavage of the leader component. No equivalents of the distinctive domain regions of the colicins are evident, although clusters of positively charged amino acids may have a role in the initial interaction with the negatively charged phospholipid head groups in the cytoplasmic membrane of sensitive bacteria (151).

The unusual amino acid residues (e.g., lanthionine and β-methyl lanthionine in lantibiotics [108]) present in some bacteriocins may function to produce a more stable conformation. Others, such as didehydroalanine, didehydrobutyrine, and cysteine, may provide reactive groupings that increase the biological activity of the molecules. Some studies have suggested that the didehydro residues of lantibiotics may have important roles in the interaction of these molecules with the sulfhydryl groupings on germinating spores (142). Similarly, the reduced -SH grouping of the cysteine may be important for the activity of "thiolbiotics" like lactococcin B (231), a role similar to that of the -SH in thiol-activated toxins (27).

Bactericidal action. It appears that two major classes of killing actions are displayed by colicins: some form ion channels in the cytoplasmic membrane, and others exhibit nuclease activity upon gaining entry to a sensitive cell (162). However, the low-molecular-weight bacteriocins of gram-positive bacteria generally appear to be membrane active (19, 24, 29, 34, 35, 117, 141). The lantibiotic subgroup of bacteriocins tends to differ from the other groups in the voltage dependence of their membrane insertion (61, 62, 123, 180–182, 185–188, 197). Barrel-stave poration complexes (7, 117, 151) have been proposed to be formed between one, two, or possibly even more species of amphipathic peptides, resulting in ion leakage, loss of proton motive force, and, ultimately, cell death.

Receptors. Each different type of colicin has been shown to adsorb to specific outer membrane receptor molecules as the first stage of its interaction with a sensitive bacterium (162). By contrast, many of the bacteriocins of gram-positive bacteria appear to exhibit relatively little adsorption specificity (19, 187, 238). The cell wall of gram-positive bacteria allows passage of relatively large molecules, so that there is unlikely to be a requirement for bacteriocin receptors analogous to those in the outer membranes of gram-negative cells. Anionic cell surface polymers like teichoic acid and lipoteichoic acid may be important in the initial interaction of cationic bacteriocins produced by gram-positive bacteria (19, 23). On the other hand, some, like lactococcin A, that have a very narrow spectrum of activity may specifically interact with cytoplasmic membrane receptors (228).

Plasmid nature. Colicin production appears to be invariably plasmid mediated. Many (but not all) of the bacteriocins of gram-positive bacteria also seem to be encoded by plasmid-borne genes. Some, like nisin, have been shown to be transposon associated (42, 86, 166). The bacteriocin-associated genes of gram-positive bacteria appear to be characteristically arranged in multigene operon-like structures, the first gene typically (but not always) encoding the structural protein (117). Additional gene products may be required for transcriptional regulation, posttranslational modifications (e.g., in lantibiotics), processing, translocation to the exterior of the cell, and producer strain self protection (immunity).

Studies of the genetic basis of the bacteriocins of gram-positive bacteria are progressing rapidly. It is now a relatively straightforward process to isolate and purify a new bacteriocin, determine its N-terminal amino acid sequence, design an appropriate probe, and clone the equivalent structural gene (117).

Lethal biosynthesis. The production of colicins is repressed in most Col^+ cells, with only a small proportion of the cells in a culture producing detectable amounts of colicin. Colicin promoters, however, are responsive to SOS-inducing agents like mitomycin and UV irradiation, and exposure to these agents results in a massive increase in colicin production associated

with partial lysis of the producer cells (from the action of lysis proteins). With the exception of some of the defective bacteriophage-like structures and high-molecular-weight bacteriocins like helveticin J (102, 103) and bacteriocin BCN5 (62a), inducible production of bacteriocin-like agents has not been documented in gram-positive bacteria. Characteristically, the lower-molecular-weight bacteriocins of gram-positive bacteria are initially produced as prepeptides with short N-terminal leader sequences that are cleaved during maturation. Secondary-structure predictions of the leaders are for helical arrays typically broken at the processing sites by either proline (lantibiotics) or glycine (non-lanthionine-containing bacteriocins) residues positioned near the cleavage site to facilitate access by the appropriate peptidases. The leader sequence probably has an important role in directing the processing reactions. These leaders do not conform to typical signal sequences (lack a hydrophobic core), indicating that they probably utilize novel secretory mechanisms. Some of the bacteriocin transport-associated genes that have been detected encode membrane transporters of the ATP-binding cassette type (117).

Because of the considerable discrepancies from the colicin prototype model displayed by most of the bacteriocins of gram-positive bacteria, some authors have preferred to use qualifying terms such as "bacteriocin-like substance" or "bacteriocin-like inhibitory substance." One suggestion has been to apply the acronym BLIS (for bacteriocin-like inhibitory substances) to bacterial antibiotic substances that are produced by gram-positive bacteria and that share with the colicins the general characteristic of being proteinaceous bacterial-produced antibiotics but may differ from them in lacking other properties including SOS inducibility, receptor-mediated attachment, and a functional domain structure (209).

A wide variety of bacterial products of gram-positive bacteria have been referred to as bacteriocins, and various attempts have been made to classify these agents. Bradley considered defective bacteriophages, which lack the ability to multiply intracellularly but are able to lyse a sensitive cell, to be physiologically identical to bacteriocins (28). In other cases, enzymes with either hemolysin, phospholipase, or bacteriolytic activities have also been categorized as bacteriocins (64, 211).

Klaenhammer (117) recently defined four distinct classes of lactic acid bacterial bacteriocins: class I, lantibiotics; class II, small (<10-kDa), relatively heat-stable, non-lanthionine-containing membrane-active peptides, subdivided into *Listeria*-active peptides with the N-terminal consensus sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys- (class IIa), poration complexes requiring two different peptides for activity (class IIb), and thiol-activated peptides requiring reduced cysteine residues for activity (class IIc); class III, large (>30-kDa), heat-labile proteins; and class IV, complex bacteriocins that contain essential lipid or carbohydrate moieties in addition to protein.

Class I, the lantibiotics (lanthionine-containing peptides with antibiotic activity), are small peptides that have been differentiated from other bacteriocins by their content of didehydroamino acids and thioether amino acids (lanthionine and 3-methyllanthionine) (105, 108). Two subgroups have been defined on the basis of their distinctive ring structures (106): type A comprises screw-shaped, amphipathic molecules with molecular masses of 2,164 to 3,488 Da and with two to seven net positive charges, and type B consists of more-globular molecules with molecular masses of 1,959 to 2,041 Da and with either no net charge or a net negative charge. However, other lantibiotics like mersacidin and actagardine do not readily seem to conform to either of these groups. Klaenhammer divided the class II bacteriocins, the small non-lanthionine-containing peptides, into three subclasses on the basis of either their distinctive N-terminal sequence, their formation of bicomponent pores, or the presence of a functional sulfhydryl grouping. As further data become available, the value of these criteria as a sound basis for a natural classification scheme of the low-molecular-weight bacteriocins will become more evident. The high-molecular-mass, heat-labile molecules in class III include many bacteriolytic extracellular enzymes (hemolysins and muramidases) that may mimic the physiological activities of bacteriocins.

In the present review, the more detailed discussion relates principally to some of the more thoroughly characterized bacteriocins of the small cationic, amphiphilic polypeptide types that correspond to Klaenhammer's classes I and II. For convenience and because of current custom, we have sometimes discussed the better-characterized bacteriocins as two separate groups according to whether they contain lanthionine and are thus, by definition, lantibiotics and non-lanthionine-containing bacteriocins. However, in view of their broadly similar characteristics, it seems that the presence of a lanthionine-related structure should probably not in itself be taken as a sufficient basis for a major subdivision within the bacteriocin-like substances.

Indeed, it is our view that although information in this field is rapidly accumulating, there may not yet be sufficient data about the complete repertoire of antibacterial proteinaceous molecules produced by gram-positive bacteria to attempt to formulate a definitive and enduring natural classification scheme.

CHARACTERISTICS OF BACTERIOCINS

Bacteriocin Prepeptides

Ingram (92, 93) initially reported that the synthesis of nisin in strains of *L. lactis* differed from that of previously studied bacterial peptide antibiotics in that it was interfered with by inhibitors of protein synthesis. The reason for this difference is that unlike other peptide antibiotics such as bacitracin and gramicidin, which are sequentially assembled by a series of reactions on multienzyme complexes, nisin is ribosomally synthesized as a precursor peptide (prepeptide) that is then enzymatically modified (90). The precursor peptides of the non-lanthionine-containing bacteriocin pediocin AcH (31) and of the lantibiotics Pep5 (189, 235) and nisin (228a, 228b) have now been either isolated or detected in gel, supporting the general thesis that the low-molecular-weight bacteriocins of gram-positive bacteria are typically first formed as ribosomally synthesized precursors (prepeptides). These bacteriocin precursors appear not to be biologically active and contain a C-terminal propeptide domain which, sometimes following a variety of posttranslational modification reactions, is cleaved from the N-terminal leader sequence to yield the mature, antimicrobial molecule (39, 196). Amino acid sequences of prepeptides of several bacteriocins from gram-positive bacteria are presented in Table 1.

Prebacteriocin leader sequences. The first lantibiotic prepeptide to be isolated from the producing cell was pre-Pep5 (235). Cells of the Pep5 producer strain, *Staphylococcus epidermidis* 5, were mechanically disrupted in the presence of a protease inhibitor and the cytosolic, cell wall, and membrane fractions were separated by centrifugation. Western immunoblot analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separations of these fractions, using antisera raised against synthetic leader sequence peptides, showed that most of the pre-Pep5 was associated with the cytosol. Examination of the purified prepeptide by ion

TABLE 1. Amino acid sequence of prepeptides (precursors of bacteriocins) of gram-positive bacteria

Bacteriocin	Leader peptide sequence						Propeptide sequence											Reference(s)
	−30	−25	−20	−15	−10	−5	−1	+1	5	10	15	20	25	30	35	40	45	50	55	
Lantibiotics																				
Nisin A				MSTKDFNLDLVSVSKKDSGAS			P R	I	TSISLCTPGCKTGALMGCNMKTATCHCSIHVSK											30, 42, 110
Nisin Z				MSTKDFNLDLVSVSKKDSGAS			P R	I	TSISLCTPGCKTGALMGCNMKTATCNCSIHVSK											40
Subtilin				MSKFDDFDLDVVKVSKQDSKIT			P Q	W	KSESLCTPGCVTGALQTCFLQTLTCNCKISK											13b, 112, 119
Pep5				MKNNKNLFDLEIKKETSQNTDELE			P Q	T	AGPAIRASVKQCQKTLKATRLFTVSCKGKNGCK											115, 116
Epidermin[a]/staphylococcin 1580[a]			MEAVKEKNDLFNLDVKVNAKESNDSGAE				P R	I	ASKFICTPGCAKTGSFNSYCC											4, 183, 194, 196
Gallidermin			MEAVKEKNELFDLDVKVNAKESNDSGAE				P R	I	ASKFLCTPGCAKTGSFNSYCC											114, 195
Salivaricin A			MNAMKNSKDILNNAIEEVSEKELMEVA				G G	K	RGSGWIATITDDCPNSVFVCC											179
SA-FF22				MEKNNEVINSIQEVSLEELDQII			G A	G	KNGVFKTISHECHLNTWAFLATCCS											95, 214
Lacticin 481[a]/lactococcin DR[a]				MKEQNSFNLLQEVTESELDLIL			G A	K	GGSGVIHTISHECNMNSWQFVFTCCS											158, 177
Non-lanthionine-containing bacteriocins																				
Pediocin AcH[a]/pediocin PA-1					MKKIEKLTEKEMANII		G G	K	YYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC											82, 138, 143, 145
Sakacin 674[a]/sakacin P[a]					MEKFIELSLKEVTAIT		G G	K	YYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK											84, 218a
Leucocin A				MMNMKPTESYEQLDNSALEQVV			G G	K	YYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW											1, 79
Sakacin A[a]/curvacin A[a]					MNNVKELSMTELQTIT		G G	A	RSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM											13, 83, 217, 218
Lactacin F					MKQFNYLSHKDLAVVV		G G	R	NNWQTNVGGAVGSAMIGATVGGTICGPACAVAGAHYLPILWTGVTAATGGFGKIRK											56, 147
Carnobacteriocin A[a]/piscicolin 61[a]					MNNVKELSIKEMQQVT		G G	D	QMSDGVNYGKGSSLSKGGAKCGLGIVGGLATIPSGPLGWLAGAAGVINSCMK											84a, 237
Carnobacteriocin B2					MNSVKELNVKEMKQLH		G G	V	NYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP											163
Lactococcin A				MKNQLNFNIVSDEELSEAN			G G	K	LTFIQSTAAGDLYYNNTNTHKYVYQQTQNAFGAAANTIVNGWMGGAAGGFGLHH											85, 208, 226
Lactococcin B				MKNQLNFNIVSDEELAEVN			G G	S	LQYVMSAGPYTWYKDTRTGKTICKQTIDTASYTFGVMAEGWGKTFH											225, 226
Lactococcin M				MKNQLNFEILSDEELQGIN			G G	I	RGTGKGLAAAMVSGAAMGGAIGAFGGPVGAIMGAWGGAVGGAMKYSI											225, 226

[a] Prepeptides with same amino acid sequence.

spray mass spectrometry clearly demonstrated that pre-Pep5 was present in various stages of dehydration (189). The highest proportion was fully dehydrated, indicating that posttranslational modification occurs very rapidly within the cell. Furthermore, amino acid sequencing of the isolated prepeptide showed that no posttranslational modifications (of even serine or threonine residues) had occurred in the leader region of the prepeptide, even though they had occurred within the propeptide domain. Thus, at least for Pep5 formation, the dehydration reactions appear to be extremely specific, involving only the amino acids in the propeptide region of the molecule. These results, supported by similar findings obtained in a subsequent study of nisin formation (228a, 228b), indicate that cleavage of the leader peptide may be the last step in lantibiotic processing. For pediocin AcH, the leader peptide is removed from the prepeptide during transmembrane translocation, probably by the same protein that is associated with the transport (31).

By comparing the actual (Edman degradation-derived) N-terminal amino acid sequences of purified preparations of the biologically active bacteriocins with amino acid sequences deduced from DNA sequence data, it has been possible to predict the complete amino acid sequences of a number of bacteriocin prepeptides, several of which are presented for comparison in Table 1. The leader sequences of the lantibiotics contain 24 to 30 amino acid residues (5, 12, 30, 42, 95, 110, 114, 115, 146, 158, 179), and those of the non-lanthionine-containing bacteriocins have 18 to 24 residues (13, 56, 79, 84, 138, 143, 145, 151a, 163, 208, 217, 218, 225, 227, 237). The two pediocin prepeptide molecules are identical (138, 143, 145), as are the sakacin A and curvacin A prepeptides (217, 218), lacticin 481 and lactococcin DR (158, 177), sakacin 674 and sakacin P (84, 218a), and carnobacteriocin A and piscicolin 61 (84a, 237). The extremely high degrees of homology between the various lactococcin leader sequences and between those of the carnobacteriocins suggest that for similar types of bacteriocins from the same species, the leader peptide amino acid sequences may be particularly highly conserved. Moreover, if consideration is given to both the hydrophobicity and charges of the individual amino acid residues, it is apparent that there has been a remarkably consistent conservation of these characteristics in the corresponding positions within the leaders of many of these bacteriocins, both lantibiotics and non-lanthionine-containing bacteriocins.

One feature of the leader peptides of all of the non-lanthionine-containing bacteriocins characterized thus far and of the lantibiotic salivaricin A is the presence of Gly residues in positions −2 and −1 relative to the processing sites (Table 1). In addition, some leader peptides of non-lanthionine-containing bacteriocins contain a Lys residue after the N-terminal Met (138, 145, 147, 225, 227). Furthermore, whereas the prepeptides of the lantibiotics nisin A (30, 42, 110), nisin Z (146), subtilin (13b), Pep5 (189), epidermin (107, 196), and gallidermin (195) have a charged or polar amino acid (Arg or Gln) in the −1 position and a hydrophobic residue at position +1, other lantibiotic prepeptides, such as SA-FF22 (91), salivaricin A (179), and lacticin 481 (158), have noncharged amino acids in the −1 position and either a charged or neutral residue (Lys or Gly) in the +1 position. The presence of either Gly or Pro in the −2 position probably induces turn formation, making the processing site accessible to the leader peptidase (105, 106).

