

Bacteriocins: Applications in Prevention and Treatment of Mastitis

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Introduction

Bacteriocins are antibacterial peptides or proteins that generally affect members of related genera or species. They constitute a family of compounds that arose during the evolution of Microbial Defense Systems, which comprise many families of molecules used by bacteria to compete successfully for survival. While bacteriocins were discovered around the same time as classical small molecule antibiotics (Rogers, 1928), they were not developed as actively as medicines due to the comparatively narrow spectrum of activity of bacteriocins. While the use of bacteriocins in prevention and treatment of mastitis is by no means a new idea, recent focus on managing subclinical mastitis and minimizing antibiotic use in food animals has led to a renewed interest in evaluating bacteriocins as a tool in managing mastitis. This paper will present an overview of bacteriocin structure and function, mastitis applications that exploit their attributes, potential drawbacks in their use, and current status of bacteriocin-based mastitis products.

Bacteriocins

While the main differences between bacteriocins and traditional antibiotics are the protein or peptide nature of bacteriocins and the fact that they are characterized by narrow target range, the mechanism of action of bacteriocins span a very wide spectrum, from enzymatic action to pore formation of the target cell. Most bacteriocin-producing organisms also possess ‘immunity’ to their own bacteriocin, allowing the producer, which would otherwise be susceptible, to survive and compete. Bacteriocin production has been identified in virtually every lineage of prokaryotes studied. Bacteriocin genes are located on plasmids, transposons, or bacterial chromosomes, and generally consist of an operon of several genes allowing for biosynthesis, post-translational modification, transport, as well as the immunity gene, and in some cases, a lysis gene causing the producer to die and release the mature bacteriocin. Given the complexity and diversity of bacteriocin production and the ubiquity of these molecules, they must have a critical function in establishment and preservation of microbial communities in order to stably maintain such energetically costly machinery. Ecological studies of bacteriocins have shown a very complex relationship between producer strains, sensitive strains and resistant strains; producers don’t necessarily dominate a given population since there are significant costs in fitness to maintain the production machinery. One such model, the ‘Rock-Paper-Scissors’ model, demonstrates that a producer can beat a sensitive strain by toxin production, a resistant strain can beat a producer strain due to lower fitness of the producer (‘cost’ of maintaining the elaborate production system), and a sensitive strain can beat a resistant strain due to the higher fitness/lower cost of the sensitive strain. This

model allows for all three types of strains to coexist but provides for periodic fluctuations and selective pressure for strain evolution (Riley, 2002).

Gram negative bacteriocins (colicins, pyocins)

Gram negative bacteriocins are almost always large proteins, ranging in size from roughly 400 to 700 amino acids. Colicins, produced by *E. coli*, can be pore-forming toxins or nuclease-type toxins. Their operons are on plasmids and consist of the colicin toxin gene, lysis gene, and immunity gene. Target specificity is governed by a receptor domain on the colicin protein that binds a specific cell surface receptor found only on certain strains of the *Escherichia* genus or other enterobacteria. Pyocins, nuclease proteins produced by pseudomonads, have sequence similarity to the colicins, and have been hypothesized to have arisen through genetic recombination with colicin producers, and the pyocin genes reside on the chromosome. The colicin and related bacteriocins have typical domain structure where there is generally a receptor-recognition domain, translocation domain, and a toxin domain, all of which have sequence similarity to their counterparts in other gram negatives.

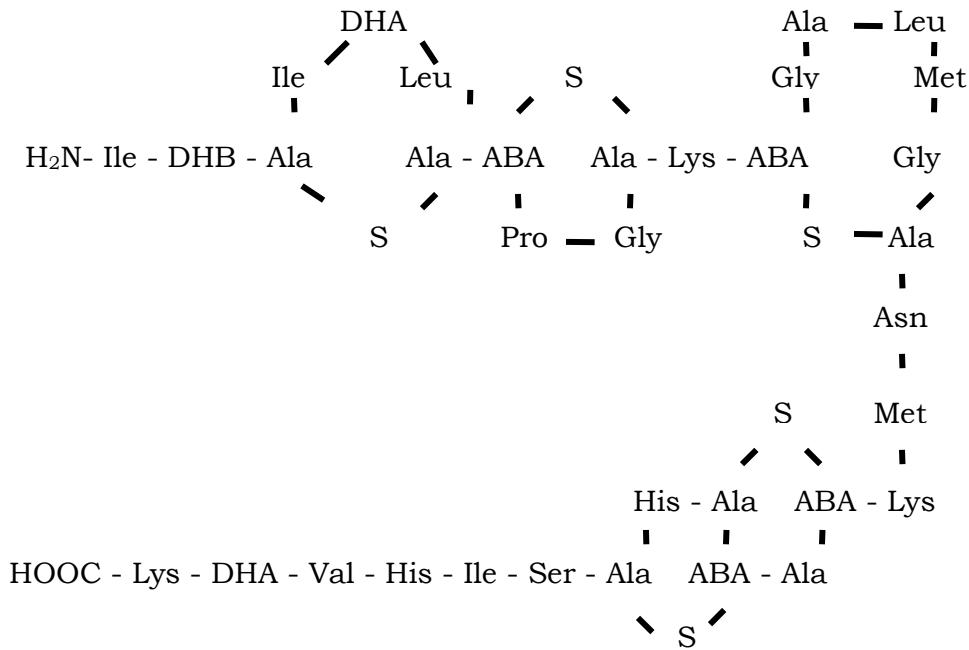
Archaea (halocins)

