Bacteriocins of Streptococcus pneumoniae and its response to challenges by antimicrobial peptides
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Chapter 1

Introduction
**S. pneumoniae, the pathogen**

*S. pneumoniae* (the pneumococcus) was first identified in 1881 simultaneously by L. Pasteur and G. M. Sternberg (540). The pneumococcus is a Gram-positive bacterium that belongs to the genus *Streptococcus*. The term *Streptococcus* means literally, “strepto”-twisted and “coccus”- from the Greek word “kokkus” meaning berry or grain. The genus consists of bacteria of round, spherical shape that occur single and/or in pairs, and/or in short chains (Fig. 1). The former name of *S. pneumoniae* was *Diplococcus pneumoniae*, since it mostly grows in pairs. *S. pneumoniae* is an aerotolerant anaerobe, but some of fresh clinical isolates are obligate anaerobes.

The main characteristics distinguishing *S. pneumoniae* from other streptococci are the production of alpha hemolysis (a green zone) when grown in blood, bile solubility, inulin hydrolysis and sensitivity to optochin (513). The genus *Streptococcus* represents part of the nasopharyngeal microflora of human and some of the *Streptococcus* species can be pathogenic. The upper respiratory tract can be colonized asymptomatically by the pneumococcus but the colonization rate varies between individuals and depends on the geographical region and population group. However, when conditions are favourable to *S. pneumoniae*, such as in young children, elderly and people with immunodeficiency disorders, the pneumococcus might relocate to other parts of the human body, *e.g.* lungs, ears, sinusitis, blood or brain, which may eventually cause diseases such as pneumonia, otitis media, sinusitis, bacteremia or meningitis. More than one million people each year suffer from *S. pneumoniae* infections, of which over 800 thousand children from developing countries, younger than 5 years old, die annually (465).

Interactions between *S. pneumoniae* and other streptococcal species during the nasopharyngeal colonization have not been studied extensively (11,54,97,317,318,402). Nevertheless, it was shown that during otitis media, *S. pneumoniae* is able to cohabit in a biofilm with *Haemophilus influenzae* and *Moraxella catarrhalis* (362). Sometimes, three latter species and *Staphylococcus aureus* might together colonize asymptomatically the
nasopharynx of young children (412), but upon the occurrence of unknown triggering conditions, they will start to compete with each other. The factors of the competition mechanism between microorganisms colonizing a human body are not exactly identified but *in vitro* and *in vivo* data showed that bacteriocins, hydrogen peroxide, pili, host immune responses and/or other (unknown) factors play a role in *S. pneumoniae* competition with other respiratory pathogens such as *Neisseria meningitidis*, *H. influenzae*, *M. catarrhalis* and *S. aureus* (97,317,318,402,434,435,437,503). Bacteriocins are antimicrobial peptides (AMPs) produced by bacteria. The genus *Streptococcus* produces a great number and diversity of known bacteriocins and one of the first published articles concerning AMPs in this genus goes back to the 1960s, when the AMPs were described in *D. pneumoniae* and in the group A streptococci (298,349).

A capsular polysaccharide (CPS), composed of carbohydrate polymers, encloses the pneumococcus cell. CPS was the first factor shown to be important in a *S. pneumoniae* virulence (132). CPS prevents phagocytosis and aggregation, affects colonization and adhesion, helps the pneumococcus to survive in the lungs and spread to bloodstream, and contributes to antibiotic tolerance (137,240,356,367,541). Based on the CPS composition, *S. pneumoniae* strains are divided into 91 serotypes (399). Consequently, virulence of the *S. pneumoniae* strains depends primarily on a type of the serotype. Interestingly, some of the pneumococcus clones, e.g. (serotype indicated in a superscript) *S. pneumoniae*Spain23F ATCC700669, are able to switch their capsule type (84,85,263). There are a few completely assembled genome sequences of the pneumococcus available in NCBI database, *i.e.* D392, TIGR44, G5419F, CGSP14, Hungary19A-6, Taiwan19F-14, P10311, JJA14, ATCC 70066923F, 705855 and R6 unencapsulated strain derivative of D39, and many more are in sequencing progress. These genome sequences have made it possible to compare DNA sequences of not-and/or closely related species. Furthermore, the genome content variability of *S. pneumoniae* strains of the same or different serotypes was demonstrated (94,179,380). Notably, it was shown for eight *S. pneumoniae* clinical isolates of different serotypes that 15.6% of the sequence was unique to the reference strain TIGR4 and 5.5% was unknown for the sequenced pneumococcus strains (472). Additionally only 46% of the homologous gene clusters were common between 17 *S. pneumoniae* strains of distinct serotypes (205).