The net charges at pH 7.0 in the leader peptides may be estimated from the number of Lys, Arg, Asp, and Glu residues present. In the non-lanthionine-containing leader peptides, the net charges range from +1 for pediocin AcH and pediocin

PA-1 to −3 for the lactococcins, whereas in the lantibiotics they range from +1 in nisin to −4 in both SF-FF22 and lacticin 481. Although the exact function(s) of these leader peptides has not yet been determined, they do differ in their chemical structure and function from typical signal peptides that direct polypeptides into *sec*-dependent excretion pathways (39). Possible functions include stabilizing a prepeptide during translation, keeping the molecule biologically inactive against membrane, maintaining the specific conformation of the propeptides during processing, and assisting with the translocation of the prepeptides by specific transport systems (39, 41, 87, 99, 169).

An analysis of hydropathy profiles of the leader sequences of lantibiotics and non-lanthionine-containing bacteriocins indicates that, in general, they are relatively hydrophilic, particularly in the vicinity of their N termini (167). Secondary-structure predictions suggest that the leader sequences should adopt α-helical conformations in lipophilic environments (32, 167). Furthermore, peptides corresponding to both the leader and propeptide regions of several lantibiotics have now been chemically synthesized, and structural analyses of these by circular dichroism and nuclear magnetic resonance spectroscopy (NMR) have confirmed that the leader peptides form amphiphilic helical structures in lipophilic solutions (14, 32, 106). By contrast, the prolantibiotic regions tend to be more lipophilic and have a greater content of turn structures and/or random coil (105, 106).

Specific mutations within the sequence of the leader region of the lantibiotic nisin (228b) have been created in an attempt to further define the role of this segment in the biosynthesis of both prenisin and mature nisin. Mutations in the vicinity of the cleavage site (e.g., Arg-1 to Gln and Ala-4 to Asp) resulted in accumulation of a mutant nisin in which the fully modified propeptide region remained linked to the leader peptide. Furthermore, this variant form of nisin was found in the culture supernatant, indicating that removal of the leader peptide is the final step in nisin modification and demonstrating that it is not an essential step for effective secretion to occur. By contrast, some mutations in the middle regions of the leader peptide sequence had little or no effect on nisin production and maturation. Mutations of the amino acids in the region from −15 to −18 resulted in strains of *L. lactis* which neither produced active nisin nor accumulated any nisin precursor. These findings indicate that these amino acids may be essential for the correct biosynthetic processing required for nisin translation and maturation.

Propeptide domains and the structure of active bacteriocins. The primary amino acid sequences of the propeptide components of many of the low-molecular-weight bacteriocins produced by gram-positive bacteria (Table 1) have now been obtained either by complete N-terminal sequencing of purified bacteriocin preparations, by deduction of the complete sequence from the corresponding DNA sequences of the structural genes, or by a combination of both approaches. The combined method has been used for a number of bacteriocins, including pediocin AcH (145), pediocin PA-1 (82, 135, 138), leucocin A (79), mesentericin Y 105 (81), sakacin A (83), lactacin F (147), curvacin A (218), the three carnobacteriocins (163, 237), lactococcins A, B, and M (85, 208, 225, 227), sakacin 674 (84), nisin A (30, 42, 110), nisin Z (68, 146), subtilin (13b, 149), epidermin (196), gallidermin (195), Pep5 (111), SA-FF22 (91), salivaricin A (179), and lacticin 481 (158).

The number of amino acid residues in the propeptide domains of the non-lanthionine-containing bacteriocins prepeptides ranges from 36 to 57. Although the influence of chain length on the antibacterial effectiveness of the mature (processed) bacteriocin molecules is not known, those with fewer amino acid residues, such as pediocin AcH (18, 169), have tended to have a relatively wider antibacterial spectrum than those with larger numbers of residues, such as lactacin F (147).

Sakacin A (13, 83) and curvacin A (217, 218) appear to be identical in spite of being produced by different *Lactobacillus* spp. Similarly, leucocin A and mesentericin Y 105, although produced by different *Leuconostoc* spp., vary only in the amino acids at positions 22, 26, and 37. Furthermore, although pediocins AcH and PA-1 (82, 135, 145), leucocin A (and mesentericin Y 105) (79, 81), and sakacin P (217) are produced by bacteria of three different genera, their propeptide domains show high degrees of homology, especially toward the N-terminal regions.

In contrast, very little homology exists among several bacteriocins produced by different strains of *Lactobacillus sake* (13, 83, 217). For example, whereas sakacin A is identical to curvacin A (from *Lactobacillus curvatus*), it has no homology with sakacin P (217), which is produced by a separate strain of the same species. Furthermore, although three lactococcins are produced by the same strain of *L. lactis* subsp. *cremoris* and three carnobacteriocins are produced by variants of the same parent strain of *Carnobacterium piscicola*, little or no homology can be found between the bacteriocins originating from the same (or very similar) parent strain (85, 163, 208, 225, 227, 237).

Other readily identifiable characteristics of the propeptide domains of these bacteriocins relate to their contents of nonpolar, polar, acidic, and basic amino acids and to their net charges at different pH values (Table 2). The bacteriocins presented differ widely in many characteristics including molecular weight, pI, presence of particular groups of amino acids, numbers of amino acid residues with ionizable side chain groups, net positive charge, and absence of certain amino acids from their sequences. However, no particular combination of these factors appears to account for the observed differences in antibacterial activities of the respective mature bacteriocins.

One important feature of many of the bacteriocins of gram-positive bacteria is their cysteine content, a feature which could be used as the basis for a subgrouping scheme (Table 3). Those in which one or more cysteine residues have linked to dehydrated serine or threonine residues to form the thioether-linked amino acids Lan or MeLan are referred to as lantibiotics (99, 106, 108, 196). Alternatively, bacteriocins in which pairs of cysteine residues undergo modification to form disulfide bridges may be referred to as cystibiotics (cystine-containing antibiotics). Examples of these include pediocin PA-1 (also pediocin AcH) (with two disulfide bonds between cysteines at positions 9 and 14 and positions 24 and 44) and leucocin A (with one bond between positions 9 and 14) (79, 82, 145). Other possibilities for formation of one disulfide bond exist in mesentericin Y 105, sakacins A and P, lactacin F, curvacin A, and carnobacteriocins A, B1, and B2 (Table 1). A third subgroup of the bacteriocins, of which lactococcin B is an example, can be designated thiolbiotics, since they contain only a single cysteine residue, and this is required to be present in the reduced thiol form for bactericidal activity (231). Bacteriocins containing no cysteine, such as lactococcins A, M, and G and plantaricin A, can be grouped separately. Although the role of cysteine residues in the antibacterial effectiveness of the non-lanthionine-containing bacteriocins has not yet been fully defined, it is of interest that pediocin AcH or PA-1 (with two disulfide bonds) has a wider antibacterial spectrum than leucocin A (containing a single disulfide bond), which in turn has a wider spectrum than both lactococcin B and A, which contain either a single or no cysteine residue (18, 66, 207, 225–228,

TABLE 2. Some characteristics of non-lanthionine-containing bacteriocins and lantibiotic nisin A

Bacteriocin	Mol mass (kDa)	pI	% of amino acid groups				Amino acid(s) absent	No. of amino acids:					Positive charge at pH[a]:			
			Nonpolar	Polar	Acidic	Basic		K	R	H	D	E	4	5	6	7
Pediocin AcH	4.6	9.6	25	57	2	16	LPEFR	4	0	3	1	0	6	6	3	3
Leucocin A	3.9	9.5	30	54	2	14	IPMQD	2	1	2	0	1	5	4	2	2
Mesentericin Y 105	3.7	9.5	28	55	3	14	PMQD	2	1	2	0	1	5	4	2	2
Sakacin A	4.3	10.0	34	54	2	10	PFDH	2	2	0	0	1	4	3	3	3
Lactacin F	5.6	11.3	46	55	0	9	DE	2	2	1	0	0	5	5	4	4
Carnobacteriocins																
A	5.1	9.3	39	49	4	8	ERH	3	0	0	2	0	1	1	1	1
B1	4.3	9.2	31	52	5	12	PFWDR	4	0	1	0	2	5	3	2	2
B2	5.0	10.4	31	57	2	10	LMDH	2	3	0	0	1	5	4	4	4
Lactococcins																
A	5.8	8.6	37	52	2	9	PCER	2	0	3	1	0	4	4	1	1
B	5.3	9.1	32	49	6	13	N	4	1	1	2	1	4	3	2	2
M	4.3	10.2	54	40	0	6	CNQDEH	2	1	0	0	0	3	3	3	3
G	4.4	10.0	33	38	8	21	LPFCE	4	3	1	3	0	5	5	4	4
Nisin A	3.5	10.1	32	53	0	15	FWYQDER	3	0	2	0	0	5	5	3	3

[a] pH of ionizable side chain groups: D, above 3.7; E, above 4.3; H, below 6.0; K, below 10.5; and R, below 13.2.

231). Preliminary studies have indicated that the thioester linkage in pediocin AcH could be necessary for its antibacterial property. Heating of pediocin AcH with β-mercaptoethanol and dithiothreitol at 60°C reduced its activity by 50% or more (167). Recently, Chikindas et al. (34) reported that pediocin PA-1 loses its activity when heated with dithiothreitol. However, the activity of those (such as leucocin A) that can form one disulfide bond is not adversely affected by dithiothreitol treatment (167).

At pH 7.0, many of the low-molecular-weight bacteriocins are cationic, and this seems to be a unifying feature of both the lantibiotic and non-lanthionine-containing bacteriocins. Two important characteristics of these molecules are related to their net charge. The first is that many of these bacteriocins have greater antibacterial activity at lower pH values (pH 5 and below) than at physiological pH (18, 90, 186). The other is that their adsorption to the cell surface of gram-positive bacteria, including the producing cells, is pH dependent, with maximum adsorption at or above pH 6 and very little adsorption at about pH 2 (13a, 19, 90, 95, 97, 238). In general, each of the bacteriocins should have a higher positive charge at pH 5.0 and below than at pH 6.0 and above (Table 2). Thus, at least for the non-lanthionine-containing bacteriocins, the increased antibacterial activity observed at low pH may be the result of any

TABLE 3. Groups of non-lanthionine-containing bacteriocins on the basis of cysteine content

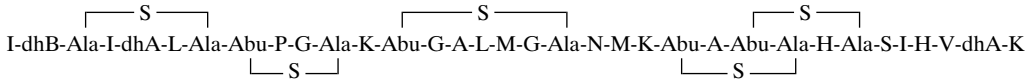
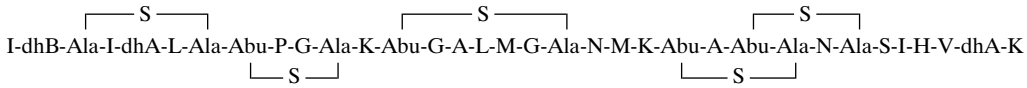
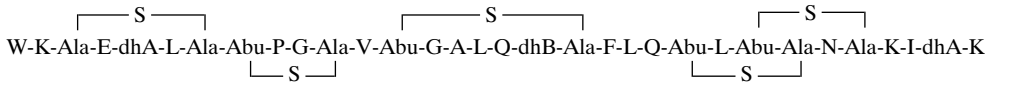
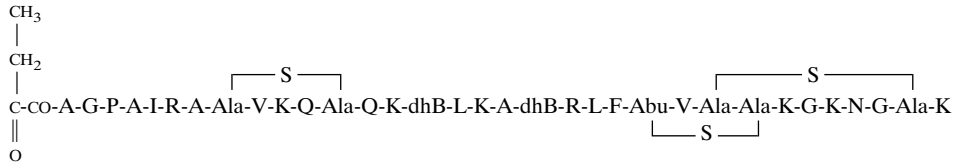
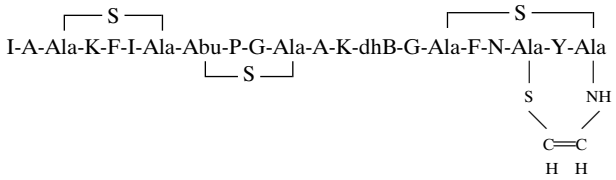
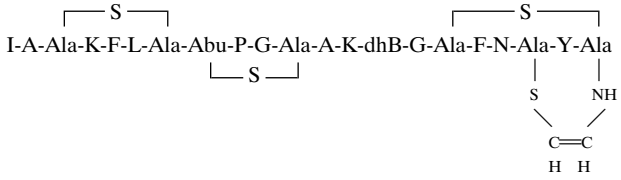
Bacteriocin	Producer strain	Antibacterial efficiency
Two or more cysteines for disulfide bridge (cystibiotic)		
Pediocin AcH	*Pediococcus acidilactici* H	Wide
Pediocin PA-1[a]	*Pediococcus acidilactici* PAC1.0	Wide
Leucocin A[a]	*Leuconostoc gelidum* UAL 187	Wide
Mesentericin Y 105	*Leuconostoc mesenteroides* Y105	Wide
Sakacin A	*Lactobacillus sake* LB 706	Medium
Sakacin P[b]	*Lactobacillus sake* LTH 673	Medium
Lactacin F	*Lactobacillus acidophilus* 11088	Narrow
Curvacin A	*Lactobacillus curvatus* LTH 1174	Medium
Carnobacteriocin A	*Carnobacterium piscicola* LV17A	Medium
Carnobacteriocin B1	*Carnobacterium piscicola* LV17B	Medium
Carnobacteriocin B2	*Carnobacterium piscicola* LV17B	Medium
One cysteine (thiolbiotic)		
Lactococcin B	*Lactococcus lactis* subsp. *cremoris* 9B4	Narrow
No cysteine		
Lactococcin A	*Lactococcus lactis* subsp. *cremoris* 9B4 *Lactococcus lactis* subsp. *cremoris* LMG 2130 *Lactococcus lactis* subsp. *lactis* bv. diacetylactis WM4	Narrow
Lactococcin M[c]	*Lactococcus lactis* subsp. *cremoris* 9B4	Narrow
Lactococcin G[c]	*Lactococcus lactis* subsp. *lactis* LMG 2081	Narrow
Plantaricin A[b,c]	*Lactobacillus plantarum* C-11	Narrow

[a] The presence of disulfide bonds has been demonstrated. Others with two or more cysteines in the propeptides are included as cystibiotic because they have the potential of forming disulfide bridges.

[b] Complete amino acid sequences are not currently available.

[c] Arbitrarily grouped as narrow against three or fewer genera, medium against four to five genera, and wide against six or more genera.

TABLE 4. Positions of lanthionine and β-methyl lanthionine formation through thioether linkage in several lantibiotics

Lantibiotic	Structure[a]
Nisin A	
Nisin Z	
Subtilin	
Pep5	
Epidermin	
Gallidermin	

[a] dhA, didehydroalanine; dhB, didehydrobutyrine.

one of a number of factors, including the following: (i) aggregation of hydrophilic peptides is less likely to occur, and, thus, more molecules should be available to interact with sensitive cells; (ii) fewer molecules will remain bound to the wall, making more molecules available for bactericidal action; (iii) hydrophilic bacteriocins may have an enhanced capacity to pass through hydrophilic regions of the cell wall of the sensitive bacteria; and (iv) interaction of the non-lanthionine-containing bacteriocins with putative membrane "receptors" may be inhibited at higher pH values.

The biologically active forms of the lantibiotic subclass of bacteriocins are notable for their content of novel, posttranslationally modified amino acids; for additional details, reference should be made to the excellent reviews by Jung (105, 106). With the notable exception of salivaricin A (179), the biologically active forms of all of the presently characterized lantibiotics (Table 4) contain different numbers of the α,β-unsaturated amino acids didehydroalanine (dhA) and didehydrobutyrine (dhB) (5, 72, 73, 94, 96, 114, 116, 146, 158). These unsaturated amino acids are formed from the dehydration of the hydroxylamino acids serine and threonine, respectively; however, the specific enzyme(s) responsible for these reactions is currently unknown. The previously characterized serine and threonine dehydratases have all been found to be active only on the free amino acids (71), suggesting that the dehydrating enzyme(s) involved in lantibiotic formation may represent a new class of enzyme (105, 106).

Lanthionine (Lan) and β-methyllanthionine (MeLan) (Fig. 1) are thought to arise from the spontaneous electrophilic addition of the thiol group of cysteine residues to specific didehydroalanine and didehydrobutyrine residues, respectively (73, 105, 106). These reactions are stereospecific since the α-carbons of the N-terminal portions of Lan and MeLan are found in the D configuration, while the C-terminal portions (so far always deriving from cysteine residues) remain in the L configuration. Similarly, the β-carbon atoms of the MeLan residues are in the L configuration following sulfur addition (60, 127). Because of the apparent scarcity of Lan and MeLan in other proteins or peptides, it seems that their formation may be specific to certain (mainly gram-positive) bacteria.