The only well-characterized archaeocin is the halocin family, produced by the halobacteria. These are extremely stable peptides derived from a larger pro-protein, appear to block the action of ion channels in sensitive cells, and are generally produced during stationary phase of growth, and are thought to kill sensitive strains during nutrient limitation allowing for nutrient release and utilization by the producer cells.

Gram positive bacteriocins

Bacteriocins from gram positive bacteria are even more diverse than those of gram negatives. They are generally secreted by the producer cell and thus do not require death of the producer cell to release mature bacteriocin. The best studied members of this group of molecules are from lactic acid bacteria (LAB), -prolific in their use in cultured food products. There are numerous classification schemes for gram positive bacteriocins, and at least 3 broad classes of LAB bacteriocins; Class I, II, and III. Class I bacteriocins constitute the lantibiotics; relatively small peptides (19-38 amino acids) that possess at least one lanthionine ring (a cyclic structure comprised of one dehydrated serine or threonine thioether bonded to a cysteine, Fig. 1). Within the lantibiotics, there are further subdivisions, based on biosynthetic pathway and other structural features. Class II bacteriocins are small, non-lanthionine containing peptides that share a conserved N-terminal sequence, are pore-formers, and have anti-*Listeria* activity. Class III bacteriocins are large, heat labile proteins; notable in this group is lysostaphin, an enzyme specific for the cell wall of staphylococci.

Figure 1: Structure of the lantibiotic, Nisin A



Legend:

- meso-Lanthionine (Ala-S-Ala):
- threo-B-methylanthionine (ABA-S-Ala)
- Dehydroalanine (DHA):
- Dehydrobutyrine (DHB):
- Amino butyric acid (ABA):

Lantibiotics

Unquestionably, the most studied and best characterized of these LAB bacteriocins are the lantibiotics. More than 60 lantibiotics have been described to-date. The best known of the lantibiotics is Nisin (Fig.1). This molecule is produced by certain strains of *Lactococcus lactis*, originally identified as a group N *Streptococcus*, from which came the name **N-Inhibitory Substance** or Nisin. Nisin exemplifies the group of lantibiotics that use two enzymes to introduce the lanthionine ring (dehydratase and cyclase, LanB and LanC), whereas a second group of lantibiotics including Mersacidin and Lacticin 3147 are modified by a single, large dehydratase-cyclase complex (LanM). This second group of lantibiotics is unique in that it also contains so-called two component lantibiotics such as Lacticin 3147, requiring two peptides to assemble together to form a functional toxin. Partly as a result of different biosynthetic pathways, type A lantibiotics (Nisin, Subtilin, Epidermin, etc.) tend to have a more linear, flexible, rod-shape (and thus tend to be more of the pore-forming variety) than the type B lantibiotics (Mersacidin, Lacticin 3147, Haloduracin), which are typically shorter and more globular.

Lantibiotic operons can reside on transposons (eg. Nisin), chromosome (eg Subtilin) or plasmid (eg Epidermin). The Nisin operon contains 11 genes, encoding leader sequence, structural gene, dehydratase, cyclase, translocase, and release peptidase, as well as the

immunity gene, NisI, which binds nascent Nisin at the producer cell surface and reduces its local concentration, preventing it from attacking the producer cell. There are at least 5 different Nisins, differing from each other by as little as one AA substitution (Nisin A, Z, F and Q) or as many as 4 substitutions (Nisin U, -produced by some strains of *S. uberis*). These substitutions do not appear to dramatically affect the antimicrobial activity, but can affect the compound's stability and solubility.

Most lantibiotics bind to Lipid II, a highly conserved lipo-glyco-peptide essential for biosynthesis of the cell wall in both gram positive and gram negative bacteria. Sequestration of lipid II is one mechanism of toxicity from the lantibiotics. In addition, type 1 lantibiotics (eg Nisin) are long enough and flexible enough to assemble into a complex of 8 Nisin:4 Lipid II molecules, resulting in a 2-2.5 nm pore in the membrane, causing lysis of the cell (Breukink, 1999).