The *S. pneumoniae* serotypes dissemination among people differs and depends on such factors as age and geographical area (189,190). Roughly 10% of the carriers that is people colonized by the pneumococcus, can be colonized by more than one pneumococcus strain at the same time if the strains are not particularly of the same serotype (54,183,221,247). It has been shown that 95% of children below the age of two, in Birmingham, Alabama, have been colonized by up to six various pneumococcal serotypes (158). *S. pneumoniae* of serotype 3, 6A, 11A, 19F, 23F, and/or 14, depending on geographical region, are commonly found at the same time in healthy carriers (52,53). Nevertheless, isolates of these serotypes (and additionally the ones of 6B, 9V and 19A) are
the most common cause of otitis media in children younger than 18 years old (442). In Denmark serotypes 3, 10A, 11A, 15B, 16F, 17F, 19F, 31 and 35F are related to high mortality among children older than 5 (182). In the Netherlands serotypes 1, 5, 7F, 15B, 20 and 33F are the ones with the lowest mortality, serotypes 4, 6A, 8, 9V, 10A, 11A, 12F, 14, 19A, 22A, 22F, 23F, and 24F have intermediate mortality rates, and serotypes 3, 6B, 9N, 16F, 18C, 19F, and 23A have the highest rates (234). In 2008 serotypes 1, 3, 7, 14, and 19 were the most common cause of the pneumococcal infectious diseases in Europe (133). All together, the serotype distribution among countries may vary but those with a high invasive diseases potential, e.g. of serotype 3, 7, 19 and 23, are the same around the world.

Penicillin, an antibiotic of the beta-lactam family, has been used to treat pneumococcal infection diseases since 1940. The resistance of a S. pneumoniae clinical isolate to penicillin was described as early as 1967 (181). Since then, the resistance of the pneumococcus to commonly used antibiotics, i.e. beta-lactams, macrolides, chloramphenicol, tetracyclines and fluoroquinolones, has increased worldwide. This increase has varied yearly for each antibiotic and also varies by country (65,133). For instance, the occurrence in European countries of S. pneumoniae strains resistant to penicillin and macrolide varies between 5% and 50% depending on the country (133,152,344). A multidrug-resistant, i.e. non-susceptible to two or more antibiotics, S. pneumoniae clinical isolate was first reported in 1977. This isolate was located in South Africa and it was resistant to penicillin, erythromycin, clindamycin, tetracycline and chloramphenicol (264). Nowadays, pneumococci of serogroups 6, 9, 14, 19 and 23F are commonly multidrug-resistant (88,138,262). Interestingly, these serotypes are more prevalent among carriers.