An interesting amino acid modification at the C terminus of epidermin and gallidermin (4, 5, 114) is *S*-[(*Z*)-2-aminovinyl]-D-cysteine] (AmiCys; Table 4). A three-step model for the formation of this novel posttranslational modification has been suggested, involving (i) formation of Lan by addition of the thiol group of the C-terminal cysteine to didehydrine alanine in position 19 (dhA-19), (ii) oxidation of this Lan by a specific enzyme, the flavoprotein EpiD, to form an unsaturated diamino acid, and (iii) spontaneous decarboxylation to form AmiCys (126).

The N terminus of Pep5 is occupied by another unusual amino acid modification, a 2-oxobutyryl group (Fig. 1; Table 4) (115, 116). This is thought to arise from the spontaneous deamination of a didehydrobutyrine residue located at position

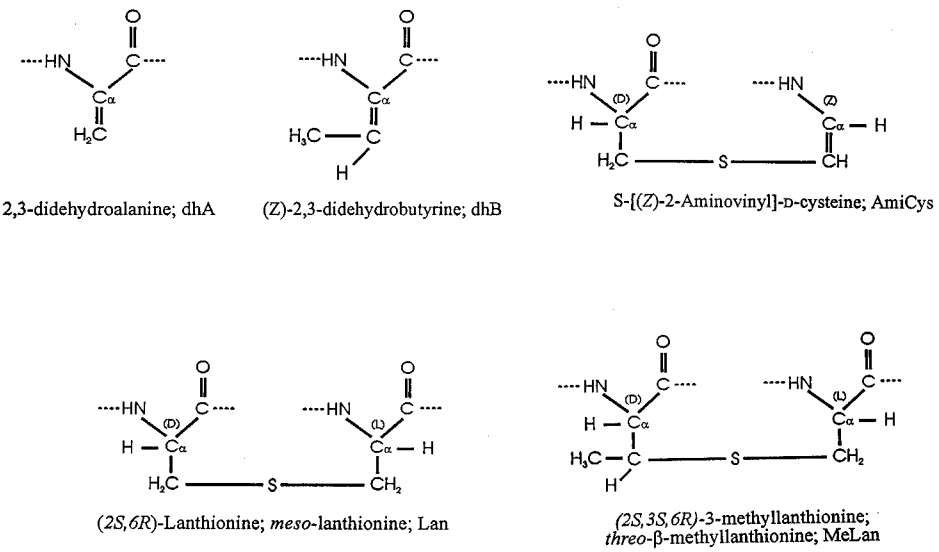


FIG. 1. Dehydration of serine and threonine and formation of lanthionine and β-methyllanthionine by thioether linkage with cysteine.

+1; this position of the propeptide domain of the prepeptide contains a threonine residue, the precursor for didehydrobutyrine formation. Similar modifications occur when α,β-unsaturated amino acids become N-terminally located during sequential Edman degradation, creating a residue that blocks further sequencing, thus complicating primary-structure elucidation (73).

Formation and release of biologically active bacteriocins. Although details of the posttranslational events occurring during the maturation of the non-lanthionine-containing bacteriocins, including propeptide modifications, cell envelope translocation, and cleavage of the leader, are not yet known, models for pediocin AcH and lactococcin A have been proposed (31, 87). One such model presented here is based on observations of pediocin AcH (Fig. 2) and predicts that since active pediocin molecules are likely to destabilize the cytoplasmic membrane (104), translocation should occur in the prepediocin form (i.e., prior to cleavage of the leader). Further evidence of this comes from the observation that pediocin producer cells have an intracellular peptidase that can inactivate pediocin AcH (104, 167, 170a). Although the modification of propediocin by the formation of two disulfide bonds is probably nonenzymatic and could thus theoretically occur either before or after translocation of the molecule, significant spatial constraints on membrane passage would probably be imposed from the folding of the molecules by the disulfide bonds, especially those between Cys-24 and Cys-44. A model developed for the lantibiotic subtilin has suggested that folding due to thioether bonding occurs following translocation of unfolded molecules through the membrane. It was argued that folding prior to export might

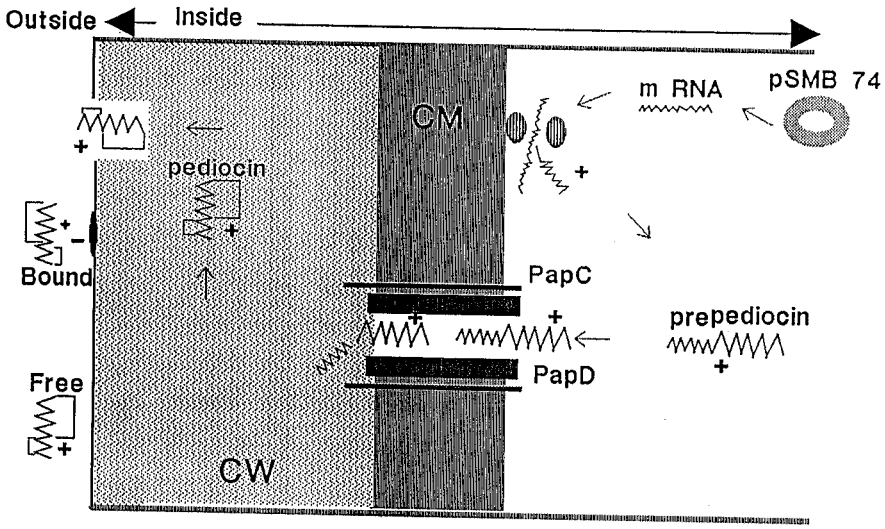


FIG. 2. Hypothetical model showing transcription of mRNA from the *pap* gene cluster in pSMB74, translation of prepediocin, translocation through the cytoplasmic membrane, processing to remove the leader peptide, formation of disulfide bonds in propediocin, and excretion of active pediocin through the wall. PapD protein has both translocation and processing functions. PapC protein may, in association with PapD, help in efficient transmembrane translocation of prepediocin. Active pediocin AcH molecules are formed away from the cytoplasmic membrane and excreted through the wall and, depending on the pH of the environment, either remain bound to cell wall or exist in the free form.

provide a barrier to traversal of the membrane by known mechanisms (77).

The endopeptidase involved in separating the leader peptide from either the propeptide or modified propeptide component at a specific cleavage site is designated the processing peptidase or leader peptidase (39). One unifying feature of the peptidases involved in the processing of the known non-lanthionine-containing bacteriocins and of the lantibiotic salvaricin A may be the ability to recognize the Gly residues in positions −2 and −1 of the cleavage sites. Some limited information is available on the properties of the enzyme(s) involved in the processing of prepediocin to pediocin AcH. The enzyme appears to act most efficiently at pH 5.0 or below (25, 104, 170a), whereas its activity is inhibited by an acid peptidase inhibitor, pepstatin A, as well as by β-mercaptoethanol (167). Recent evidence suggests that both transmembrane-translocation and peptidase action are carried out by the same protein (31). A separate study reported that this protein has only a transport function and has structural homology with several ATP transporter membrane proteins (138).

Three-Dimensional Structures

The complete primary structures of the lantibiotics nisin A (73), nisin Z (146), subtilin (72), epidermin (4, 5), gallidermin (114), and Pep5 (115, 116) have been determined. Only the arrangements of the thioether bonds remain to be elucidated for SA-FF22 (95), salivaricin A (179), and lacticin 481 (158). Attempts by NMR analysis to derive the bridging arrangements of the structurally similar lantibiotics lacticin 481 (158) and SA-FF22 (95) have not yet succeeded, because of severe line broadening of the amide resonances involved in the cyclic structures. In addition, NMR has been used to determine the three-dimensional solution structures of several of these lantibiotics. Three-dimensional structures of other types of bacteriocins have not yet been reported.

Because of the commercial significance of the lantibiotic nisin, considerable effort has been expended in deriving details of its three-dimensional structure and in assessing the impact of the local environment on this structure. It appears that in aqueous solution, nisin forms a relatively flexible structure, whereas in more lipophilic solvents such as trifluoroethanol or dimethyl sulfoxide, several more-constrained regions can be defined (130, 154, 202, 228c). The amino acids in positions 3 to 19 (forming the first three thioether rings) and positions 23 to 28 (forming the final two rings) appear to form amphiphilic helices in which the hydrophobic residues are exposed on one face and the charged amino acids (Lys and His) are orientated on the opposite face. The amino acids in positions 1 and 2 (at the N terminus) and 29 to 34 (at the C terminus) and in the central "hinge" region between the two helical components are considerably more flexible. Nisin in solution has an overall length of ca. 50 Å (5 nm), is ca. 20 Å (2 nm) in diameter, and has a net dipole moment of greater than 50 D.

Fragments of nisin, found after prolonged storage of the peptide (17) or after acid treatment (1 M HCl in 20% aqueous acetonitrile for 6 days at room temperature) have also been characterized by NMR (33). Nisin1–32-amide (i.e., [*des*-ΔAla-33–Lys-34; Val-32NH_2]nisin) showed similar antimicrobial activity to that of native nisin and, as might be expected since such modifications do not disrupt the integral ring structure of the peptide, also retained similar three-dimensional characteristics to those of the parent bacteriocin. These results confirm earlier observations (90) that nisin is a particularly stable bacteriocin and can be heated in dilute acid without significant loss of biological activity. A second product isolated by Chan et al. (33), [*des*-ΔAla-5]nisin1–32-amide], has lost the didehydroalanine at position 5, and as a result, the first ring of the peptide has been opened. Interestingly, this peptide showed little or no biological activity. However, what remains to be determined is whether the loss of bactericidal activity results from loss of an essential didehydroamino acid or from the changes in conformational stability resulting from the opening of the first ring.

Although the lantibiotic gallidermin (114) is considerably smaller (overall length, ca. 30 Å [3 nm]) than nisin, it has also been found to adopt an "extended corkscrew-like conformation," especially in the lipophilic solvent trifluoroethanol (57–59). In water, the peptide undergoes slow conformational change and additional flexibility is evident, especially between positions 11 and 16, which acts as a pseudo-hinge region. Interestingly, this region contains a potential cleavage site (Lys-13) for the endoproteolytic enzyme trypsin. In practice, trypsin acts very slowly at this site, and molecular modelling has indicated that the more flexible conformation in water is essential if the peptide is to fit the active site of the enzyme.

Preliminary investigations of the three-dimensional solution structure of Pep5 have also been reported (57). Circular dichroism analysis suggests that in aqueous solution the peptide is extremely flexible and unordered, findings further confirmed by NMR studies. However, during solvent titration from water to trifluoroethanol, Pep5 undergoes a two-state, random-coil-to-helix transition. Similar results have been obtained from circular dichroism measurements of SA-FF22 during solvent titration and in the presence of artificial phospholipid vesicles and SDS micelles (94, 95).

Purification

Early studies of nisin production showed that essentially all of the nisin activity in *L. lactis* fermentor cultures maintained at pH 6.7 remained associated with the cells and that subsequent cold-acid extraction yielded nisin preparations of high specific activity (13a). It has also been shown that the lantibiotic SA-FF22 occurs predominantly as a cell-associated, acid-extractable form when the producer strain, *Streptococcus pyogenes* FF22, is grown under pH-controlled conditions similar to those used for nisin (96). Because of their high specific activity, bacteriocin preparations obtained in this way have proved particularly useful as a starting material for subsequent purification.

A significant improvement in the yield of the lantibiotics epidermin and gallidermin has been achieved by use of a two-compartment fermentor system (222). In this system, the inner and outer chambers are separated by a low-molecular-weight-cutoff dialysis membrane which allows nutrient influx into the inner compartment in which the cells are growing and efflux of the bacteriocin into the outer chamber, from which the bacteriocins are subsequently purified. Both cells and high-molecular-weight proteins are thus excluded from the starting material used for purification. With application of optimized media and strain selection, gallidermin recovery was increased from less than 10 to as much as 720 mg/liter, making purification of large quantities of these bacteriocins possible (6, 222).

More recently, a generally applicable and commercially viable method has been developed for the recovery of several of the non-lanthionine-containing bacteriocins and nisin (17, 238, 239). The method is based on observations that the fully processed bacteriocin molecules (i) are excreted by the producer cells; (ii) are cationic; (iii) adsorb to the cell surface of the producer strains (and other gram-positive bacteria); (iv) adsorb in a pH-dependent manner, high (ca. 90%) at about pH 6.0 and low (ca. 1%) at about pH 2.0; and (v) adsorb efficiently

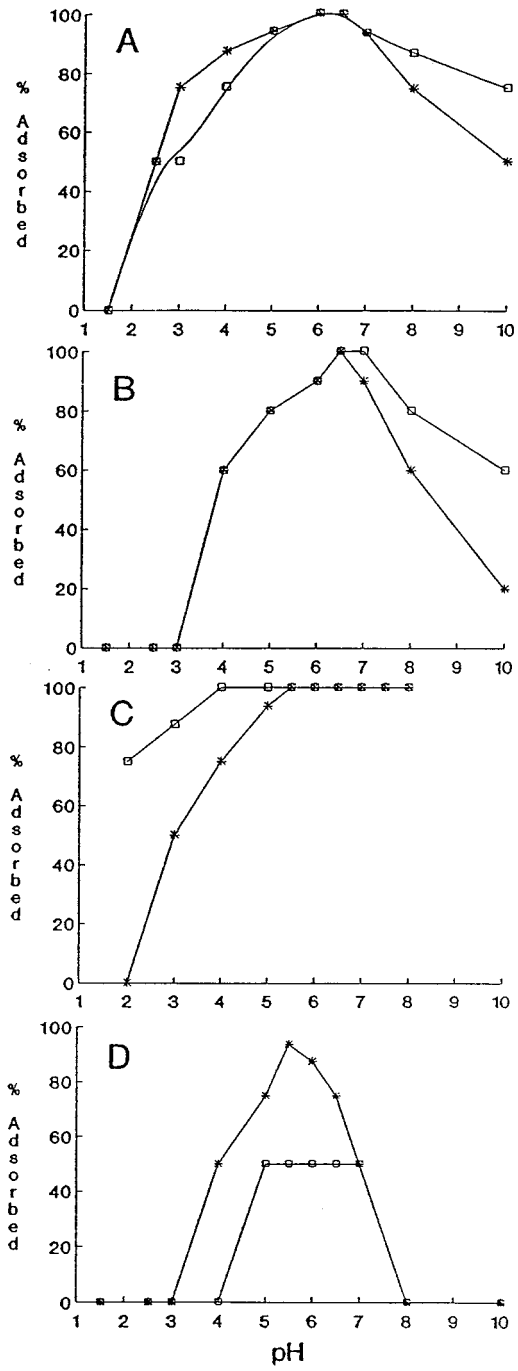


FIG. 3. Adsorption of bacteriocins onto producing and indicator bacteria. (A) Pediocin AcH adsorption on *Pediococcus acidilactici* LB42-923 (*) and *Lactobacillus plantarum* NCDO 955 (□). (B) Nisin adsorption on *L. lactis* subsp. *lactis* ATCC 11454 (*) and *Lactobacillus plantarum* NCDO 955 (□). (C) Sakacin A adsorption on *Lactobacillus sake* LB 706 (*) and *Enterococcus faecalis* MB1 (□). (D) Leuconocin Lcm1 adsorption on *Leuconostoc carnosum* Lm1 (*) and *Leuconostoc mesenteroides* Ly (□). Taken from reference 238.

to heat-killed cells. As a result, a large-scale partial-purification method was developed for three non-lanthionine-containing bacteriocins, pediocin AcH, leuconocin Lcm1, and sakacin A, and the lantibiotic nisin (Fig. 3) (238). The method consists of growing the producing strain in a fermentor under conditions optimized for production of the respective bacteriocin (terminal pH, time, temperature, medium composition, etc.) followed by pasteurization of the culture to kill the producing cells. The pH of the culture is then adjusted to pH 6.0 to allow bacteriocin adsorption to occur, and the cells are harvested and resuspended in a small volume of 100 mM saline (pH 2.0) at 4°C to release the bacteriocin molecules from the cell surface. With this procedure, yields of >95% of the starting materials with a potency of $>10^8$ activity units/g of protein for some bacteriocins have been achieved (238). The resulting material provides a convenient starting point for subsequent purification of these bacteriocins.

One of the most successful purification schemes devised for the lantibiotic-type bacteriocins takes advantage of the overall cationic nature and relative hydrophobicity of these molecules and was initially devised by Sahl and Brandis (184) for the purification of Pep5. The method, which involves sequential steps of adsorption, cation-exchange chromatography, and gel chromatography, with modifications including reversed-phase high-pressure liquid chromatography (HPLC), has subsequently been used to purify a variety of lantibiotics including subtilin (197), SA-FF22 (95, 97), salivaricin A (179), staphylococcin Au-26 (199), and staphylococcin 1580 (which is identical to epidermin [183]).