Many lantibiotics are active against a variety of gram positive bacteria, owing to the conserved nature of the Lipid II complex. Gram negative bacteria, while also possessing Lipid II, are generally resistant to the effects of lantibiotics due to the bacterial outer membrane barrier between the growing cell wall and the bacterial exterior. However, topical preparations of Nisin that contain chelators and nonionic surfactants are active against certain gram negative bacteria through disruption of the outer membrane, allowing access of the lantibiotic to the Lipid II target. Interestingly, there is a recent description of a lantibiotic produced by *Bifidobacterium longum* that appears to target gram negative species in the absence of outer membrane perturbation (O'Sullivan, 2011)

Of -interest is a series of findings showing that Nisin and other lantibiotics are very active against MRSA and Vancomycin Resistant Enterococci (VRE). The latter finding is particularly interesting, since vancomycin, a glycopeptide antibiotic, binds to Lipid II. The Lipid II binding sites for vancomycin and Nisin do not overlap, however, and binding of one does not compete with the other, supporting the assertion that the use of bacteriocins has a low likelihood of engendering cross resistance to antibiotics used in human medicine.

Use in food preservation

A Nisin preparation consisting of ~2.5% w/w Nisin in a milk-solids base, was commercialized in the 1940s as a 'natural' food preservative, being particularly useful due to its activity preventing *Clostrida* and *Bacillus* spore outgrowth. Coupled with very high stability in acidic environments, this preparation became very popular in dairy foods, achieving Generally Regarded As Safe (GRAS) status in 1988 for use in pasteurized processed cheese products (US Food and Drug Administration, 1988). Nisin is now approved for use in food preservation in over 50 countries world-wide, with hundreds of thousands of metric tons of food being preserved with Nisin preparations every year.

Bacteriocin use in bovine mastitis

Given that bacteriocins are peptides, have been shown to be safe for human consumption (in the case of Nisin), and have activity against many gram positive species, a natural application for these molecules is in the prevention and treatment of mastitis.

Lantibiotic properties that are advantageous for mastitis treatment

Broadest spectrum of most bacteriocins

Most potent against *Staphylococcus* and *Streptococcus* spp

Rapid killing kinetics

Active against antibiotic-resistant bacteria (MRSA, VRE)

Potential for minimal cross resistance (e.g. Vancomycin)

Nontoxic to mammalian cells

GRAS status in US and >50 countries

Degrades completely in environment and GI tract

Potential for zero milk and meat discard period

Figure 2 illustrates Nisin's activity against bovine mastitis isolates in a recent, pivotal field trial across 16 centers in the US.

Figure 2. Nisin MICs from ImmuCell Pivotal Effectiveness Study, 2008

Species	n	Nisin ($\mu\text{g}/\text{mL}$)		
		Mode	MIC50	Range
<i>Streptococcus agalactiae</i>	20	0.008	0.03	0.004-2.0
<i>CNS</i>	108	0.25	0.12	0.015-2.0
<i>Streptococcus dysgalactiae</i>	29	4.0	4.0	0.004-8.0
<i>Streptococcus uberis</i>	18	4.0	4.0	0.03-8.0
<i>Enterococcus faecium</i>	25	2.0	1.0	0.008-2.0
<i>Staphylococcus aureus</i>	46	0.25	0.25	0.125-2.0

The potential utility of bacteriocins in management of mastitis has not gone unnoticed over the years. Possibly the first attempt to treat mastitis with a bacteriocin occurred in 1949 when Taylor, et al formulated Nisin in peanut oil for infusion, but found the formulation had a significant irritant effect, precluding its use for safety reasons. Several other attempts were made to use Nisin infusion in lactating cows, but found some level of udder irritation.

Topical applications

In the early 1990s, topical forms of Nisin were commercialized as udder preps.

Consept™, a liquid Nisin formulation was commercialized as a pre and postmilking dip, and was shown to be equivalent to traditional iodophor in prevention of new intramammary infections caused by susceptible pathogens. Similarly, a premilking teat wipe product (Wipe Out®) was developed as a convenient 'one step' premilking prep using Nisin as the main active ingredient, and also contained chelators and surfactants to

broaden the spectrum of activity to include gram negatives. The advantage of the use of bacteriocins in these topical products include minimal chance of milk adulteration from cross contamination, and less irritation to the skin compared to certain chemical dips. These products have utility in many dairy situations but have suffered in the market due to the difficulty in competing with the price of traditional chemical dips.

