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Lactococcal bacteriocins: mode of action and immunity

Koen Venema, Gerard Venema and Jan Kok

The past several years have seen a rapid expansion in our knowledge of lactic-acid bacteria and the antimicrobial peptides, or bacteriocins, that they produce. Detailed studies have been made of these peptides, their mechanisms of action, the immunity, processing and secretion systems, and the genes involved in these processes^{1–9}. In this article, we focus on the bacteriocins that are produced by *Lactococcus lactis*. [Data on (similar) bacteriocins from other lactic-acid bacteria are given in several recent excellent reviews^{2,6,9}.]

Tagg *et al.* defined bacteriocins in 1976 as proteinaceous compounds that kill closely related bacteria¹⁰. It is tempting to assume that different strains of a species produce these substances to enable them to compete for the same ecological niche. Tagg's definition holds true for the majority of bacteriocins that have been investigated, but it has gradually become evident that certain bacteriocins, such as the lantibiotics, may also have bactericidal activity against more distantly related bacterial species. In addition, there are examples of bactericidal complexes that contain an essential lipid or carbohydrate moiety as well as a protein.

On the basis of genetic and biochemical studies, the bacteriocins of lactic-acid bacteria have been grouped into four distinct classes⁶. Class I bacteriocins, or lantibiotics, are small membrane-active peptides that contain the unusual amino acids lanthionine, β -methyl-lanthionine, dehydroalanine and dehydrobutyrine. Examples include lactocin S, carnocin U149, lactacin 481 and nisin^{4,11,12}. Class II bacteriocins are small (<5 kDa), heat-stable, non-lanthionine-containing membrane-active peptides that are characterized by a Gly⁻²-Gly⁻¹-Xaa⁺¹ processing site in the bacteriocin

Bacteriocins are antimicrobial peptides produced by bacteria. Some of those synthesized by *Lactococcus lactis* have been characterized in great detail recently.

The lactococcal bacteriocins are hydrophobic cationic peptides, which form pores in the cytoplasmic membrane of sensitive cells.

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precursor. This site is not restricted to class II bacteriocins, as it is also present in some lantibiotics¹³. Some bacteriocins of this class consist of two separately synthesized peptides. Examples include lactococcin M, lactacin F and lactococcin G (Ref. 6). Most bacteriocins that have been characterized so far belong to this class. Class III bacteriocins are large (>30 kDa) heat-labile proteins, and include helveticin J, helveticin V-1829

and lactacins A and B (Refs 4,14–16). Class IV complex bacteriocins are composed of a protein together with one or more non-proteinaceous moieties (lipid or carbohydrate), which are required for their biological activity. Examples include leucocin S, lactocin 27 and pediocin SJ-1 (Refs 17–20). However, there is controversy over whether or not this class of bacteriocins really exists, with some suggesting that the lipid or carbohydrate component is just a contaminant.

All the lactococcal bacteriocins that have been thoroughly characterized so far belong to class I or II. In this article, we highlight their role in the ecology of *Lactococcus* by discussing their mode of action and the corresponding immunity proteins.

Mode of action of lantibiotics: nisin

Nisin is the only lantibiotic produced by *L. lactis* for which the mode of action has been studied. It is active against a broad spectrum of Gram-positive bacteria; *Escherichia coli* and other Gram-negative bacteria are only affected when their outer membranes are weakened or disrupted by treatment with EDTA or osmotic shock^{21,22}, which makes their inner membrane accessible to the lantibiotic.

Nisin has a dual activity against spore-forming bacteria: it inhibits the outgrowth of spores and kills cells in the vegetative state. The 2,3-didehydroamino acid residues in nisin are thought to act against spores by interacting with the membrane sulphydryl groups of germinating spores²³. The primary target of nisin in vegetative cells is the cytoplasmic membrane. It dissipates the membrane potential of whole cells, cytoplasmic membrane vesicles and artificial membrane vesicles (liposomes)^{24,25}, indicating that the peptide does not require a specific receptor protein for activity or for membrane insertion.

Early work on the mode of action described a voltage-dependent depolarization of the membrane by nisin²⁵. Garcerá *et al.* concluded that the membrane potential is not essential, but that the total proton-motive force stimulates the action of nisin²⁶. However, recent work from the same group has shown that a membrane potential is essential in a different membrane system²⁷. Membrane disruption is believed to result from the incorporation of nisin into the cytoplasmic membrane to form an ion channel or pore. The efficiency of insertion of nisin into liposomes depends on the phospholipid composition of the liposomes. This may account for the differences in sensitivity seen be-

tween bacterial species or strains, as permeabilization only occurs in liposomes that contain zwitterionic phospholipids^{28,29}.