S. pneumoniae, vaccines

Currently, two types of vaccine against S. pneumoniae are available on the market, i.e. a non-conjugated pneumococcal polysaccharide vaccine (PPV23) and a pneumococcal conjugate vaccine (PCV). The first vaccine with commercial name Pneumovax23, is effective against 23 serotypes, i.e. 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F. Nevertheless, it is useful only for older children and adults because, in children under the age of two years, the PPV23 fails to mount an adequate immunity response (547). The PCV7 vaccine (produced by Wyeth under the commercial name Prevnar) consists of capsular poly- and/or oligosaccharide of serotypes 4, 6B, 9V, 14, 18C, 19F and 23F conjugated to a carrier protein (a nontoxic recombinant variant of diphtheria toxin) and is safe for use by infants and elderly people (546). The improved PCV7, Prevnar13, will be available on the market soon and it will give protection against six additional serotypes, i.e. 1, 3, 5, 6A, 19A and 7F. The PCV10 produced by GlaxoSmithKline, under the commercial name Synflorix, contains antigen from ten serotypes of the pneumococcus, i.e. 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F, conjugated to a carrier protein (a protein from non-typeable H. influenzae strains) (155,559).
The number of the pneumococcal invasive diseases caused by penicillin, erythromycin and cephalosporin non-susceptible strains, e.g. 6B, 9V, 19F and 23F, has decreased since the introduction of PCV7. Nevertheless, the number of invasive diseases of non-vaccine serotypes, i.e. 3, 15A, 33F, 22F, 35B and 19A, has increased. A similar situation was reported for carriage serotypes, namely a reduction of vaccine serotypes and an increase of non-vaccine serotypes in carriers (93). Nowadays, multidrug-resistant serotypes, such as 19A, are the most common cause of the pneumococcal infectious diseases (93,204). The PPV23 and PCV vaccines protect against a limited number of serotypes, and thus an increased number of the pneumococcal invasive diseases, caused by the non-vaccine serotypes, is observed. Hence, a new protein-based pneumococcal vaccine is currently in development and/or being clinically trialed, and is likely to become a universal vaccine that is effective against all serotypes for all age groups (10,266). The protein-based vaccine would consist of numerous pneumococcal virulence factors and surface proteins.

S. pneumoniae, virulence factors

Virulence factors are molecules contributing to the morbidity and mortality caused by a pathogen. Generally, virulence factors facilitate pathogen colonization, proliferation, escape from the host’s immune response and person to person spread.

One of the most important virulence factors is the pneumococcal capsular polysaccharide (CPS). Apart from CPS, S. pneumoniae has other important virulence factors, described below. Pneumolysin (Ply) is a cytoplasmic cholesterol dependent toxin that is released from a bacterial cell during lysis with help of autolysin (LytA). Once released, Ply forms pores in the cellular membrane of eukaryotic cells causing discharge of cytoplasm and consequently tissue damage (208). The non-cytotoxic activity of pneumolysin involves inhibition of complement system (567). Ply is essential for S. pneumoniae to survive in the upper and lower respiratory tracts and to disseminate from lungs to blood in an in vivo model. Thus, Ply is an important cytotoxic in invasive diseases, i.e. pneumonia, bacteraemia and meningitis (30,32,389). The major autolysin, LytA, a N-acetyl-muramyl-L-alanine amidase, is involved in the prevention of phagocytosis and production of cytokines (331) and, a lytA mutant is less virulent in pneumonia and bacteraemia murine models (58). Nevertheless, it is thought that LytA only mediates virulence by triggering the pneumococcal cell lysis and, as a consequence, release of pneumolysin, inflammatory cell wall components and teichoic acids (331). Both the NanA and NanB neuraminidases play a significant role during colonization and survival in the lungs and the blood stream (313). PavA, pneumococcal adhesion and virulence A, modulates adherence to immobilized fibronectin and plays an important role in virulence, as it is attenuated in the sepsis and the meningitis model (213,419). Notably, this protein stimulates adaptive immune response and production of cytokines, and it helps to avoid phagocytosis (378). Another adhesin, namely the pneumococcal serine-rich repeat adhesin
Introduction