Since the major objective of most of these studies has been the preparation of highly purified bacteriocins, the low yield, relatively lengthy processing time, and requirement for costly instrumentation in these purification protocols were not a primary consideration. However, with certain modifications to the procedure, Allgaier et al. (6) have developed a commercially viable procedure for the large-scale production of epidermin and gallidermin. They showed that by omission of the reversed-phase HPLC and gel filtration steps and inclusion of hydrophobic interaction and anion-exchange chromatography procedures, up to gram quantities of the pure lantibiotics can be obtained, with yields of up to ca. 50% of the starting material.

A large number of the non-lanthionine-containing bacteriocins have now also been purified. In general, the methods have consisted of growing the producer strain in a suitable nutrient medium (preferably liquid) under optimal conditions for bacteriocin production, removing the cells, and precipitating the proteins from the culture supernatant by addition of ammonium sulfate (17–19, 79, 81–83, 85, 147, 216, 217, 228, 231). The precipitated proteins are subsequently dissolved in deionized water or a weak buffer, and the bacteriocin molecules are separated by use of various procedures including hydrophobic, ion-exchange, and size exclusion chromatography. Although these techniques have facilitated production of highly purified bacteriocin preparations, the final yield has generally been below 20% and involves several days of processing. For example, several researchers (85, 151a, 216) have described a four-step method to purify several non-lanthionine-containing bacteriocins. This involved ammonium sulfate precipitation from the culture supernatants and then sequential fractionation of the bacteriocin through cation-exchange, octyl-sepharose CL-4B, and reversed-phase chromatography. This procedure resulted in ca. 20% yield and 7,000-fold increase in specific activity. Similarly, pediocin PA-1 has been purified by ammonium sulfate precipitation followed by ion-exchange and reversed-phase chromatography (82). A slightly different method has been used to purify mesentericin Y 105 (81). The culture supernatant was subjected to affinity chromatography on a blue agarose column, ultrafiltered through a 5-kDa-cutoff membrane, and finally purified by reversed-phase HPLC.

GENETICS OF BACTERIOCIN PRODUCTION

The low-molecular-weight bacteriocins of gram-positive bacteria generally appear to be translated as prepeptides that are subsequently modified to form the mature biologically active (bactericidal) molecules. Specific auxiliary functions required of bacteriocin-producing cells include mechanisms for extracellular translocation of the bacteriocin and for conferring immunity to the bactericidal activity of the molecule. Growing awareness of the potential practical applications of bacteriocins has motivated renewed attempts to characterize the complete repertoire of genetic elements required to effect bacteriocin production. Increased understanding of the mechanisms involved in bacteriocin regulation, processing, translocation, and immunity should facilitate attempts to optimize bacteriocin production and may further open the way to directed in vitro modification of their antibacterial spectra.

Location of Gene(s)

For a number of years, there was considerable debate about the location of the genetic determinants of the lantibiotic nisin. Nisin production and sucrose metabolism were shown to be linked in *L. lactis*, and observations that these characteristics were cotransducible and were coeliminated in curing studies lead to suggestions that the determinants may be encoded by a conjugative plasmid (63, 65, 113, 204, 221). Following identification of *nisA*, the structural gene for the nisin precursor (30, 42, 110), sequencing of the flanking DNA revealed that *nisA* is carried on a 70-kb conjugative transposon, designated as Tn*5301* and Tn*5276* by two groups (43, 166). The transposon was found to have a directly repeated hexanucleotide target sequence, with two specific chromosomal sites being preferred (43, 86). Subsequently, *nisA* has been localized to megabase-sized fragments of the lactococcal genome, suggesting that if these fragments were plasmid derived, they would constitute a previously unheard-of percentage of the total cellular DNA (77, 205). Likewise, the operon encoding the subtilin structural gene *spaA* has been localized to the chromosome of *Bacillus subtilis* (13b, 77, 112), but in this case no evidence of transposon involvement has been reported.

Staphylococcus epidermidis 5 has been shown to harbor five plasmids, and at least some of the genetic determinants for production of and immunity to the lantibiotic Pep5 are located on the 18.6-kb plasmid, pED503. Following elucidation of the structure of the peptide (115), Pep5-specific oligonucleotides were constructed and were shown to specifically hybridize to pED503 but not to either chromosomal DNA or the other four plasmids (111). In addition, sequencing of one region of the plasmid demonstrated that it contained the structural gene, *pepA*, along with several other Pep5-related genes, which most probably form an operon (175). By a similar approach, the genes forming the epidermin operon in *Staphylococcus epidermidis* Tü3298 (11, 12, 193, 194, 196) have been localized to the 54-kb plasmid, pTü32.

Neither of the currently documented lantibiotic-producing streptococcal strains, *Streptococcus pyogenes* FF22 or *Streptococcus salivarius* 20P3, appear to contain plasmids, prompting speculation that the determinants for the production of SA-FF22 and salivaricin A may be chromosomally encoded (201). Previous studies have demonstrated that there are similarities between nisin and SA-FF22 (215) and that the production of a specific immunity to SA-FF22 may be transferred by transduction between different *Streptococcus pyogenes* strains (213) or spontaneously lost upon aging of the producer culture (214). Hence, it appears likely that the various genetic determinants required for SA-FF22 production and immunity may be linked and could, like that of nisin, perhaps be transposon associated. By using a similar approach to that adopted for Pep5, specific oligonucleotide probes based on the peptide sequences of purified SA-FF22 and salivaricin A were used to identify, clone, and sequence the structural genes *scnA* (91) and *salA* (179) from libraries of whole-cell DNA. Although the beginning of an additional reading frame downstream of *salA* was reported, no additional genes involved in the production of either of these lantibiotics have been identified so far.

The structural genes encoding many of the currently characterized non-lanthionine-containing bacteriocins have been located on plasmids (3, 13, 36a, 37, 44, 66, 69, 88, 150, 172, 173, 177, 190, 198, 225), with the notable exceptions of those encoding lactacin F, which is carried on a recombinant plasmid or episome (147), and plantaracin A (45) and sakacin 674 (84), which are chromosomally encoded. Plasmid-curing studies and comparison of the plasmid profiles of both bacteriocin-producing and -nonproducing strains have provided further evidence for a plasmid location for a number of these bacteriocins. The plasmids isolated vary greatly in size, ranging from 6.0 kb for the pediocin SJ-1-associated plasmid (198) to 131 kb for the plasmid associated with lactococcin A production in *L. lactis* subsp. *lactis* (208). The plasmids encoding lactococcins A, B, and M and diplococcin have been shown to be conjugative, whereas conjugal transfer of a plasmid and the concomitant inheritance of a bacteriocin-producing phenotype have been used to tentatively link bacteriocin production to plasmid-encoded genes in several studies (37, 148, 190).

In some cases, a single plasmid may carry the genetic determinants for several bacteriocins; thus, p9B4-6 encodes lactococcins A, B, and M plus the corresponding immunity proteins (226, 227). Alternatively, separate plasmids carried in different strains and subspecies may encode the same bacteriocin. For example, lactococcin A has been shown to be carried on three separate plasmids ranging in size from 55 to 131 kb in two different subspecies of *L. lactis* (85, 208, 226, 227). Finally, two or more bacteriocins may be encoded by different plasmids in the same strain. Thus, *Carnobacterium piscicola* LV17 harbors pCP49, encoding carnobacteriocin A, and pCP60, encoding carnobacteriocins B1 and B2 (163, 237).

Genetic Organization

The first lantibiotic gene cluster to be characterized was that which encodes production of epidermin. The structural gene *epiA* encodes the 52-amino-acid epidermin precursor and is contained within a polycistronic operon on the 54-kb plasmid pTü32 (11, 12, 193, 194, 196). Through the use of complementation analysis of deletion mutants and heterologous expression in *Staphylococcus carnosus* (11, 12, 194), it has been shown that a minimum of six genes are required for epidermin biosynthesis (see Fig. 4). *epiB* and *epiC* immediately follow *epiA* and overlap by 122 bp with *epiC* frameshifted by −1 bp (193). They are homologous to similar genes found in the nisin and subtilin operons. *epiD* is uniquely found in the epidermin gene cluster. *epiQ* and *epiP* are also located downstream from *epiA* but are in the opposite orientation to *epiA*, *epiB*, *epiC*, and *epiD*. *epiQ* shares homology with certain regulatory genes such as *phoB* in *E. coli*, while *epiP* is homologous to several serine proteases (11, 12, 193, 194). These similarities have led to suggestions that *epiQ* may play a regulatory role in epidermin biosynthesis and that *epiP* may be involved in the cleavage of the 30-amino-acid leader from the epidermin prepeptide molecule.

Analysis of the genetic control of the epidermin operon (12, 194) has shown that *epiA* is preceded by a promoter and fol-

lowed by a terminator, while *epiB*, lacking its own promoter, is cotranscribed from the *epiA* promoter. This suggests that the terminator is "leaky" and regulates downstream transcription. Overall, two major transcripts have been found, one corresponding to *epiA* and the other probably corresponding to full-length transcription of *epiABCD.*

The nisin structural gene, *nisA*, has likewise been cloned and sequenced (30, 42, 110) and found to be part of a polycistronic operon (46, 47, 77, 205). Three additional genes lie downstream: *nisB*, which is homologous to *epiB*; *nisT*, which shares homology with the hemolysin B gene and therefore may be involved in nisin transport; and *nisC*, which is homologous with *epiC*. In addition, *nisC* overlaps *nisT*. However, because of the presence of three possible translation initiation sites, the exact amount of overlap is presently unknown. As in the epidermin operon, transcripts can be found corresponding to the structural gene either alone or together with the contiguous genes (*nisABTC*). In addition, several additional genes (*nisI*, *nisP*, and *nisR*), which also appear to be involved in the production of nisin, have been demonstrated further downstream (124).

NisB contains several putative transmembrane helical regions and appears to bind to artificial phospholipid vesicles (47), leading to the suggestion that it is membrane bound and that nisin biosynthesis occurs at the cytoplasmic membrane. Subsequently, it has been demonstrated that the homologous gene involved in subtilin production, *spaB*, is also membrane bound (74). Some discrepancies exist regarding the size of the *nisB* product (205). Western blots of SDS-PAGE gels have been used to establish that Nis B corresponds to an approximately 117.5-kDa protein (46), some 17 kDa larger than that suggested by Steen et al. (205). Such a difference may be the result of a sequencing error (46).

Other genes present in the nisin operon include *nisI*, *nisR*, and *nisP. nisI* is thought to encode a lipoprotein involved in nisin immunity, and *nisR* appears to be involved in the regulation of nisin biosynthesis (124, 228a). It has been shown that NisR has significant homology to regulatory proteins of two-component sensor kinase/response regulator systems (228a). Subsequently, the second component, NisK, which is thought to be the histidine kinase, was also identified (47). Interestingly, both NisR and NisK have considerable homology with the products of the subtilin genes *spaR* and *spaK*, which are also thought to form a response regulatory system (118), but not with the product of *epiQ*, the regulator of epidermin biosynthesis (11, 12, 193, 194). NisP shows strong homology to many of the subtilisin-like serine proteases and, as such, may be involved in nisin processing (228a).

The subtilin operon, while sharing several similarities with the nisin operon, also shows some interesting differences. *spaS* encodes the precursor peptide from which subtilin is matured (13b). Although *spaB*, *spaT*, and *spaC* share homology with their counterparts *nisB* and *epiB*, *nisT*, and *nisC* and *epiC*, respectively, they differ in their position within the operon in that they lie upstream from *spaS* (112, 119). This difference in operon arrangement indicates that although nisin and subtilin may have a common ancestral origin, they have undergone significant subsequent divergence (77). It is noteworthy that in a separate study, an additional open reading frame (ORF), *spaD*, was defined between *spaT* and a correspondingly shortened version of *spaB* (36, 77). This apparent discrepancy, which could result from alteration of only a single nucleotide, still requires resolution.

Further similarities between the nisin and subtilin operons include the presence of an overlap between *spaC* and *spaT*, as occurs between *nisC* and *nisT* in the nisin operon, and the homology of SpaT with ATP-binding cassette transporters such as HylB. Evidence of a transport role for SpaT has come from analysis of *spaT*-deficient mutants; these have altered cellular morphology and lose viability as a result of cytoplasmic accumulation of subtilin (112, 119).

Recently, Klein et al. (118) have identified two overlapping ORFs, *spaR* and *spaK*, both of which are required for subtilin production and are located 3 kb downstream from *spaS*. On the basis of their homologies with other proteins, SpaR and SpaK appear similar to several pairs of proteins which together form histidine kinase/response-regulatory systems in *E. coli* (9, 10). Indeed, production of subtilin by wild-type *B. subtilis* appears regulated to occur in the late logarithmic growth phase, and deletions in either of these two genes results in the loss of subtilin-producing ability (118). Similar findings have been obtained for mutations in *nisR* and *nisK*, the recently identified response-regulatory elements for nisin biosynthesis in *L. lactis* (47, 228a).

Analysis of the genes required for Pep5 production has revealed three ORFs that precede the structural gene, *pepA*; these are *pepI*, which has been implicated in immunity to Pep5, *pepT*, which has homology with *nisT* and *spaT*, and ORF X (174). Three other genes, *pepP*, *pepB*, and *pepC*, immediately following *pepA* and having homologies to corresponding genes in the nisin and subtilin cluster, also appear to play a role in Pep5 production (183a).

The gene clusters of several non-lanthionine-containing bacteriocins are presented in Fig. 4. Two groups have independently determined the location and sequence of the pediocin PA-1 (pediocin AcH) operon. Marugg et al. (138) cloned a 5.6-kb *Eco*RI-*Sal*I fragment from the 9.4-kb plasmid pSRQ11 of *Pediococcus acidilactici* PAC1.0 into the plasmid vector pBR322. The recombinant plasmid was transformed into *E. coli*, and the transformants produced pediocin PA-1. Subsequent studies revealed the presence of four ORFs in a cluster, with a common promoter and independent ribosome-binding sites. The genes were designated *pedA*, *pedB*, *pedC*, and *pedD*, with *pedA* encoding the prepediocin molecule and *pedD* encoding a protein associated with translocation.

In a separate study, the 8,877-bp plasmid pSMB74, which encodes pediocin AcH production and immunity in *Pediococcus acidilactici* LB42-923, has been completely sequenced (31, 143, 171). By using the partial amino acid sequence obtained from purified pediocin AcH, the location of the structural gene encoding pediocin AcH, *papA*, could be identified (145). Further sequence analysis in both directions revealed the presence of a cluster of four adjacent genes with a common promoter, a common rho-independent terminator, and independent ribosome-binding sites, initiation codons, and stop codons. The genes, *papA*, *papB*, *papC*, and *papD*, encode proteins of 62, 112, 174, and 714 amino acids, respectively. While *papA* encodes prepediocin, *papD* encodes a protein that has both translocation and endopeptidase activities. Also, *papB* could encode immunity protein, since a mutation in *papB* failed to protect cells from the lethal effect of pediocin AcH (31, 143). Comparison of these sequences with those obtained from *Pediococcus acidilactici* PAC1.0 (138) showed that the two bacteriocins are identical.