The activity of nisin can be significantly reduced by di- and trivalent cations, and activity can even be prevented by gadolinium (Gd^{3+})²⁹, a lanthanide that is known to inhibit various channels in eukaryotic and prokaryotic cells³⁰. Binding these ions neutralizes the negatively charged head groups of phospholipids and makes the lipids condense, resulting in a more rigid membrane, which probably decreases the efficiency with which nisin inserts and forms pores. Nisin also has a lower activity at temperatures below 7°C (Ref. 29), presumably because increased ordering of the lipid hydrocarbon chains in the cytoplasmic membrane inhibits nisin insertion.

Mode of action of non-lantibiotics

Diplococcin

The effect of purified diplococcin from *L. lactis* subsp. *cremoris* 346 on sensitive cells was first studied in 1981³¹. The addition of 8 arbitrary units of diplococcin to sensitive cells completely abolishes DNA and RNA synthesis within 2 min, which may partially interrupt protein synthesis. Diplococcin is now known to be

Fig. 1. Amino acid sequence (single-letter code) of mature (a) lactococcin A and (b) lactococcin B, and helical-wheel presentations of (c) the hypothetical transmembrane helix in lactococcin A and (d) the putative amphiphilic α helix in lactococcin B. Amino acids in the putative α helices are underlined in (a) and (b). The helices of both bacteriocins are thought to insert into the cytoplasmic membrane of sensitive cells using a *Lactococcus*-specific receptor (see Fig. 2).

equivalent to lactococcin A (Ref. 32), and these effects are thought to be due to an increase in the permeability of the bacterial cytoplasmic membrane (see later section).

Lactostreptocin 5

Lactostreptocin 5 (Las5) and other lactostreptocins have a strong and rapid bactericidal effect on sensitive cells³³; only Las5 has been characterized in detail. It inhibits uridine uptake and causes leakage of K^+ ions and ATP from cells. Like diplococcin, Las5 inhibits DNA, RNA and protein synthesis, probably by the inhibition of transport of precursors required for macromolecular synthesis, energy depletion of the cell and/or leakage from the cell of small solutes that are required for various metabolic activities. Las5 is equally active against energized and energy-depleted cells³³.

Lactococcins A and B

Lactococcins A and B specifically inhibit the growth of lactococci. They belong to a group of small, cationic hydrophobic peptides (including several lantibiotics) that permeabilize membranes^{28,34-36}. The mode of action of purified lactococcin A has been studied using whole cells of sensitive lactococcal strains and membrane vesicles made from such cells, and also using liposomes obtained from lactococcal phospholipids³⁷. Similar studies on whole cells have also been done using partially purified lactococcin B (Ref. 38).

At lactococcin concentrations that do not affect cells that are immune to these peptides, both bacteriocins rapidly dissipate the membrane potential (lactococcin B also dissipates the pH gradient across the cytoplasmic membrane) of energized sensitive cells^{37,38}. Furthermore, the addition of either lactococcin to sensitive cells that have accumulated glutamate or α -amino isobutyric acid (a non-metabolizable alanine analog) results in the immediate efflux of the amino acid, even

when the proton-motive force has been dissipated before the bacteriocin is added. These results indicate that both lactococcins form pores in the cytoplasmic membrane in a voltage-independent manner. Lactococcal cells that have been gently pretreated with lysozyme release the intracellular enzyme lactate dehydrogenase within a few minutes of lactococcin A treatment³⁹. Lactococcin A inhibits leucine uptake in cytoplasmic membrane vesicles from sensitive lactococcal cells, but not in vesicles derived from membranes of *Bacillus subtilis*, *Clostridium acetobutylicum* or *E. coli*³⁷. In contrast to the effect of nisin, liposomes derived from lactococcal phospholipids are not affected by lactococcin A.

From these data, and the fact that lactococcin A specifically inhibits lactococcal strains, Van Belkum and colleagues concluded that lactococcin A forms pores in the cytoplasmic membrane of sensitive cells using a *Lactococcus*-specific receptor protein³⁷. A 21 amino acid sequence between residues Ala30 and Phe50 in lactococcin A could form a membrane-spanning helix³⁹ (Fig. 1a), and lactococcin A may insert into the cytoplasmic membrane of sensitive cells with this hypothetical, transmembrane helical segment. In lactococcin B, there is a putative amphiphilic α helix between residues Ile28 and Phe46 (Fig. 1b), which is in a similar position to that of the putative transmembrane helix in lactococcin A.