P ( PsrP), is important in the development of pneumonia since it contributes to pneumococcal adherence to lung epithelial cells (447,474). The pneumococcal pili encoded either by the first pilus islet, PI-1 (also known as the rlrA islet) or by the second pilus islet, PI-2, are not present in all pneumococcal clinical isolates. Those strains with either type of the pili, PI-1 or PI-2, exhibit increased adhesion to human respiratory epithelial cells (17,366). Additionally PI-1 triggers higher host inflammatory responses than strains without pili, and they enhance the ability of *S. pneumoniae* to colonize, and to cause invasive diseases (21,153). The choline-binding protein A, CbpA, also known as the pneumococcal surface protein C, PspC, plays a role in colonization by facilitation of adherence to epithelial cells, prevents phagocytosis and avoids complement activation (178,235,383,448). Another choline-binding protein, *i.e.* pneumococcal surface protein A (PspA), is a significant factor for the pneumococcus to colonize and to cause invasive diseases as it prevents complement-mediate opsonisation and killing by lactoferrin (383,471,519).

Metal-binding lipoproteins such as pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake (PiuA) are important for *S. pneumoniae* virulence since mutation of these transporters reduces pneumococcal virulence in pneumonia, bacteraemia and additionally mutation of *psaA* reduces colonization (31,50,320). PsaA is involved in the protection from oxidative stress (517). The polyamine transporter (PotD) and immunoglobulin A protease (IgA) are important virulence factors because IgA is the pneumococcal protease that reduces efficiency of the immunity system by cleavage of a human immunoglobulin protease IgA1 (537) and a *potD* mutant is attenuated in pneumonia and the bacteraemia model (538). Pneumococcal surface proteins such as enolase (Eno) and hyaluronate lysase (HylA) contribute to *S. pneumoniae* virulence in invasive diseases (27,236). Another surface virulence factors, *i.e.* β-galactosidase (BgaA) and β- N-acetylglucosaminidase (StrH), mediate colonization of the human nasopharynx by facilitating pneumococcal adherence to the epithelial cells (256). Surface anchored proteins PhtA, PhtB and PhtD (pneumococcal histidine triad) protect against nasopharyngeal colonization, pneumonia and sepsis (1,177,382,570).

Bacteriocins are peptides with an antimicrobial activity mainly against closely related species and they are characterized in detail below. BlpM and BlpN (bacteriocin-like peptides) are bacteriocins produced by *S. pneumoniae* strains (97,300). They are important factors for *S. pneumoniae* strains that enable intra- and interspecies competition in the nasopharynx (97) and consequently survival, colonization and transmission of the pneumococcus to other parts of the human body.

Many of these virulence factors are highly immunogenic, which makes them good candidates for a vaccine. In addition, this suggests that more than one virulence factor is essential for *S. pneumoniae* virulence and therefore, for the protein vaccine. Virulence factors such as PspA, PspC, PsaA, LytA, pneumolysin, PiuA, PiaA, PotD, NanA and PhtB,
are good candidates for the vaccine because they trigger a protective immune response in animal models and are likely to increase survival (381,499). However, the optimal combination of the proteins mentioned above, which would give the best protection against pneumococcal infection diseases, is still being investigated.

**Antimicrobial peptides**

**Antimicrobial peptides, an introduction**

Antimicrobial peptides (AMPs) are small proteins produced by the majority, if not all, living organisms in order to eliminate environmental microorganisms such as viruses, bacteria, fungi and/or protozoa, while they are immune. Thus, AMPs are defensive weapons used widely by both bacteria and the human body. Regardless of their host diversity, AMPs contain common features such as they are all natural products of living organisms and they all have antimicrobial activity, and low molecular weight. AMPs are grouped independently of their producer organism, but according to the way they are synthesized and their structural characteristics. For instance, there are ribosomally and nonribosomally (nonribosomal peptides, NRP) synthesized peptides, anionic or cationic molecules, circular, linear, or globular ones and those with specific amino acids compositions. One of the best characterized and most common group of AMPs is the class of ribosomally synthesized and of cationic nature, named cationic antimicrobial peptides (cAMP). Generally, cAMPs, of both human and bacteria, are produced as inactive peptides consisting of an N-terminal leader sequence, which is cleaved off during release of the peptide from the cell, and a C-terminal cationic sequence which forms the active peptide. cAMPs become active once the N-terminus is cleaved off. Here, AMPs produced by humans and Gram-positive bacteria will be discussed.