The arrangement of the genes in the lactococcin A operon appears quite different from that in the pediocin AcH/pediocin PA-1 gene cluster. The structural gene, *lcnA*, encoding the prepeptide for lactococcin A, has been independently identified in three strains of *L. lactis* (85, 208, 226, 227). van Belkum et al. (226, 227) identified and sequenced *lcnA* (encoding the 75-amino-acid prelactococcin) and *lciA* (encoding a 98-amino-acid putative immunity protein) and showed that these two genes are located next to each other on a 1.3-kb *Sca*I-*Hin*dIII

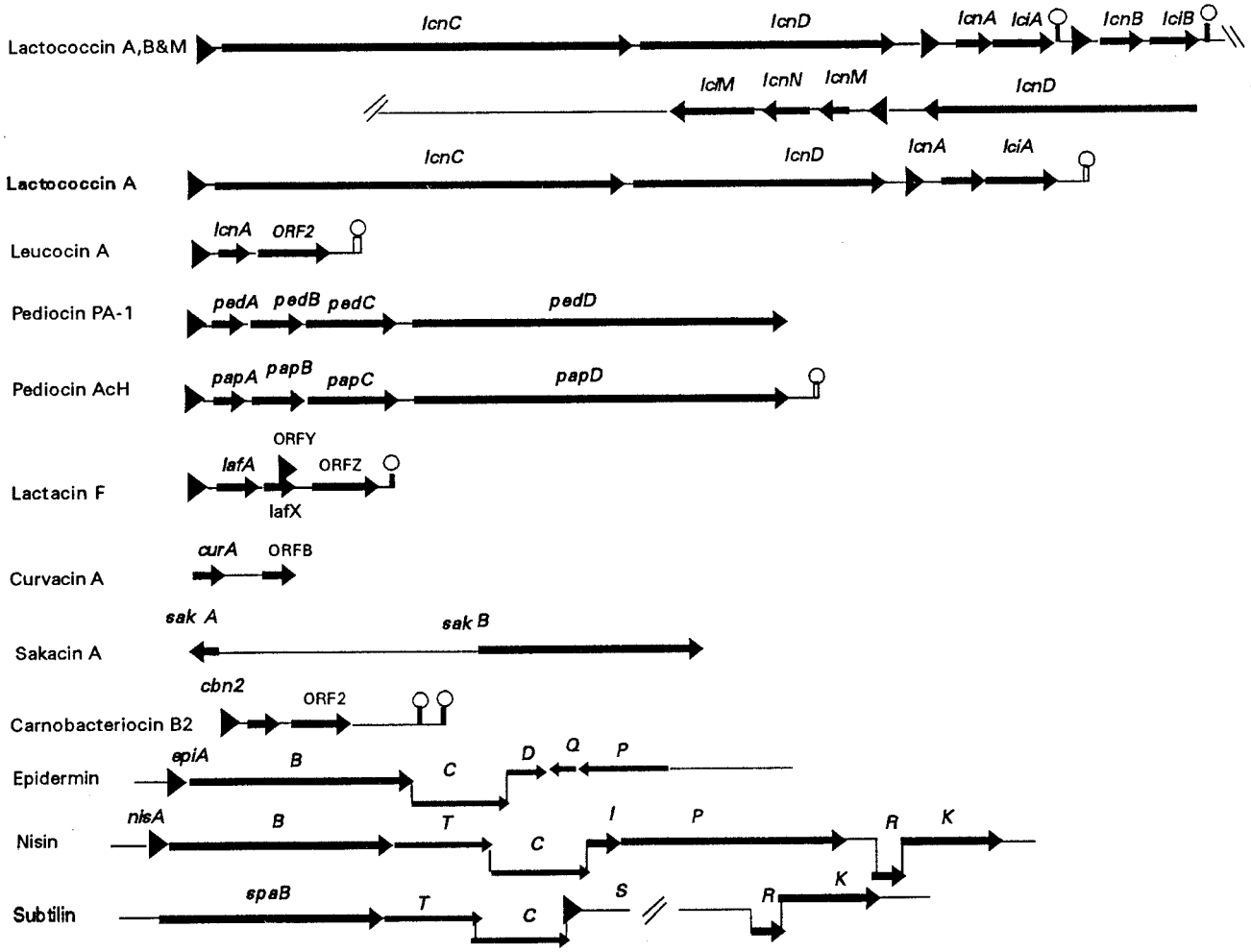


FIG. 4. Arrangement of the genes in clusters associated with the production and immunity of several bacteriocins in gram-positive bacteria. In some, only two genes have been identified. Promoters (arrowheads), terminators (lollipop-like symbols), and overlapping sequences (arrows below the line) are shown where information is available.

fragment of the 60-kb plasmid p9B4-6. The two genes have a common promoter upstream of *lcnA*, a common rho-independent terminator(s) downstream of *lciA*, and independent ribosome-binding sites. Furthermore, by mutational analysis, these authors were able to confirm the functions of the proteins encoded by the two genes. Both genes were also identified and sequenced by Holo et al. (85) with a 1.2-kb *Rsa*I-*Hin*dIII fragment from a 55-kb plasmid. They identified the function of the *lcnA* gene but not of the second gene (ORF2). Similarly, Stoddard et al. (208) sequenced a 5.6-kb *Ava*II fragment from the 131-kb plasmid pNP2 and located a cluster of four ORFs present in the order *lcnC*, *lcnD*, *lcnA*, and *lciA*. This cluster was shown to contain two promoters, one upstream of *lcnC* and the other upstream of *lcnA*, and two stem-loop terminators downstream of *lciA*. The 716-amino-acid LcnC and the 474-amino-acid LcnD proteins were thought to be involved in translocation of lactococcin A through the cytoplasmic membrane. In addition to *lcnA* and *lciA*, van Belkum et al. (226, 227) identified and sequenced two other gene clusters located on p9B4-6 that were associated with production of and immunity to lactococcin B and lactococcin M. *lcnB* and *lciB* were found downstream of the lactococcin A operon and encoded the 68-amino-acid prelactococcin B molecule and the corresponding 91-amino-acid immunity protein. *lcnB* and *lciB* have a common promoter and a common terminator but have independent ribosome-binding sites. Further downstream, the gene cluster *lcnM*, *lcnN*, and *lciM* was detected and was shown to have an orientation opposite to that of the lactococcin A and B clusters. Both the 69-amino-acid LcnM and the 77-amino-acid LcnN proteins were associated with the antimicrobial activity, while the 154-amino-acid LciM protein could confer immunity to lactococcin M.

Recently, Havarstein et al. (80) reported sequencing the genes involved in the production of lactococcin G, whose antimicrobial activity is also dependent on the combined action of two peptides, LagA and LagB. The *lag* operon consisted of five genes in the same orientation, each preceded by its own ribosome-binding site. The first two genes, *lagA* and *lagB* encode the 54- and 60-amino-acid precursors of LagA and LagB, respectively. The *lagC* gene, downstream of *lagB*, probably encodes the immunity protein. *lagD* and *lagE*, located further downstream, are thought to be involved in membrane translocation of the bacteriocin.

In a similar study, Rince et al. (177) have sequenced the genes located on a 70-kb plasmid from *L. lactis* that are involved in the production of lactococcin DR. The first gene

downstream of the common promoter encodes a 51-amino-acid prelactococcin molecule and is followed by a gene that encodes a 944-amino-acid protein apparently associated with the expression of lactococcin DR.

Some of the genetic determinants of lactacin F production by *Lactobacillus johnsonni* VPI 11088 have also been sequenced (2, 7, 56, 147). The gene cluster consists of *lafA*, *lafX*, ORF Z, and an additional gene, ORF Y, which overlaps *lafX*. While *lafA* is the structural gene for lactacin F, *lafX* encodes a protein that enhances the action of the bacteriocin. The operon has a promoter upstream of *lafA* and a rho-independent terminator downstream of ORF Z. It has also been suggested that other genes, which encode additional proteins necessary for processing, maturation, and translocation of lactacin F, may be present upstream and downstream of the lactacin F operon (56).

The structural gene for the leucocin A-UAL 187 (or leucocin A) prepeptide is located on an 11.4-kb plasmid in a *Leuconostoc gelidum* strain (79). Nucleotide sequence analysis of a 1.1-kb fragment revealed that the structural gene for preleucoccin (*lcnA*) is preceded by an additional gene, ORF2, encoding a 113-amino-acid protein. The two genes in this cluster have a promoter upstream of *lcnA*, a rho-independent terminator downstream of ORF2, and separate ribosome-binding sites. The function of the ORF2 protein has not yet been determined but is hypothesized to be related to development of immunity. The locations of other genes associated with maturation, processing, and translocation were not established.

The gene encoding production of the precursor of curvacin A (*curA*), a bacteriocin produced by *Lactobacillus curvatus*, has been localized to a 1.2-kb *Ace*I-*Eco*RI fragment of a 60-kb plasmid and was found to precede an additional gene, ORF2 (218). Although the function of the 51-amino-acid ORF2 protein was not defined, it was predicted to have a role in producer cell immunity. A promoter was identified upstream of *curA* along with a potential ribosome-binding site; however, no ribosome-binding site was identified for ORF2. No other genes that could be implicated in peptide processing, maturation, and translocation were present in the sequenced portion. In another study, the structural gene for sakacin A, *sakA*, was identified on a 1.4-kb *Eco*RI fragment present in a 60-kb plasmid from *Lactobacillus sake* LB 706 (13, 191). The nucleotide sequence of *sakA* was subsequently shown to be identical to that of *curA*. Sequencing of a separate 1.8-kb *Hin*dIII-*Bgl*I fragment from the same plasmid revealed the presence of another gene, *sakB*, with its own promoter and ribosome-binding site but in the opposite orientation to *sakA* and separated from it by 1.6 kb. The 430-amino-acid SakB is thought to provide immunity to sakacin A.

By contrast, the plantaricin A prepeptide structural gene has been located in a 5-kb chromosomal fragment isolated from *Lactobacillus plantarum* C11 (151a). However, there was no report of additional genes necessary for processing, maturation, translocation, and/or immunity. Similarly, the gene encoding presakacin 674 has recently been identified and localized to a 3.6-kb *Eco*RI-*Cla*I fragment of the chromosome of *Lactobacillus sake* LB 674 (83). Once again, no additional genes associated with the production of or immunity to sakacin 674 have been reported.

Three structural genes, encoding carnobacteriocins A, B1, and B2, have been located in two separate plasmids in a *Carnobacterium* strain (163, 237). The *carA* gene was contained on a 2.0-kb *Eco*RI fragment of a 74-kb plasmid, and its presumptive immunity gene was detected on a separate 5.4-kb *Xba*I-*Pst*I fragment. Two further genes, *carB1* and *carB2*, encoding the prepeptides for carnobacteriocins B1 and B2, respectively, were identified on an additional 60-kb plasmid harbored in the same strain. Nucleotide sequence analysis of a 1.9-kb *Hin*dIII fragment revealed the presence of a clustered arrangement of two genes, *carB2* and ORF2, with a common promoter upstream of *carB2* and separate ribosome-binding sites for each gene. The ORF2 protein was presumed to be the immunity protein for carnobacteriocin B2. Once again, the putative genes necessary for the processing, maturation, and translocation of carnobacteriocin A, B1, and B2 have not yet been identified.

Naturally Occurring Variants

Nisin Z is a naturally occurring variant of nisin, differing only in the exchange of asparagine for histidine at position 27 (H27N-nisin) as the result of a single-base substitution (C to A) in the first position of codon 27 (40, 125, 146). Although this represents only a minor change to the DNA sequence, the ramifications at the peptide level are highly significant; H27N-nisin is significantly more water soluble and heat stable at elevated pH values than nisin yet retains comparable biological activity. By contrast, nisin is poorly soluble in water and is inactivated at or above neutral pH, especially at elevated temperatures (90). Furthermore, H27N-nisin shows better diffusion properties in solids, probably because of the increased hydrophilicity of the peptide (89). Together, these factors may prove relevant in its future applications to food preservation.

Similarly, the lantibiotics epidermin and gallidermin differ only by a single, conservative amino acid exchange (L7I), suggesting that they may be natural variants (4, 114). However, for historical reasons, they have both retained their original names.

Associated Genes and the Functions of Their Products

Epidermin contains, in addition to Lan and MeLan, the C-terminal modification AmiCys (4, 5). Furthermore, analysis of the epidermin gene cluster suggests that the gene *epiD* is not homologous to any of the genes currently implicated in the biosynthesis of either subtilin or nisin, two lantibiotics that lack AmiCys residues (72, 73). Recently, Kupke et al. (126) overexpressed EpiD in *E. coli* as a maltose-binding protein fusion product and were able to demonstrate that it plays a role in the formation of AmiCys. Furthermore, they demonstrated that the *epiD* gene product is a flavoprotein requiring the cofactor flavin mononucleotide, prompting the suggestion that the enzyme is responsible for the removal of two reducing equivalents during the formation of AmiCys.

EpiQ has previously been implicated in the regulation of epidermin biosynthesis (11, 12, 193, 194). More recently, Peschel et al. (157) have shown that EpiQ is a transcriptional activator of the *epiA* promoter, and putative operator sites for EpiQ action have been located upstream of *epiA*. Furthermore, the derivation of a recombinant epidermin-producing strain in which excess EpiQ was produced led to considerably increased production of epidermin, suggesting that enhancement of EpiQ levels might prove useful in generating epidermin-overproducing strains of *Staphylococcus epidermidis.*

As previously mentioned, the gene *nisP* has been identified within the nisin operon, and NisP has homology to several previously described subtilisin-like serine proteases (228a). Furthermore, deletion of the *nisP* gene led to formation of fully matured nisin, which was still attached to the leader peptide, suggesting that removal of the leader peptide occurs after nisin maturation. As further evidence for this role, Nis P expressed in *E. coli* is able to specifically cleave the leader

peptide of purified prenisin. Interestingly, NisP has some homology to EpiP but also contains a putative membrane-anchoring sequence not found in EpiP (200), suggesting that the location of leader peptide cleavage differs in the formation of nisin and epidermin (228a). Such a membrane location for the peptidase lends further support to the notion that nisin biosynthesis occurs at, or very close to, the cytoplasmic membrane (46, 228a).

One of the definitive features of a bacteriocin (117, 167, 211) is the ability to resist the action of its own inhibitory substance through a specific immunity mechanism. Both production of and immunity to Pep5 in *Staphylococcus epidermidis* 5 have been shown to be associated with the presence of plasmid pED503 (111). Subsequently, the structural gene *pepA* has been localized to this plasmid (111) along with an adjacent, separately transcribed gene, ORFI (*pepI*), which has been shown to be essential for Pep5 immunity (175). By use of deletion mutants, it has been shown that neither *pepI* nor *pepA* alone is sufficient to confer immunity to the bacteriocin, but, together, these genes could restore immunity in sensitive mutants to levels similar to those of the wild-type strain. It was not clear, however, whether there was a need for *pepA* per se or some hitherto unidentified transcriptional factors. Analysis of the putative gene product of *pepI* suggests that it is a 69-amino-acid peptide, both terminal domains of which may form membrane-associated helical structures, and that the peptide is most probably loosely attached to the outer side of the cytoplasmic membrane (174). From these studies, it is suggested that the peptide may interact with the cytoplasmic membrane and provide immunity by a mechanism similar to that of some colicin immunity proteins (175, 203).

Recently, the gene encoding immunity to nisin, *nisI*, was identified (124). In contrast to the Pep5 immunity gene, *pepI* (175), *nisI* encodes a 32-kDa protein (245 amino acids unprocessed) which contains a consensus lipoprotein signal sequence (19 amino acids), suggesting that NisI is extracellular, membrane anchored, and modified by lipid components. In addition, expression of NisI in *L. lactis* cells led to a significant increase in their level of immunity to endogenously applied nisin, confirming that the gene product is involved in the immunity process. Interestingly, expression of *nisI* in a mutant *L. lactis* strain carrying a truncated *nisA* (the structural gene for nisin) produced significantly less immunity to endogenous nisin, suggesting that immunity development was dependent on expression of both *nisA* and *nisI*. This could be further confirmed by complementation of the truncated *nisA*, a process which restored immunity to levels comparable to those obtained in the wild type. Thus, as has been shown for Pep5 immunity (175), complete nisin immunity occurs in *L. lactis* only when mature nisin is produced and *nisI* is present (124).

Similarly, the cells of non-lanthionine-containing, bacteriocin-producing strains are immune to their own bacteriocin because of their ability to produce a specific immunity protein(s). In addition, strains producing one specific bacteriocin may or may not be sensitive to other similar bacteriocins. Thus, *Leuconostoc carnosum* Lm1, producing leuconocin Lcm1, is sensitive to pediocin AcH produced by *Pediococcus acidilactici* H but *P. acidilactici* H is resistant to leuconocin Lcm1 (238, 240). Resistance to the activity of a bacteriocin can also develop when a normally sensitive strain is grown in the presence of a bacteriocin. For example, *Leuconostoc carnosum* Lm1 has been shown to develop resistance to pediocin AcH following growth in a broth containing the pediocin (75, 153). Furthermore, some strains of bacteria can produce more than one type of bacteriocin, as well as the corresponding specific immunity proteins responsible for producer self-protection to each of the bacteriocins produced. An example of this phenomenon occurs in *L. lactis* subsp. *cremoris* 9B4, which produces three different bacteriocins, lactococcins A, B, and M, and three correspondingly different specific immunity proteins (226, 227).

DNA sequence analysis has enabled researchers to determine the putative amino acid sequences of additional proteins encoded by genes located in the same gene clusters and adjacent to the structural genes in several bacterial strains. Some of these proteins are assumed to be immunity proteins against the respective bacteriocins and include a 91-amino-acid protein found in a lactococcin B-producing strain, the 154-amino-acid lactococcin M immunity protein (226, 227), a 114-amino-acid leucocin A immunity protein (79), a 112-amino-acid protein protective against pediocin PA-1/AcH activity (31, 82, 143), and the 111-amino-acid carnobacteriocin B2 immunity protein (163). Recently, a 430-amino-acid protein suspected to be the sakacin A immunity protein has also been described (13); however, there are several differences between this protein and the other putative immunity proteins. The structural gene for presakacin A and the gene for the immunity protein are separated by 1.6 kb, and the gene product is comparatively large (430 amino acids) and has putative transmembrane regions in the N-terminal half. In addition, the sakacin A immunity protein is homologous to transmembrane protein kinases involved in various adaptive response systems in bacteria (13).