A large number of pore-forming toxins are known to form channels by the molecules aggregating like barrel staves around a central water-filled pore^{40,41} (Fig. 2a), and nisin and the lactococcins A and B are thought to act in this way. The size of the pore would be determined by the number of molecules involved (Fig. 2b). Low concentrations of lactococcin B allow leakage of protons and ions, whereas 150-fold more bacteriocin is needed for leakage of glutamate to occur³⁸, which indicates that pores of different sizes can exist.

Immunity and resistance to bacteriocins

Immunity to bacteriocins is defined as the production of a so-called immunity protein by a bacterial strain to protect itself.

Nisin immunity and resistance

There are several mechanisms by which bacteria protect themselves against nisin. Nisin resistance (Nis^r) is not genetically linked to nisin production. In the only study of the mechanism of Nis^r carried out so far, which involved the resistance of *Bacillus cereus* to nisin, the target organism was found to inactivate nisin by modifying one or more of its dehydroamino acid residues by means of a reductase activity⁴².

Nis^m is a spontaneously appearing mutation that causes nisin resistance⁶. Spontaneous nisin-resistant mutants can be selected by growing nisin-sensitive strains in the presence of nisin, and such mutations have greatly hampered the use of nisin as a food biopreservative.

True nisin immunity is encoded by the nisin operon. $NisI$ is a lipoprotein that is anchored to the outside of the cytoplasmic membrane⁴³. Cloning and expression of $nisl$ in *E. coli* or in *L. lactis* protects the cells from nisin, but the exact mechanism has not been investigated^{43,44}.

Recently, three other genes ($nisFEG$) involved in nisin immunity have been identified⁴⁵. The nig gene encodes a hydrophobic protein that might act in a simi-

lar manner to the immunity proteins against several colicins (bacteriocins produced by *E. coli*)⁴⁶. These immunity proteins interact directly with the colicin molecules, closing the pores formed by these bacteriocins. The proteins $NisE$ and $NisF$ have sequence similarity to ATP-binding cassette (ABC) transporters. Such an ABC transporter might transport the lantibiotic out of the cell⁴⁵, but the exact details of this mechanism have not been investigated. Interestingly, mutants with mutations in the nisin structural gene $nisa$ are also more sensitive to nisin than is the wild-type strain^{43,45}, which may be due to a general stimulation of expression of the gene cluster ($nisBTCIPRKFE$) downstream of $nisa$ (Ref. 45).

LciA: the lactococcin A immunity protein

So far, the only detailed investigation of immunity against class II bacteriocins has involved the immunity protein LciA against lactococcin A (Ref. 47). In one study, LciA, apparently present in the membrane of cells immune to lactococcin A, protected them from the effect of lactococcin A (Ref. 37). However, a study using monoclonal antibodies against LciA found similar amounts of the protein in the cytosolic, membrane and membrane-associated fractions⁴⁷.

Epitope mapping and enzyme-linked immunosorbent assay experiments on normal and inside-out vesicles

Fig. 3. (a) Amino acid sequence (single-letter code) of the lactococcin A immunity protein LciA, and (b) the putative amphiphilic α helix [underlined in (a)] that spans the cytoplasmic membrane. (c) Model for the mode of action of the lactococcin A immunity protein. Interaction with the lactococcin-A-specific receptor (R; light gray) allows the amphiphilic α helix (black) in LciA (I; dark gray) to span the cytoplasmic membrane (CM). The carboxyl terminus of the immunity protein is outside the cell; the amino terminus (N) is in the cytoplasm. By binding the receptor, LciA prevents lactococcin A (L) from inserting into the membrane, although binding of lactococcin A to the receptor still occurs.

indicate that the carboxyl terminus of LciA is on the outside of the cytoplasmic membrane⁴⁷. Normal membrane vesicles derived from a strain producing both lactococcin A and the immunity protein do not react with a monoclonal antibody against LciA. This also occurs when lactococcin A is added externally to normal vesicles derived from a strain producing only LciA (Ref. 47), suggesting that the bacteriocin shields the epitope in LciA from binding the monoclonal antibody. Treatment of membrane vesicles of both immune and sensitive cells with proteinase K made the leucine uptake insensitive to the bacteriocin, suggesting that proteinase K digests both LciA and the putative bacteriocin receptor protein.

LciA could have one of two possible mechanisms of action: the immunity protein might bind to and neutralize the bacteriocin, or alternatively, it might interact with and block the bacteriocin receptor. If LciA were to bind and neutralize lactococcin A, then fusion of immune and sensitive vesicles would result in immune fusion vesicles. However, if LciA were to block the bacteriocin receptor, then the fused vesicles would be sensitive, as the unblocked receptors from the sensitive vesicles would still interact with lactococcin A. Leucine uptake in the fused vesicles is sensitive to the addition of lactococcin A, and so it has been concluded that LciA interacts directly with the lactococcin A receptor to prevent the insertion of the bacteriocin into the cytoplasmic membrane⁴⁷.