**cAMPs of the human host**

In the human body, the first defense barrier against pathogenic microorganisms is the innate immune system. It is composed of multiple components: a mechanical barrier of skin epithelium, tissues of epithelial cells producing mucus, lysozyme, neutrophils, dendritic cells, macrophages, natural killer cells, phagocytes and/or effector molecules such as antimicrobial peptides. In humans, the following distinct groups of cationic AMPs have been identified: cathelicidin, defensins and histatins (19,109,345).

**Cathelicidin**

The cathelicidin family/group includes one member, the 18 kDa hCAP-18. The hCAP-18 is produced by neutrophils, monocytes, NK cells, T and B cells and by epithelial cells, as an inactive preproprotein (42,124). The serine proteinase cleaves the precursor protein, cathelin, from the carboxyterminal peptide, which is then named LL-37 (486). The
Introduction

11 kDa cathelin displays antimicrobial activity against bacterial species that are resistant to LL-37. However, the lack of a net-positive charge and structural similarity to the cystatin family disqualifies cathelin from the cAMP group. LL-37 is a linear 37 amino acid cationic peptide with activity against both Gram-positive and Gram-negative bacteria (20,124). It has been shown that bactericidical action of LL-37 is due to immobilization of the peptide into the membrane and as a consequence destabilization of the bacterial membrane (388,522). In addition to killing pathogens, this peptide has multiple roles, such as immune activation, proliferation of the inflammatory cells, chemotaxis, angiogenesis, wound healing and antitumor activity (20,42,124).

Defensins

In humans, defensins are produced by epithelial cells of the skin, gastrointestinal, urogenital, and respiratory systems, and immune cells. Defensins are small 3.5-6 kDa peptides, of which cysteine residues form three to four disulfide bridges within the molecule. Based on the arrangement of the bridges and their structure, human defensins are divided into two classes, namely α-defensins and β-defensins. Both types of defensins can protect the human body against Gram-positive and Gram-negative bacteria, viruses including the immunodeficiency virus HIV-1 and pathogenic yeasts (19,103,428). In addition to their antimicrobial activity, defensins participate in many other processes such as chemoattraction and activation of the immune or inflammatory responses to infection sites, wound healing, acceleration of angiogenesis, promotion of the production and release of cytokines and chemokines and neutralization of bacterial lipopolysaccharides (110,375,376,428).

Histatins

Histatins are peptides rich in histidine residues and are found in human saliva. They contribute significantly to a healthy oral cavity because they have antibacterial and strong antifungal activity, and they inhibit plaque formation. Importantly, histatins prevent inflammation and inhibit host and bacterial enzymes involved in periodontal diseases. As a consequence, histatins are under clinical investigation as treatment for oral fungal infections (103,117,251).

The nonribosomal peptides (NRP) of Gram-positive bacteria, bacitracin

The NRPs are a class of secondary metabolites of microorganisms. Bacitracin, an example of the NRP, is an antimicrobial substance produced by Bacillus licheniformis and some strains of Bacillus subtilis. Bacitracin is synthesized as a mixture of closely related cyclic dodecylpeptides, by a specialized nonribosomal peptide synthetase (NRPS) complex. In general, the NRPS complex is organized in several modules, e.g. an initiation, an elongation and a termination module. Each of these modules is responsible for the introduction of one additional amino acid. Bacitracin is used as an antibiotic for treatment
of skin and eye infections as well as for prevention of wound contagions caused by Gram-positive cocci and bacilli. However, this AMP has a rather narrow antimicrobial spectrum, for which it requires a divalent metal ion (271,314,315,464).