Some of the characteristics of the protein considered responsible for mediation of immunity to pediocin AcH in *Pediococcus acidilactici* H, PapB, have been determined (31). *Pediococcus acidilactici* LB 42, a strain sensitive to pediocin AcH, failed to produce transformants with a recombinant plasmid carrying a mutation in the *papB* gene. However, strain LB 42 produced transformants with plasmids carrying mutations in the other three genes (31). The protein has an estimated molecular mass of 12,993 Da and a pI of 7.4 and contains 37.5% nonpolar, 34.6% polar, 11.6% acidic, and 16.1% basic amino acids. In addition, it does not contain Arg or Cys. The hydropathy curve suggests that it is principally hydrophilic and contains no large hydrophobic regions to span the cytoplasmic membrane (Fig. 5).

The lactococcin A immunity protein (ImmA) from *L. lactis* subsp. *lactis* LMG 2130 has been purified and characterized (150). The deduced amino acid sequence revealed that it is made up of 98 amino acids and is also homologous to the lactococcin A immunity proteins affording immunity in *L. lactis* subsp. *cremoris* 9B4 and *L. lactis* subsp. *lactis* bv. diacetylactis WM4 (150, 208, 226). In addition, ImmA appears to be a major cell protein, because a single cell can contain as many as 10^5 molecules. However, it seems that immunity is not effected simply by binding of the ImmA protein to lactococcin A molecules to neutralize their bactericidal action, since sensitive cells exposed to excess ImmA are still killed on subsequent treatment with exogenous lactococcin A. Furthermore, ImmA molecules do not appear to protect specific components of the cell surface as a means of counteracting the bactericidal action of lactococcin A, because, at least in the producing cells, ImmA appears to be associated with the cytoplasmic membrane. This association probably prevents destabilization of the cytoplasmic membrane and thus protects the cell against the bactericidal action of lactococcin A (150). However, analysis of the hydropathy curves of this protein produced in *L. lactis* WM4 suggests that it is a hydrophilic protein with no hydrophobic region to span the membrane (208), and therefore the nature of its association with the cytoplasmic membrane is not clearly understood.

It is probable that for each bacteriocin there may be a relatively specific membrane protein, whose function is to trans-

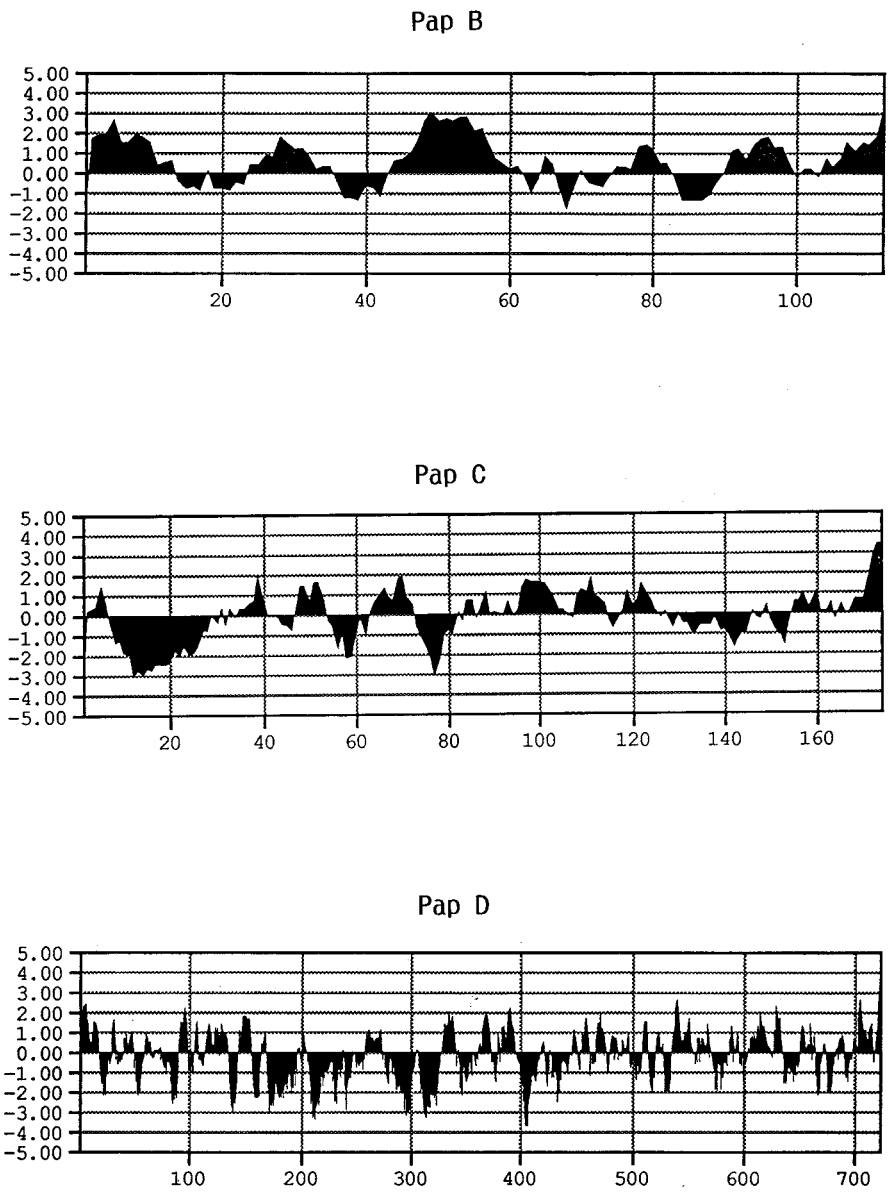


FIG. 5. Hydropathy plots of deduced amino acid sequences of proteins PapB, PapC, and PapD encoded by *papB*, *papC* and *papD*, respectively, in plasmid pSMB74. Hydropathy plots were prepared with a calculation interval of 7 amino acid residues, using MacVector 3.5 sequence analysis programs (Version 3.5.6; International Biotechnologies, Inc., New Haven, Conn.). The *x* axis shows amino acid residues; the *y* axis shows the hydropathy index. Hydrophobicity is below and hydrophilicity is above the baseline on the *x* axis. PapD protein (724 amino acids) has four (or more) large hydrophobic regions of between about 170 and about 450 amino acid residues. PapB (112 amino acids) does not have a large hydrophobic region, but PapC (174 amino acids) has one N-terminal and one C-terminal hydrophobic region large enough to span the membrane.

locate a precursor form of the bacteriocin across the cytoplasmic membrane to the outside of the cell. Although none of these putative translocating proteins has yet been purified, DNA sequence analysis has enabled predictions to be made about the amino acid sequences of the proteins probably associated with translocation of prelactococcin A in *L. lactis* subsp. *lactis* bv. diacetylactis WM4 (208) and prepediocin in *Pediococcus acidilactici* H and PAC1.0 (31, 82, 138, 143, 145). DNA sequence analysis of *L. lactis* WM4 has revealed a cluster of four genes with a common promoter associated with the production, translocation, and processing of prelactococcin A (228). The 715-amino-acid protein LcnC has pronounced amino acid sequence homology with several proteins implicated in the signal sequence-independent translocation of proteins across the cytoplasmic membranes of gram-negative bacteria. This protein, like some other secretory proteins in the HylB family of ATP-dependent membrane translocators, has a 200-amino-acid region that contains a conserved ATP-binding domain located at the C terminus. In addition, there are three hydrophobic regions located at the N terminus that might be capable of spanning the cytoplasmic membrane. Another gene in this operon, *lcnD*, has been found to encode a signal sequence-independent protein of 474 amino acids that is mostly hydrophilic but has a hydrophobic region of 43 amino acids at the N terminus. This protein resembles some of the inner membrane proteins of *E. coli*, such as HylD, that are associated

with transport systems in gram-negative bacteria. In the gram-negative cell, these proteins are also signal sequence independent and are mostly hydrophilic, although each has an N-terminal hydrophobic region with which it binds to the inner membrane. Thus, it is postulated that in *L. lactis* WM4, translocation of prelactococcin A across the cytoplasmic membrane is mediated by both the 715- and 474-amino-acid proteins (208).

Similarly, *Pediococcus acidilactici* PAC1.0 and H (producing pediocin PA-1 and pediocin AcH, respectively) have been shown to contain a cluster of four genes with a common promoter and terminator which are associated with pediocin production, translocation, and processing (31, 138, 143). A 724-amino-acid protein encoded by the gene *papD* (PedD) has a hydrophilic C terminus and six or more potential hydrophobic membrane-spanning regions toward the N terminus (Fig. 5). Amino acid homology comparison suggests that this protein has homology with certain ATP-binding proteins as well as ATP-dependent transport proteins, such as HylB in *E. coli* and ComA in *Streptococcus pneumoniae* (138). Recent studies have shown that PapD protein has both translocation and processing functions, at least in *E. coli* (31). In the same cluster, there is another protein of 174 amino acids, encoded by the gene *papC* (PapC), which has a hydrophilic C-terminal region and a hydrophobic N-terminal region that also might span the membrane (Fig. 5) (31, 138, 143). The PapC protein, like LcnD and HylD, is thought to be involved, along with the PapD protein, in facilitating or accelerating the transmembrane translocation of prepediocin in *Pediococcus acidilactici* (31).

Similar mutation studies of several genes involved in the production of lactacin F suggest that the gene *lafA* encodes a prepeptide which is later processed to biologically active lactacin F (56). However, an additional protein encoded by *lafX* appeared to enhance the antibacterial spectrum of lactacin F. In this respect, it may be related to lactococcin M and lactococcin G, since the antibacterial activities of the latter (M and G) appear to be dependent on the presence of two peptides (151). In addition, another protein, encoded by ORF Z, appears to have some similarities to other proteins associated with immunity against bacteriocins.

MODE OF ACTION

Antimicrobial Spectrum

The low-molecular-weight bacteriocins of the gram-positive bacteria demonstrate bactericidal activity which is directed principally against certain other gram-positive bacteria (211). For example, the prototype lantibiotic nisin has been shown to be effective against many strains of gram-positive bacteria, including staphylococci, streptococci, bacilli, clostridia, and mycobacteria (90). However, the degree of sensitivity of these genera varies, mycobacteria being approximately 100 times less sensitive than the others.

Similarly, among the non-lanthionine-containing bacteriocins, some, like pediocin AcH, have a wide range of action against gram-positive bacteria, while others, such as lactococcin A, have a much narrower range and are effective against only a few strains of *L. lactis* (18, 41, 88, 144, 226) (Table 3). Several other general observations may be made which apply to the antibacterial activities of the low-molecular-weight bacteriocins: (i) within a given species, some strains may be sensitive and others may be resistant to a particular bacteriocin (90, 169); (ii) a strain that appears to be sensitive to a bacteriocin may also have some cells in the population that are resistant to it (75, 153, 168); (iii) a strain can be sensitive to one bacteriocin while being resistant to a similar type of bacteriocin (75, 238, 240); (iv) cells of a strain producing one bacteriocin can be sensitive to another bacteriocin (75, 240); (v) although the spores of a strain whose cells are sensitive to a bacteriocin are resistant to that bacteriocin, they become sensitive following germination (90, 169, 170); and (vi) under normal conditions, gram-negative bacteria are not sensitive to bacteriocins produced by gram-positive bacteria (18, 90, 169).

Some of the lantibiotics such as nisin and some of the non-lanthionine-containing bacteriocins such as pediocin AcH have been shown to act on several species of gram-negative bacteria, provided that the integrity (or barrier functions) of the outer membrane is first disrupted (26, 109, 168, 169, 206). The details of the mechanism(s) by which gram-negative bacteria and certain gram-positive bacteria manifest resistance to bacteriocins are generally not well understood. However, this resistance does appear to differ from the specific immunity displayed by a producer strain to its own bacteriocin product. The possible mechanism of bacteriocin resistance of gram-negative and some gram-positive bacteria has been suggested to be associated with the barrier properties of the outer membrane and cell wall (26, 168, 206).

Primary Modes of Action

Despite the widespread use of nisin as a biopreservative, until recently relatively little was known about the mechanism by which it is able to kill susceptible bacterial cells. Initially, nisin was thought to act as a surfactant because of its cationic nature and because treatment of cells with nisin caused leakage of UV-absorbing material (164). Later, Gross and Morrell (73) suggested that the didehydroamino acids could be involved in the antibacterial activity of nisin because of their possible reaction with enzyme sulfhydryl groups. Nisin has also been implicated as an inhibitor of bacterial cell wall biosynthesis (132, 176); however, since very high concentrations of the peptide were used in these experiments, doubts have been raised about whether this is a primary mode of action (182).

The lantibiotic Pep5 (184) has, like nisin, been shown to have a concentration-dependent mode of action, which is also affected by physiological conditions such as ionic strength, temperature, and pH, as well as by the growth phase of the target cells (90, 185). Similarly, both of these lantibiotics inhibit the biosynthesis of DNA, RNA, protein, and polysaccharides, leading to speculation that treated cells no longer have sufficient energy to carry out biosynthetic processes and that the energy-transducing cytoplasmic membrane may be the primary biochemical target (181, 182, 187). Similar findings have subsequently been reported for the lantibiotics subtilin, epidermin, gallidermin, and SA-FF22 (16, 95, 182).

When *Staphylococcus simulans*, *B. subtilis*, and *Micrococcus luteus* are grown in the presence of chloramphenicol, the cells are able to import and accumulate radiolabelled amino acids but cannot incorporate them into intracellular protein. Treatment of such cells with low concentrations of either nisin (180), Pep5 (185, 186), subtilin (197), epidermin and gallidermin (182), or SA-FF22 (95) results in release of the labelled compounds from the cells. Similarly, accumulated Rb^+ (a K^+ analog) was shown to be released following nisin or Pep5 treatment (180, 185, 186), indicating that energy transduction mechanisms had been interfered with. Furthermore, following Pep5 treatment, ATP (for which no known transport system exists) could be detected extracellularly, prompting speculation that the lantibiotic formed pores in the bacterial cytoplasmic membrane (186). The observed efflux of ATP was significantly reduced in starved cells when compared with cells that had

been energized with glucose prior to the addition of the peptide, indicating that the energization state of the membrane was an important determinant of lantibiotic action. Two further observations support the view that lantibiotics form defined pores in membranes, rather than producing the generalized membrane disruption that would be expected of detergents: (i) Pep5 stimulates the phosphoenolpyruvate-dependent phosphotransferase system of cells in a manner similar to that of known protonophores (122), and (ii) larger molecules such as sugars and proteins have not been found outside the cell following Pep5 treatment (182).

Confirmation of the membrane-deenergizing action of the lantibiotics has been achieved by direct measurement of the membrane potential ($\Delta\Psi$) of lantibiotic-treated cells (1, 29, 95, 121, 187, 197). The level to which the membrane is energized prior to lantibiotic treatment appears to be critical, indicating that a minimum membrane potential is required for lantibiotic pore formation to occur. Studies with artificially energized cytoplasmic membrane vesicles of *B. subtilis* clearly show that nisin, Pep5, subtilin, and SA-FF22 induce the efflux of preaccumulated radiolabelled amino acids (95, 123, 181, 185, 197). However, this activity differs significantly from that produced by protonophores such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (182). Cells pretreated with CCCP do not energize and do not accumulate the label; cells pretreated with lantibiotics accumulate label until a certain level of energization is achieved, after which efflux occurs (95, 123, 181, 185, 197). Further investigations with uncoupled cells or artificial phospholipid vesicles, artificially energized to various levels with valinomycin, have established that a minimum membrane potential is required for lantibiotic action and suggest that the peptides act on membranes in general, with no requirement for specific membrane receptors (123). Similar studies of the action of nisin on liposomes have also confirmed that nisin dissipates $\Delta\Psi$ by increasing membrane permeability (61). In addition, a recent study of the action of nisin on liposomes composed of *E. coli* phospholipids and loaded with a fluorophore has indicated that while $\Delta\Psi$ potentiates nisin activity, pore formation may, under certain circumstances, also occur in the absence of a membrane potential (62).

To understand details of the mechanism of pore formation by the lantibiotics, significant use has been made of artificial membrane systems, especially the so-called black lipid membranes (BLM). The elegant BLM system uses two buffer-filled compartments in a Teflon block, separated by a phospholipid bilayer which has been formed across a small circular hole. Defined membrane potentials are applied via electrodes, and since intact BLM are efficient insulators, current flow occurs only when the integrity is disrupted by the formation of the pores (15). Current-voltage curves for nisin (121, 188) demonstrated that pore formation occurs only when the voltage is applied in a *trans*-negative orientation (the same as occurs in bacterial cells, with hydrogen ions accumulating outside). By contrast, subtilin (197), epidermin or gallidermin (16), and SA-FF22 (94) appeared to form pores irrespective of the orientation of the applied potential. The threshold potential for pore formation (i.e., the minimum potential required for a significant current to begin flowing through the membrane, indicating that *trans*-membrane channels had opened) can also be measured. For nisin and Pep5, this threshold potential is ca. −80 mV, for subtilin it is ca. +80 mV, for epidermin or gallidermin it is ca. +50 mV, and, for SA-FF22, it is ca. +100 mV. The current-voltage curves for each of these lantibiotics show hysteresis, indicating that once formed, the pores are relatively stable and remain intact, even upon significant reduction of the applied potential (16).