Dry cow treatment

Lacticin 3147 was formulated with an inorganic salt (bismuth) to be used as a dry cow treatment/teat sealant. This formulation appeared to be non-irritating and had activity against *S. dysgalactiae* (Ryan 1998). Additional studies showed potential to reduce new IMI caused by *S. dysgalactiae* and *S. aureus*, but some udder irritation was observed. This work has not progressed noticeably, perhaps due to difficulties producing pharmaceutical grade bacteriocin at an acceptable cost.

Clinical Mastitis

Nisin Z was recently compared to gentamycin in the treatment of clinical mastitis, and was shown to be statistically equivalent in clinical and bacteriological cure (Cao, 2007). Some elevation in somatic cell counts was noted in the Nisin treatment group, but it was not significantly higher than the gentamycin group. Unfortunately, a negative control was not used in this study.

Subclinical mastitis

Recombinant Lysostaphin was used to treat staphylococcal mastitis in an experimental challenge study, and was compared to traditional antibiotics (Oldham, 1991). Cure rates were somewhat lower than approved antibiotics in this study.

Sears, et al, have been pioneers in the area of bacteriocin use for mastitis and have shown that Nisin and lysostaphin have the potential as a combination treatment, in hopes of optimizing the cure rates against staphylococci and streptococci (Sears, 1992).

Recently, controlled field studies of Nisin in subclinical mastitis have been performed. In one study using Nisin Z, statistically significant cure rates were observed compared to negative controls (Wu, 2007). Unfortunately, the Nisin used in this study was only ~50% pure, and some udder irritation was observed. Pharmaceutical-grade Nisin A has been produced and formulated for intramammary use (Mast Out®, ImmuCell Corp). This material showed statistically significant cure rates compared to control in a dose-ranging study (Coughlin, 2004). Notably, the udder irritant in the older Nisin preparations was shown to be a contaminant from the growth media and not Nisin itself. The Mast Out® Nisin formulation has recently proceeded through pivotal effectiveness, target animal safety and human food safety studies, showing statistically highly significant effectiveness, no irritation, and has achieved zero milk and meat withhold (subject to full FDA approval)(unpublished data).

Potential drawbacks of bacteriocin use in mastitis

Effects on dairy starter cultures

The safe concentrations of Nisin (and presumably other lantibiotics) for human consumption lead to the potential for zero milk and meat withhold, and could potentially open up the current unmet area of a subclinical mastitis treatment without the associated costs of discarding milk. However, the relatively high potency of the compound against gram positive organisms could lead to some interference with cultured dairy products (certain cheeses, yogurts) if a high enough proportion of animals are treated at any one time. Selection of treatment candidates in any given herd using SCC records and culture data are anticipated to result in treatment decisions that would not lead to Nisin concentrations that would threaten to interfere with dairy cultures at the processing facility. However, Blitz-type treatment strategies would not be advisable without a milk discard.

Stability

Nisin-based intramammary formulations are expected to be most stable at refrigerated temperatures, which could be an inconvenience to certain producers. Other bacteriocin preparations may or may not be shelf-stable at room temperature.

Resistance development

While an advantage of bacteriocins is the unlikely contribution to cross resistance to antibiotics used in human medicine, bacteriocin resistance has been described in the literature. It is not known how rapid or widespread bacteriocin resistance will develop after commercial introduction of a mastitis treatment. Given the widespread use of Nisin in food preservation and the lack of noticeable development of Nisin-resistant food pathogen strains, it appears unlikely that rapid resistance will develop to a prescription intramammary bacteriocin formulation.

Cost

Possibly the biggest drawback to commercially successful bacteriocin-based mastitis products is the comparatively higher cost needed to manufacture pharmaceutical-grade bacteriocins compared to traditional antibiotics. While these costs would be expected to come down dramatically following development of large scale manufacturing and optimization of bacteriocin expression from the producer strains, the initial phase of commercialization will be expected to cause bacteriocin-based products to be premium priced. Therefore, careful determination of the producer's return on investment (ROI) will be critical to the commercial success of such products. For example, use of bacteriocin-based treatments for subclinical mastitis can cite savings from no milk discard, better somatic cell premiums, reduced incidence of clinical flare-ups, higher productivity, etc. to generate a positive ROI.

Conclusions

- Bacteriocins have been shown to be as effective as traditional antibiotics in mastitis treatment.
- Bacteriocin use can reduce on-farm use of traditional antibiotics.
- Zero Discard status could allow for widespread use in subclinical disease (need to identify treatment candidates)

- Blitz treatment cannot be used without milk discard (could affect dairy starter cultures).
- Higher relative cost will necessitate evaluation of producer ROI in subclinical mastitis.

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