**cAMPS of Gram positive bacteria**

AMPs produced by bacteria are called bacteriocins. In general, they are short (between 30 and 60 amino acids), hydrophobic and/or amphipathic peptides. Bacteriocins are ribosomally synthesized as an inactive precursor peptide (prepeptide) that consists of an N-terminal leader sequence and a C-terminal propeptide. The leader sequence targets bacteriocins to a dedicated transporter and keeps bacteriocins in an inactive form until they are secreted (121,131,374). As there were previously diverse classifications of bacteriocins, N. C. K. Heng and J. R. Tagg proposed a universal classification consisting of four classes, namely i) modified bacteriocin named lantibiotics, ii) unmodified peptides, iii) large proteins and iv) cyclic peptides (203,258). However, the majority of bacteriocins belong to the first two classes and thus these classes will be discussed in more detail below (Table 1).
Table 1. Overview of some of the class I and class II bacteriocin peptides mentioned in this introduction

Table 1. Overview of some of the class I and class II bacteriocin peptides mentioned in this introduction

a synthesized without the leader sequence; +, feature present/used; ND, not determined

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Producer strain</th>
<th>Mass (Da)</th>
<th>Leader type</th>
<th>Modification enzyme</th>
<th>Processing and transport</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PR-type</td>
<td>GG-type</td>
<td>LanB, LanC LanM LanP, LanT LanT(P)</td>
<td></td>
</tr>
<tr>
<td>Nisin</td>
<td>Lactococcus lactis</td>
<td>3353</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(161)</td>
</tr>
<tr>
<td>Subtilin</td>
<td>Bacillus subtilis</td>
<td>3317</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(159,160)</td>
</tr>
<tr>
<td>Pep5</td>
<td>Staphylococcus epidermidis</td>
<td>3488</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(244)</td>
</tr>
<tr>
<td>Mutacin I</td>
<td>Streptococcus mutans</td>
<td>2364</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(423,424)</td>
</tr>
<tr>
<td>Epidermin</td>
<td>S. epidermidis</td>
<td>2164</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(3)</td>
</tr>
<tr>
<td>Mersacidin</td>
<td>Bacillus ssp.</td>
<td>1825</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(68,69)</td>
</tr>
<tr>
<td>Lacticin 481</td>
<td>L. lactis</td>
<td>2901</td>
<td></td>
<td>+</td>
<td>+</td>
<td>(413)</td>
</tr>
<tr>
<td>Salivaricin A</td>
<td>Streptococcus salivarius</td>
<td>2315</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(449)</td>
</tr>
<tr>
<td>Streptococcin A-FF22</td>
<td>Streptococcus pyogenes</td>
<td>2795</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(225)</td>
</tr>
<tr>
<td>Cytolysin Lα/Lβ</td>
<td>Enterococcus faecalis</td>
<td>4164/2631</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(154)</td>
</tr>
<tr>
<td>Lacticin 3147 A/B</td>
<td>L. lacti</td>
<td>3322/2847</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(453)</td>
</tr>
<tr>
<td>Staphylococcin C55α/C55β</td>
<td>Staphylococcus aureus</td>
<td>3339/2993</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(364)</td>
</tr>
</tbody>
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## Class II – unmodified bacteriocins

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Producer strain</th>
<th>Mass (Da)</th>
<th>Leader type</th>
<th>Processing and transport</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No leader/other leader</td>
<td>GG-type</td>
<td></td>
</tr>
<tr>
<td>pediocin PA-1</td>
<td><em>Pediococcus acidilactici</em></td>
<td>4629</td>
<td>+</td>
<td>+</td>
<td>(198,332)</td>
</tr>
<tr>
<td>leucocin A</td>
<td><em>Leuconostoc gelidum</em></td>
<td>3390</td>
<td>+</td>
<td>+</td>
<td>(187,397,530)</td>
</tr>
<tr>
<td>mesentericin Y105</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>3868</td>
<td>+</td>
<td>+</td>
<td>(9,194,195)</td>
</tr>
<tr>
<td>sakacin A</td>
<td><em>Lactobacillus sakei</em></td>
<td>4306</td>
<td>+</td>
<td>+</td>
<td>(13,14)</td>
</tr>
</tbody>
</table>