Analysis of a single channel formed in BLMs by nisin, subtilin, Pep5, and SA-FF22 suggests that the peptides form irregular, unstable pores, since current flow was observed to fluctuate in millisecond timescale burst or spikes (94, 123, 180, 181, 197). The information gained from these same single-channel measurements could be used to estimate the size of the pores, assuming that they are cylindrical and uniformly filled with the same fluid that bathes the membrane (15); nisin and Pep5 form channels ca. 1 nm in diameter, subtilin forms channels of ca. 2 nm in diameter, and SA-FF22 channels are somewhat smaller, ca. 0.5 to 0.6 nm in diameter. However, similar analysis of epidermin and gallidermin pores indicated some significant differences. The mean pore lifetimes (i.e., pore stabilities) of these lantibiotics were considerably higher, in some cases up to 30 s, and the pore diameter appeared to increase with the applied potential, suggesting that the number of gallidermin or epidermin molecules involved in pore formation could directly increase together with the applied potential (16).

Several features of the mode of action of the non-lanthionine-containing bacteriocins of gram-positive bacteria require further explanation: (i) the reason why, for two sensitive strains, one undergoes lysis following treatment with a particular bacteriocin while the other does not is not known (19); (ii) for a bacteriocin to come into contact with the cytoplasmic membrane of sensitive cells, the molecules must first pass through the cell wall; the mechanism of this translocation remains to be understood; and, finally, (iii) there is evidence that non-lanthionine-containing bacteriocin molecules may be adsorbed on the surface of most gram-positive bacterial cells, including sensitive, resistant, and producer strains; the influence of this is not yet fully understood (19, 238).

In general, the non-lanthionine-containing bacteriocins also appear to effect their bactericidal action by destabilizing the cytoplasmic membrane of sensitive cells; however, the mechanism through which they achieve this appears to differ somewhat from that described for the lantibiotics. Treatment of whole cells with low concentrations of pediocin PA-1 or AcH and lactacin F (19, 29, 141) or pediocin JD (35) increases the permeability of the cytoplasmic membrane, as determined by the increased influx of small molecules and efflux of UV-absorbing materials (e.g. amino acids, K^+, *o*-nitrophenol) from the cytoplasm. In addition, these bacteriocins dissipated the proton motive force (PMF) of the target cells, as shown by their influence on the uptake of amino acids whose influx is mediated by secondary and phosphate-bond-driven transport systems. However, unlike the lantibiotics, these bacteriocins appear to act on sensitive cells regardless of their degree of prior energization, suggesting that the loss of permeability of the cytoplasmic membrane occurs in a voltage-independent manner.

In addition, dithiothreitol-reduced pediocin PA-1, in which the Cys is present in the reduced, thiol form, has been shown to be inactive, suggesting that intact disulfide linkages are essential for the activity of this bacteriocin (34). Treatment of pediocin AcH with β-mercaptoethanol, especially at higher temperature, also reduced its bactericidal efficiency (167). The same study also found that the transmembrane pores produced by pediocin PA-1 increased in size with increasing pediocin concentration. Furthermore, fluorescence spectroscopy of the tryptophan residues of pediocin PA-1 has demonstrated that the peptide can associate intimately with phospholipid bilayers prior to insertion and pore formation and that pore formation probably occurs in conjunction with a protein "receptor" in the cytoplasmic membrane of the susceptible cells (34). Similarly, the lantibiotics SA-FF22 and subtilin have been shown to as-

sociate closely with phospholipid bilayers prior to pore formation and in the absence of a suitable membrane potential (95, 197).

The mechanism of action of lactococcin A against both whole cells and membrane vesicles prepared from bacteriocin-sensitive cells has been extensively studied (88, 228). In immune cells, dissipation of PMF and increase in membrane permeability occurred only at very high concentrations of lactococcin A. Treatment of cytoplasmic membrane vesicles of sensitive cells with low concentrations of lactococcin A inhibits both influx and efflux of leucine, again suggesting that dissipation of PMF and loss of the permeability barrier of the membrane had resulted from lactococcin A treatment. This increase in membrane permeability was also found to occur in a voltage-independent manner. In addition, treatment of the membrane vesicles from immune cells with low concentrations of lactococcin A did not inhibit either PMF-driven uptake or efflux of leucine, indicating that the immunity factor in these cells acts at the level of the cytoplasmic membrane. It was also observed that membrane vesicles from lactococcin A-resistant cells (including *Clostridium acetobutylicum*, *B. subtilis*, and *E. coli*) and liposomes derived from phospholipids purified from sensitive cells of *L. lactis* (and thus not containing membrane-associated proteins) were not affected by lactococcin A. The high degree of specificity of lactococcin A action could be taken to indicate that its action is exerted through interaction with membrane-associated binding sites in the sensitive cells. Electron-microscopic investigation of sensitive cells following treatment with lactococcin A revealed no loss of membrane integrity, supporting the theory that pore formation without loss of membrane integrity (as might be expected from a detergent-like action) by lactococcin A is the cause of membrane permeabilization and cell death.

Similarly, mesentericin Y 105 was shown to inhibit transport of both leucine and glutamate by dissipating $\Delta\Psi$ and to induce efflux of preaccumulated amino acids in sensitive *Listeria monocytogenes* cells (136). However, mesenterocin Y 105 also inhibited ADP-stimulated respiration and ATP synthesis in rat liver mitochondria and adenine nucleotide translocase in beef heart mitochondria through pore formation in the energy-transducing membranes. These latter results would suggest that if "receptor" proteins in the membrane are associated with the action of the nonlantibiotic bacteriocins, mitochondria may also have receptors for mesentericin Y 105, perhaps suggesting that these "receptors" are not specific for bacteriocins.

Recently, Nissen-Meyer et al. (151) reported that the bactericidal action of lactococcin G requires the complementary action of two peptides, α1 and β. The α1 peptide, with 39 amino acid residues, has been designated lactococcin G (Table 1); the β peptide has 35 amino acid residues. The N-terminal halves of the two peptides are hydrophilic and form amphiphilic α-helices, a structural characteristic that is thought to allow the peptides to form membrane channels (pores) via a barrel-stave mechanism. A similar mechanism which requires the complementary action of two peptides, α and β, for bactericidal action has been suggested for plantaricin A (151a). In this case, both of the peptides are the products of the same gene, with the β peptide lacking only the first amino acid, an Ala residue, of the α peptide. It has been suggested that both α and β may form amphiphilic α-helices and that composite membrane channels are created by means of a barrel-stave mechanism.

The bactericidal activity of lactacin F has likewise been shown to be dependent on the cooperative activity of two peptides, LafA and LafX (56, 117). Analysis of the mode of action of this bacteriocin has shown that the two peptides form an active lactacin F complex, capable of inducing efflux of intracellular potassium and organic phosphates, dissipation of the PMF, and hydrolysis of internal ATP in susceptible bacterial strains but not in the bacteriocin-immune producing strain (2). In addition, lactacin F action was enhanced at acidic pH values and inhibited by di- and trivalent cations and reduced temperature. Furthermore, since the bacteriocin also induced the same effects in protonophore-treated cells, it was concluded that lactacin F action is energy independent.

The bactericidal effect of low concentrations of lactococcin B on sensitive cells has also been found to be produced by dissipation of the PMF and loss of the permeability barrier function of the membrane (231). Furthermore, the membrane functions of strains carrying specific immunity protein genes were not affected by lactococcin B even at high concentrations, while the insensitivity of sensitive cell membrane-derived liposome vesicles once again indicates the probable presence of protein "receptors" for lactococcin B in the membrane. However, a further requirement for the bactericidal effect of lactococcin B is that Cys-24 be in the reduced state. It is suggested that the reduced state of the single sulfhydryl group either may be necessary for the interaction of lactococcin B with a receptor molecule or could alter the structure of lactococcin B in such a way as to enable it to participate in pore formation. Furthermore, the immunity protein, instead of either interacting with lactococcin B or making the receptor protein unavailable to lactococcin B, probably inhibits pore complex formation in the membrane.

In general, it appears that the bactericidal action of the non-lanthionine-containing bacteriocins against sensitive cells is produced principally by destabilization of membrane functions such as energy transduction rather than by disruption of the structural integrity of the membrane. This effect results from the energy-independent dissipation of the PMF and loss of the permeability barrier of the cytoplasmic membrane and contrasts with the energy-dependent bactericidal action of the lantibiotics (16, 182).

Both the lantibiotic and non-lanthionine-containing bacteriocins seem to affect the membrane permeability barrier by forming water-filled membrane channels or pores, probably by a barrel-stave mechanism (16, 117). In addition, prior to the formation of pores, all of the non-lanthionine-containing bacteriocins appear to interact with membrane-associated receptor proteins, again in direct contrast to the lantibiotic-type bacteriocins, which appear to have no such requirement (16, 182, 197). Furthermore, producer strains harboring an immunity gene appear to produce specific immunity proteins that prevent pore formation by the bacteriocin by an as yet unidentified mechanism(s). However, this might be accomplished either by shielding of the receptor protein, by competitive interaction with the bacteriocin molecules, or by closing or blocking the pores (150, 231).

Secondary Modes of Action

In addition to their membrane pore-forming capabilities (16, 182), both Pep5 and nisin have been shown to induce autolysis in *Staphylococcus simulans* 22 (23), an effect that results from their strongly cationic nature. Indeed, even synthetic peptides, such as polylysine, can bind to teichoic, lipoteichoic, and teichuronic acids in the cell wall of this bacterium, releasing and thereby activating autolytic enzymes that are normally bound to these substrates (21–23, 187). Since the amount of autolytic activity depends on the degree of cationicity of the peptides interacting with the cells, it appears that enzyme release results from an ion-exchange-like process (20, 23). In addition, elec-

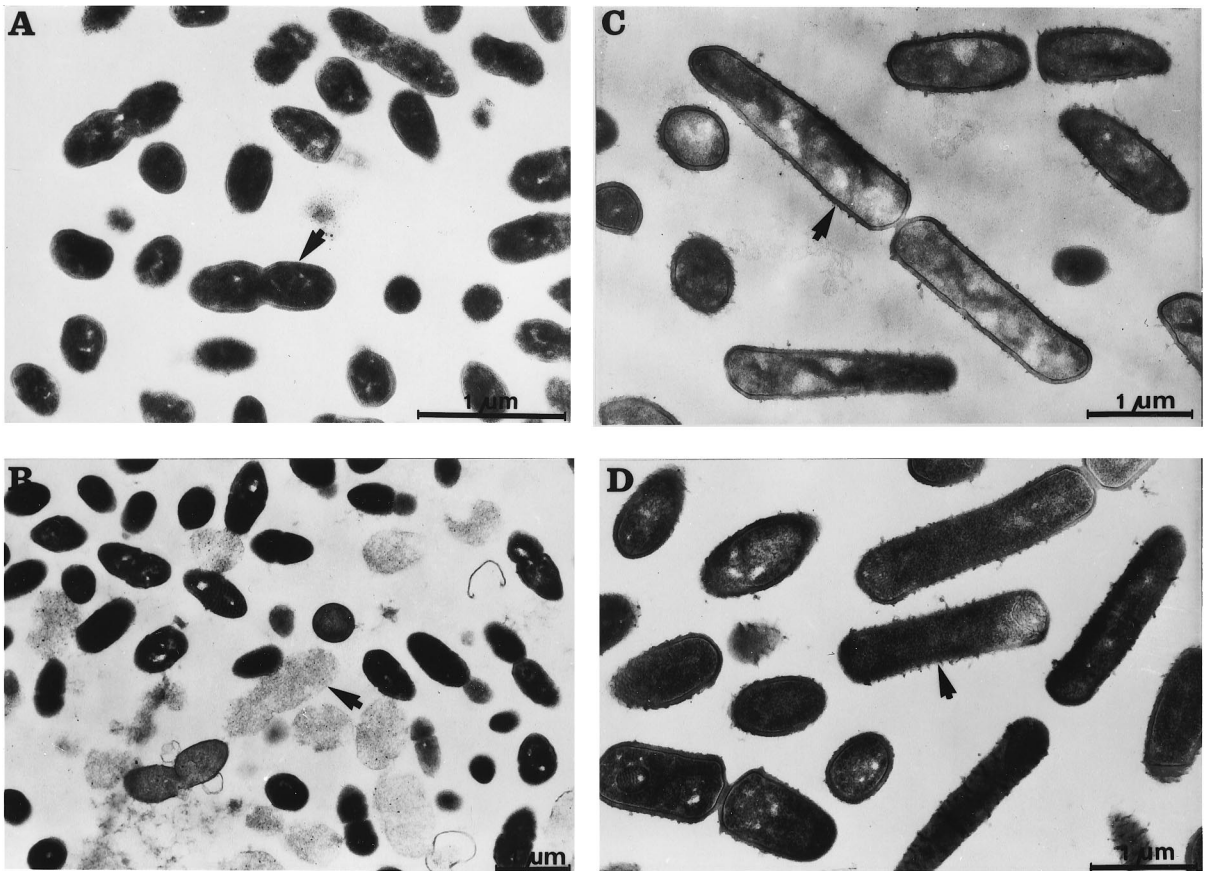


FIG. 6. Transmission electron micrographs of cells of *Leuconostoc mesenteroides* Ly (A and B) and *Lactobacillus plantarum* NCDO 955 (C and D). Pediocin-treated samples show lysed ghost cells (B) and darker cytoplasm (D). Taken from reference 19 with permission of the publisher.

tron-microscopic studies have shown that activation of the autolytic enzymes appears to be predominant in the area of the cell wall septum between two daughter cells (23, 24). Depolarization of the cytoplasmic membrane alone (with protonophores) is insufficient to cause lysis and does not prevent induction of autolysis by the cationic peptides, whereas preenergized cells, in which pore formation and subsequent depolarization is accelerated, show increased cell lysis (21, 22).

Taken together, it is suggested that following membrane depolarization by pore formation, any damage caused by the induction of autolytic enzyme activity in the region of the septum cannot be repaired. In addition, since the pores formed in the membrane are not sufficiently large to allow efflux of high-molecular-weight components, there should be enhanced osmoinduced influx of water through the pores. The resulting increase in osmotic pressure will encourage subsequent cell lysis (23).

Conflicting results have been reported concerning the ability of non-lanthionine-containing bacteriocins to induce lysis of sensitive cells. Pucci et al. (160) reported that treatment of growing sensitive cells of *Listeria monocytogenes* LM 01 and *Pediococcus pentosaceus* FBB63 with pediocin PA-1 resulted in cell lysis as observed by the reduction of optical density (OD). The two strains differed in lysis rate and in the minimum concentrations of pediocin PA-1 that were capable of inducing lysis, with the more sensitive cells lysing at a higher rate. In contrast, Bhunia et al. (19) demonstrated that treatment of growing sensitive cells of *Lactobacillus plantarum* NCDO 955 with pediocin AcH prevented any further increase in OD and resulted in a loss of cell viability but did not decrease the OD of the culture. However, under similar conditions, the OD of pediocin AcH-treated *Leuconostoc mesenteroides* Ly cells decreased significantly, suggesting that cell lysis had occurred. Transmission electron microscopy of the two strains after treatment further confirmed that lysis occurred only in *Leuconostoc mesenteroides* Ly (Fig. 6). In a separate study, three of seven sensitive strains of *Listeria monocytogenes* treated with pediocin AcH showed lysis (reduction in OD_{600}); in the other four strains, there was viability loss but the OD_{600} remained unchanged (144).

Recently, van Belkum et al. (225) reported that following treatment with lactococcin A, sensitive *L. lactis* cells and membrane vesicles derived from these cells showed neither lysis nor other morphological alterations when examined by electron microscopy. They suggested that the loss of barrier functions of the membrane of sensitive cells occurs not as a result of lysis but as a result of pore formation. Bhunia et al. (19) have also suggested that the primary killing action of non-lanthionine-containing bacteriocins, such as pediocin AcH, is destabilization of cytoplasmic membranes but that cell death may activate autolytic systems and bring about lysis in some strains.