The 11 kDa LciA protein has been purified by exploiting the physicochemical characteristics derived from its deduced amino acid sequence⁴⁸. Its amino acid composition and sequence suggest that LciA is not post-translationally modified. LciA is predicted to have an amphiphilic α helix with a strong hydrophobic moment of 0.52 between amino acids 29 and 47 (Fig. 3a)⁴⁷. These results have been united in a model for LciA topology (Fig. 3b). The carboxy-terminal residues 48–98 are on the outside of the cell. Residues 29–47 are considered to span the cytoplasmic membrane as an amphiphilic α helix by interacting with another transmembrane protein, possibly the lactococcin A receptor. The amino terminus of LciA is considered to be on the cytoplasmic face of the membrane.

This model explains the observation that only part of the LciA molecule pool is present in the cytoplasmic membrane fraction (see previous discussion). Apparently, this fraction is enough to interact with all the receptors. The cytoplasmic and membrane-associated fractions of LciA may form a continuously available reservoir from which molecules can be drawn rapidly to prevent newly synthesized receptors from interacting with bacteriocin molecules⁴⁷.

Conclusions and perspectives

The past few years have seen significant progress in our understanding of nisin and the lactococcins. The structural and immunity genes and the genes encoding the secretion and post-translational modification machinery have been cloned, and we are now beginning to understand the modes of action of nisin and the lactococcins A and B, and the way in which the lactococcin A

immunity protein LciA works. The immunity protein probably interacts with the as-yet-unidentified lactococcin A receptor. The molecular details of the mechanism of immunity to nisin will no doubt be resolved in the near future. This knowledge, combined with structure–function studies of the bacteriocins, should allow the construction of molecules with enhanced or altered activities and broader specificities for use as, for example, food preservatives.

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Phenotypic variation of carbohydrate surface antigens and the pathogenesis of *Haemophilus influenzae* infections

R. John Roche and E. Richard Moxon

H*aemophilus influenzae* is a nonmotile Gram-negative bacterium with a tropism for the mucous membranes of the human respiratory tract; indeed, the upper respiratory tract of humans is virtually the sole reservoir for this organism. Although it is usually a commensal, it is potentially pathogenic and is a frequent cause of respiratory tract infection and invasive systemic disease, particularly in children^{1–3}. Nonencapsulated, so-called nontypeable *H. influenzae* is important as a cause of upper and lower respiratory tract infections^{2,3}, while encapsulated *H. influenzae*, usually of serotype b, is the major cause of invasive systemic disease, such as meningitis¹. Infection by *H. influenzae* illustrates the complex interplay that can occur between the host and the pathogen, in a relationship that does not always culminate in disease⁴.

Definitions of bacterial virulence often include infectivity (minimal infectious dose) and the ability to damage host tissues. These characteristics do not necessarily include a measure of the longer-term fitness (reproductive success) of the bacterium^{5,6}. Thus, although virulence factors may be important for survival and spread within and between hosts, pathogenicity determinants may expand the population of bacteria within host microenvironments, even when this does not increase

Phenotypic variation of two major carbohydrate surface antigens of *Haemophilus influenzae*, the capsule and lipopolysaccharide, exemplifies some of the genetic mechanisms used by pathogenic bacteria in interacting with host microenvironments. The ability to generate phenotypic variety at high frequency within clonal populations of microorganisms provides an adaptive mechanism to combat the polymorphisms and immune repertoires of the host.

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the probability of transmission to another host⁶.

Two major surface-exposed carbohydrate structures of *H. influenzae*, the capsule and lipopolysaccharide (LPS), are critical in the pathogenesis of infection^{7,8}. Surface determinants that are essential for the organism at one stage of host colonization may be unnecessary or detrimental at a later stage, either because they inhibit specific cell–cell interactions, or because they provide targets for immune attack. The mechanisms that have evolved for phenotypic variation of the

capsule and LPS in *H. influenzae* illustrate some general principles of the adaptive potential of surface determinants and their role in commensal, as well as pathogenic, behaviour.

Capsule

Different strains of *H. influenzae* each may express one of six serologically distinct types of capsule, designated a–f (reviewed in Ref. 7). Extensive epidemiological and seroepidemiological data link the expression of capsule, especially that of serotype b [polyribosylribitol phosphate (PRP)], to invasive disease of humans caused by *H. influenzae*¹. Furthermore, experimental data from an infant-rat model of invasive *H. influenzae* infection suggest that capsule expression is directly involved in