### Class IIa

<table>
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<tr>
<th>Peptide</th>
<th>Producer strain</th>
<th>Mass (Da)</th>
<th>Leader type</th>
<th>Processing and transport</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>lactococcin A</td>
<td><em>L. lactis</em></td>
<td>5778</td>
<td>+</td>
<td>+</td>
<td>(216,528)</td>
</tr>
<tr>
<td>lactococcin 972</td>
<td><em>L. lactis</em></td>
<td>7500</td>
<td>+</td>
<td>(QA-site)</td>
<td>ND (327,329)</td>
</tr>
<tr>
<td>lactococcin Q</td>
<td><em>L. lactis</em></td>
<td>5926</td>
<td>+</td>
<td>ND</td>
<td>(144)</td>
</tr>
<tr>
<td>Blp</td>
<td><em>S. pneumoniae</em></td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>(97,307)</td>
</tr>
</tbody>
</table>

### Class IIb

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Producer strain</th>
<th>Mass (Da)</th>
<th>Leader type</th>
<th>Processing and transport</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>lactococcin G α/β</td>
<td><em>L. lactis</em></td>
<td>4346/4110</td>
<td>+</td>
<td>+</td>
<td>(373)</td>
</tr>
<tr>
<td>Mutacin IV NlmA/NlmB</td>
<td><em>S. mutans</em></td>
<td>4169/4826</td>
<td>+</td>
<td>+</td>
<td>(278,424)</td>
</tr>
<tr>
<td>termophilin 13 ThmA/ThmB</td>
<td><em>Streptococcus thermophilus</em></td>
<td>5776/3910</td>
<td>+</td>
<td>ND</td>
<td>(316)</td>
</tr>
<tr>
<td>CibAB CibA/CibB</td>
<td><em>S. pneumoniae</em></td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>(168,193)</td>
</tr>
</tbody>
</table>
**Introduction**

**Class I - lantibiotics**

Bacteriocins of the class I were named lantibiotics because they contain unusual amino acids *i.e.* lanthionine (Lan) and/or methyllanthionine (MeLan). Additionally these bacteriocins can contain unsaturated amino acids such as 2,3-didehydroalanine (Dha) and/or (Z)-2,3-didehydrobutyryne (Dhb), as well as structures such as lysinoalanine, β-hydroxy-aspartate, D-alanine, 2-oxobutyrate, 2-oxopropionate, 2-hydroxypropionate, S-aminovinyl-D-cysteine, and/or S-aminovinyl D-methylcysteine (67,553).

The inactive form of the lantibiotic prepeptide is generally called LanA (“Lan” is a general abbreviation for proteins involved in lantibiotics biosynthesis). The leader sequence serves as recognition and a redirection site of the prepeptide to dedicated modification(s) (LanBC or LanM and/or LanD) and transport (LanT) proteins. The LanBC or LanM enzymes initiate the amino acid modifications, which results in the Lan and MeLan residues. Modifications occur as follows: serine and threonine residues of the propeptide are dehydrated to Dha and Dhb, respectively, by LanB or LanM. Subsequently, in the propeptide, lanthionine or methyllanthionine might be formed by a cyclization reaction of Dha or Dhb with a cysteine residue performed by either LanC or LanM (Fig. 2). Once the dedicated modification enzymes have posttranslationally transformed the C-terminus of the peptide, the peptide is released from the cell by an ABC transporter (LanT) with or without the N-terminus. Such a peptide becomes an active lantibiotic, once the leader sequence is removed by a dedicated protease (LanP) or a LanT variant that contains a protease domain (Fig. 2 and 3). The leader peptide is cleaved off behind characteristic GG, GA, GS, GI, or PR or PA cleavage sites. However, some lantibiotics are processed in other, more uncommon sites (67,163,553).