Passage across the Cell Wall

The loss of viability of sensitive cells of gram-positive bacteria following treatment with a number of the non-lanthionine-containing bacteriocins occurs very rapidly, perhaps

within 1 min (19). Since cell death appears to occur through destabilization of the cytoplasmic membrane, the bacteriocin molecules must cross the cell wall before establishing contact with the membrane. However, the mechanism(s) of bacteriocin passage through the cell wall has not yet been critically studied. Simple diffusion models fail to entirely account for many of the observations regarding the action of these bacteriocins, including: (i) why they are more bactericidal at a low pH, (ii) why some gram-positive non-bacteriocin-producing strains are resistant to a bacteriocin, (iii) why a gram-positive strain can be sensitive to one bacteriocin but resistant to another, (iv) why some cells in the population of a sensitive strain are resistant to a bacteriocin, (v) why a sensitive strain can acquire resistance by growing in the presence of a bacteriocin but revert when grown in the absence of bacteriocins (153), (vi) why gram-negative cells are normally resistant to these bacteriocins, (vii) why sublethally injured bacterial cells become sensitive to a bacteriocin to which the uninjured cells are resistant (168, 169), and (viii) how *E. coli* cells transformed with recombinant plasmids carrying the gene cluster of a gram-positive bacterial bacteriocin then secrete the bacteriocin through the outer membrane (31).

Many bacteriocins have been reported to have greater bactericidal activity at low pH. The peptides carry both positive and negative charges with a net positive charge below pH 7 (Table 2). At pH values in the range of 3 to 7, Lys and Arg should not influence any change in the net charge of the peptides; however, as the pH drops below 6, the net positive charge should increase because of His, and as the pH drops below 4, the net charge due to Asp and Glu residues should also decrease. It has been suggested that the pH-induced alterations in net charge might facilitate translocation of some bacteriocin molecules through the cell wall (169, 170a). In addition, both non-lanthionine-containing bacteriocins and lantibiotic molecules are adsorbed to the cell surfaces of gram-positive bacteria, irrespective of their being bacteriocin producer, nonproducer, resistant, or sensitive. A recent study of several of these bacteriocins has shown that their degree of adsorption is pH dependent, with a maximum at about pH 6.0 and a minimum at or below pH 2.0 (238). These observations further support the suggestion that initial adsorption occurs through ionic attraction between the bacteriocin molecules and the cell surface. The molecular components on the cell surfaces of gram-positive bacteria to which bacteriocin molecules are adsorbed are thought to include teichoic and lipoteichoic acids (19, 23, 95).

Several reports have indicated that although nisin is not effective against gram-negative bacteria, membrane vesicles derived from gram-negative cells are sensitive to it (61, 185). This indicates that the resistance of gram-negative bacteria to bacteriocins could be due to the relative impermeability of their outer membranes. Both gram-negative and resistant gram-positive bacteria can be made sensitive to pediocin AcH (and nisin) following exposure to sublethal stresses (26, 109, 168, 169, 206). Many sublethal stresses are known to increase the permeability of the cell walls of gram-positive bacteria and the outer membranes of gram-negative bacteria, thus interfering with their barrier properties (26, 109, 168, 169, 206). Therefore, it has been suggested that adsorption of bacteriocin molecules can induce a change in cell wall barrier functions only in sensitive gram-positive bacteria, rendering the wall more permeable to the bacteriocin. No such change occurs following adsorption to bacteriocin-resistant cells (109, 168, 169).

Importance of Amino Acid Sequence(s)

Although the lantibiotic subtilin has a broad spectrum of inhibitory activity and marked structural similarities to nisin (90), it has proved to have little commercial application, in part because of its lack of stability (100). Recently, Liu and Hansen (133) demonstrated that two distinct types of activity could be differentiated for this bacteriocin: one against vegetative cells by the pore formation mechanism described above, and the other directed against the outgrowth of spores. Furthermore, the difference in these activities has been suggested to be due to the presence of the unsaturated amino acid didehydroalanine in position 5. Subtilin antispore activity has a half-life of only around 0.8 day, and the loss of this component, as well as its biological activity, is concomitant with the saturation of dhA5, as demonstrated by proton NMR studies (133). Through the application of site-directed mutagenesis, it has been demonstrated that the mutant subtilin, dhA5Ala-subtilin, while active against vegetative cells, was devoid of detectable antispore activity. Furthermore, by altering the residues preceding the didehydroalanine at position 5 from glutamate to isoleucine (E4I-subtilin), it was possible to demonstrate a 57-fold increase in the half-life of the antispore activity, suggesting that the glutamate at position 4 participated in the nucleophilic saturation of didehydroalanine at position 5. This substitution of the isoleucine for glutamate is the same as that found in the structurally similar lantibiotic, nisin; didehydroalanine at position 5 in nisin is not readily saturated (133). Therefore, it has been concluded that although didehydroalanine at position 5 may not be necessary for pore formation, it could participate directly in prevention of spore outgrowth, perhaps through the formation of a covalent linkage(s).

While the mode of action of many of the lantibiotics has been shown to be the formation of pores in the cytoplasmic membrane, little is known of their actual mechanism of insertion. Jack and Tagg (96) isolated a truncated form of the bacteriocin SA-FF22, SA-FF22/5–26, which had no detectable biological activity associated with it; the mode of action of the intact peptide was shown to be the formation of voltage-dependent ion-permeable channels in the cell membrane (95). Although the peptide was devoid of the N-terminal 4 amino acids (Gly-Lys-Asn-Gly), in all other respects tested (post-translational modifications, amino acid composition, and sequence) it appeared identical to the parent peptide. Since this region of the N terminus is not involved in bridging, loss of these amino acids is unlikely to significantly change the conformation of the peptide. This finding may suggest that either the peptide becomes too short to span the membrane or the loss of one positive charge results in the loss of pore-forming ability. Alternatively, it may be that the peptide can insert the N terminus only through the membrane and that any changes in this region are thus detrimental to the biological activity. Similarly, a nisin derivative in which ring A was opened by hydrolysis of the peptide bond between residues 4 and 5 also showed little or no activity (33).

Currently, nisin is the only lantibiotic that has been chemically synthesized de novo, a formidable task because of the complex chemistry of its novel ring structures and α,β-unsaturated amino acids (234). The resulting product was indistinguishable from native nisin in its amino acid sequence, content of modified amino acids, and three-dimensional structure, and it also showed identical biological activity to that of native nisin. During this synthesis of nisin, it was observed that nisin1–19 (i.e. the N-terminal half of native nisin) also demonstrated nisin-like antimicrobial activity, albeit at slightly higher peptide concentrations. Thus far, the mechanism of action of

this fragment has not been further characterized; however, these results further confirm that C-terminal modifications in nisin appear to be less detrimental to biological activity.

Importance of Three-Dimensional Structure(s)

Hydropathy plots of lactococcin A indicate that the carboxy terminus has a hydrophobic region of 21 amino acid residues between Ala-30 and Phe-50 which is thought to form an amphiphilic membrane-spanning helix (87, 208). The bacteriocin appears to interact with "receptor" proteins in the cytoplasmic membrane of the sensitive cells, form a helical transmembrane structure, and, probably in conjunction with additional lactococcin A molecules which might form a water-filled barrel-stave-like structure, increase the membrane permeability. In contrast, hydropathy plots indicate that lactococcin B does not contain any hydrophobic regions that could span the cytoplasmic membrane. Thus, the mechanisms of pore formation by lactococcins A and B appear to differ.

The known three-dimensional structures of the lantibiotics share several common characteristics. Each is an amphiphilic helical peptide with higher degrees of flexibility in aqueous solution than in lipophilic solution (57–59, 67, 130, 154, 202, 228, 229). Additionally, each has been shown to have potential membrane-spanning sequences and a high dipole moment, both of which are necessary to support models for voltage-induced pore formation in lipid membranes (16, 58, 165). Interestingly, all form alternate faces with hydrophobic residues on one side of the helix and hydrophilic residues on the other, while they also show some flexibility in the central region of the peptide (57–59, 67, 130, 228c, 229). Recently, Vogel et al. (232) suggested that such bends or flexibility in transmembrane helical structures may be necessary for the stabilization of channels or pores.

Current models of pore formation by lantibiotics (16, 24, 182) are based on structural data (57–59, 228c, 232) and suggest that cationic lantibiotics are attracted to the membrane by ionic interaction. If a sufficiently large $\Delta\Psi$ is present, the peptides adopt a transmembrane orientation, aggregating with their hydrophobic faces toward the bilayer and their charged, hydrophilic faces toward the channel center. When sufficient peptides come together, electrostatic repulsion between the positive charges may be responsible for pushing open the transmembrane channel. It is not yet clear when the peptides might aggregate (i.e., before or after they adopt a transmembrane orientation), which terminus remains on the outside of the membrane or whether insertion is bidirectional, and how many peptides represent the minimum requirement for pore formation.

CONCLUSIONS

It is now evident that the bacteriocin-like products of gram-positive bacteria, especially those with a relatively broad antibacterial spectrum, will continue to be an active area of applied research. The potential for either the discovery or genetic engineering of novel peptides with commercially desirable antibacterial activities offers an irresistible lure (134). With a view to the anticipated increased research activity in this field, it seems sensible to attempt to establish a more rational scientific basis for the definition, classification, and nomenclature of these substances. An early effort to adopt uniform guidelines may help minimize the introduction of further discrepancies and confusions.

General Definition and Specific Nomenclature for the Bacteriocin-like Peptides Produced by Gram-Positive Bacteria

Should the term "bacteriocin," originally defined in terms of the colicin-like molecules produced by gram-negative bacteria, continue to be used with reference to the small, relatively broadly active antibacterial peptides produced by gram-positive bacteria? It is our suggestion that the definition of the term "bacteriocin" be broadened somewhat to encompass extracellularly released primary or modified products of bacterial ribosomal synthesis, which can have a relatively narrow spectrum of bactericidal activity, characterized by inclusion of at least some strains of the same species as the producer bacterium and against which the producer strain has some mechanism(s) of specific self protection.

Classification Schemes

Since the term "lantibiotic" was introduced to specifically categorize bacteriocins of gram-positive bacteria that contain lanthionine and/or β-methyl lanthionine residues, it is becoming customary to treat these molecules as a quite separate group from other essentially similar molecules that do not have these particular residues. Indeed, a recent suggestion for classification of the bacteriocins of lactic acid bacteria incorporated a primary grouping of the low-molecular-weight inhibitory peptides as either lantibiotics or nonlantibiotics (117). However, such a grouping system introduces some potential conflicts. First, the presence of lanthionine may or may not be related to the antibacterial property of these molecules. Second, the prefix "non" could imply possible inferiority of "nonlantibiotics" by comparison with lantibiotics. This clearly would be inappropriate, since, for example, if the breadth of inhibitory activity is taken as one measure of possible commercial utility, some "nonlantibiotics" like pediocin AcH (pediocin PA-1) may have a greater potential than some of the lantibiotics (18, 158, 196). Finally, there could be some other molecules (such as toxins and antibiotics) that contain lanthionine but are quite different from the currently known bacteriocins of gram-positive bacteria. On the other hand, if the presence of particular chemical groups is considered to be an appropriate basis for a classification scheme, some other groups might also be proposed, such as "thiolbiotics" (having an active -SH group) and "cystibiotics" (with a cystine residue). Each of these groups could in turn be subgrouped into types A, B, etc., on the basis of other characteristics, as has been recommended for the "lantibiotics" (106, 108).

It has been suggested that a subgroup of "*Listeria*-active peptides" could be established within the "nonlantibiotic" lactic acid bacterial bacteriocins. This subgroup comprises peptides that have a particular sequence of 7 amino acids and apparent widespread inhibitory activity against *Listeria* strains (117). However, not all of these bacteriocins have been tested against large collections of *Listeria* species and strains. There are some indications that several *Listeria* species and strains could be resistant to such bacteriocins while other strains are sensitive (78a, 98a, 163a, 169). Also, *Listeria* strains sensitive to some of these bacteriocins have resistant cells in the population (75, 144). More importantly, it has not yet been established that this particular amino acid sequence is the peptide domain to which the *Listeria* strains are sensitive. Many *Listeria* spp. are also sensitive to lantibiotics, such as nisin, which logically should then also be included in this group. Introduction of a target bacterium-based classification system ("x-species active," "y-species inactive," and so on) opens up the potential for creation of subgroupings comprising collections of possibly

distantly related molecules that have as their principal unifying feature inhibitory activity against a particular range of target species. Another proposed subgrouping of the bacteriocins of lactic acid bacteria is that of "complex bacteriocins" (117). The loss of antibacterial activity of some bacteriocins following treatment with either carbohydrate- or lipid-hydrolyzing enzymes has been considered in some studies to indicate the presence of a carbohydrate or lipid moiety which is essential for antibacterial activity. However, in most cases, only partially purified bacteriocin preparations have been used and the possibility of contamination of the enzyme preparations with amylases or lipases has not been eliminated. Chemical analysis of the purified antibacterial components will be necessary to confirm that the presence of a carbohydrate or lipid component is essential to the biological activity of these molecules, thus justifying the establishment of a "complex bacteriocin" subgroup.

Peptide Nomenclature

The current basis for allocating a name to the agent responsible for bacteriocin-like activity produced by a gram-positive bacterium has been to adopt some derivation of either the genus or the species name of the producer strain together with an alphabetical and/or numerical code designation specifying that strain. All too often, the appellation "bacteriocin" has been prematurely used to account for uncharacterized interspecific bacterium-inhibitory activities on agar media that are eliminated by treatment with proteolytic enzymes. Examples of inconsistencies previously arising in the naming of bacteriocin-like agents of gram-positive bacteria include epidermin and staphylococcin 1580 (both are the same) and Pep5 from *Staphylococcus epidermidis* strains; mesentericin Y 105, leucocin A UAL-187, and leuconocin Lcm 1 from *Leuconostoc mesenteroides*, *Leuconostoc gelidum*, and *Leuconostoc carnosum* strains, respectively; carnobacteriocin, carnocin, piscicolin, and piscicocin from different strains of *Carnobacterium piscicola*; pediocin A and pediocin PA-1 (or pediocin AcH), respectively, from *Pediococcus pentosaceus* and *Pediococcus acidilactici* strains (pediocin PA-1 and pediocin AcH are chemically identical peptides); lactococcin A and diplococcin from different *L. lactis* subspecies (they are the same peptide); and curvacin A and sakacin A from *Lactobacillus curvatus* and *Lactobacillus sake* strains, respectively (they are the same peptide) (Table 1).

It is our recommendation that in the future, the specific naming of any newly discovered bacteriocin should be delayed until details of its amino acid sequence and the nucleotide sequence of the corresponding structural gene are known. Until then, the term BLIS followed by the producer strain designation may be used (e.g., BLIS HL1 from *Pediococcus acidilactici* HL1). When sequence information establishes that the bacteriocin differs significantly from those previously reported in the literature, naming should be based on the genus, or preferably the species, designation of the producer strain.

Bacteriocins that have only minor conservative differences in their amino acid sequences, resulting in no significant change in their secondary (e.g., bridging) structures, activity spectra, and cross-specificity of producer strain self protection, should be referred to as natural variants. For example, nisin A and nisin Z could be considered natural variants, as could gallidermin and epidermin. Also, irrespective of the species of origin, bacteriocins having the same amino acid sequences should have one name (the first one published).

Structural-Gene Nomenclature

The available information suggests that the bacteriocins of gram-positive bacteria are translated as prepeptides from the nucleotide codes in the structural genes. Although structural genes for only a limited number of these prepeptides have as yet been identified, some discrepancies in their naming are already apparent. For example, *sakA* and *curA*, which encode sakacin A and curvacin A, respectively, have the same nucleotide sequences; *pedA* and *papA*, which encode pediocin PA-1 and pediocin AcH production, respectively, also have the same nucleotide sequences; *lcnA* is used for both lactococcin A and leuconocin A UAL-187; *spaS* is used for subtilin; and *pepA* is used for Pep5. Also, the structural gene for epidermin is *epiA*, but the immunity gene for lactococcin A is *lciA* (the structural gene is *lcnA*).

Nomenclature of Associated Genes and Their Protein Products

It is accepted now that the structural genes encoding the bacteriocins of gram-positive bacteria are present in operon-like gene clusters that also harbor genes encoding proteins for immunity, processing, maturation, regulation, and translocation functions. There have been differences in the manner in which the genes (ORFs) in these clusters have been designated (Fig. 4).

For naming both structural and associated genes, methods suggested for lantibiotics can be used for all bacteriocins (39). Following this method, three initial letters of a bacteriocin can be used to identify its genes (e.g., *nis* for nisin). The structural gene is designated A, and if more than one peptide component is required for the function, these can be designated as A1, A2, and so on. For other genes, the following designations can be used: immunity, I; transport, T, E, F, and G; protease, P; modification, B, C, D, and M; regulators, R, K, and Q. Prior to identification of the functions, they can simply be designated as ORF 1, 2, 3, etc., in sequence.

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