In addition to the modifications carried out by LanB and LanC or LanM, some lantibiotics possess a LanD enzyme that performs the oxidative decarboxylation of LanA. The LanD proteins oxidize and decarboxylate the C-terminal cysteine residues, before they are coupled with Dha or Dhb, forming S-[(Z)-2-aminovinyl]-D-cysteine (AviCys) or to S-[(Z)-2-aminovinyl]-3S)-3-methyl-D-cysteine (AviMeCys) (291-296,311).

Based on their structure, lantibiotics are divided into type A (elongated peptides), type B (globular peptides) and type C (multi-component peptides) (203). Lantibiotics can be further classified according to the enzymes used for their modifications. Thus, there are lantibiotics that use two distinct proteins, LanB and LanC, and others that use only one protein, namely LanM, which combines the function of both LanB and LanC (67,163,553).

**Class IA of lantibiotics**

The IA class includes elongated lantibiotics modified by both the LanB and LanC proteins or only by LanM. Nisin (161) (Fig.2), subtilin (159,160), epidermin (3), gallidermin (252), staphylococcin T (146), mutacin 1140 (206), mutacin B-Ny266 (358), mutacin I and III (422,422,423), Pep5 (244) and epicidin 280 (197) are modified by LanB.
Chapter 1

and LanC. The LanM protein transforms bacteriocins such as lactocin S (480,481), plantaricin C (521) and nukacin ISK-1 (460).

Figure 2. Posttranslational modification process of l antibiotics based on a representative bacteriocin i.e. nisin A. The prepeptide NisA is synthesized; subsequently, NisB catalyzes dehydration of underlined serine and threonine residues, S and T, respectively, which is followed by a cyclization, in the prepeptide part of the NisA, carried by NisC. In the cyclization reaction the Lan and/or MeLan are made. After that, NisP proteolytically removes the leader peptide and a mature nisin is formed.

Nisin, produced by Lactococcus lactis, is one of the best-characterized lantibiotics. Nisin has been used successfully for over 45 years in the food industry as a preservative (105). There are three known forms of nisin namely nisin A, nisin Z and nisin Q. The two latter forms differ from nisin A by their amino acid sequence, namely nisin Z by one amino acid and nisin Q by four (104,359,568). The nisin biosynthetic gene cluster, located on a conjugative transposon, is composed of genes encoding proteins involved in synthesis of the structural peptide (NisA; Nis, is the abbreviation for proteins engaged in nisin production), nisin modification (NisB and NisC), transport (NisT), processing (NisP), regulation (NisR and NisK) and immunity (NisI, NisF, E and G). The NisBTC proteins
I
ntroduction

Form the modification and transport membrane-associated complex (67,70,306). The structural prepeptide of nisin (NisA; Fig. 2) is composed of 57 amino acid residues. The mature and active form of nisin has 21 common amino acids, 1 lanthionine, 4 methylanthionines, 1 dehydrobutyryne, and 2 dehydroalanines, making a total of five lanthionine rings. A schematic representation of the posttranslational modifications and processing of nisin is shown in Fig. 2.

Class IB of lantibiotics

The type IB includes globular lantibiotics such as mersacidin (68,69), cinnamycin (143,243), duramycin, duramycin B and C (143), lacticin 481 (413), salivaricin A (449), streptococcin A-FF22 (225), butyrvibriocin OR79A (246), variacin (420), mutacin II (556) and actagardine (also known as gardimycin (254)). These lantibiotics have single LanM protein to catalyze dehydratation and cyclization reactions. Mersacidin is produced by Bacillus strains and contains three MeLan rings and one Dha, and AviMeCys. Interestingly, mersacidin and cinnamycin do not have a typical processing site, instead these lantibiotics are cleaved off after an EAA and AFA site, respectively (34,548).

Class IC of lantibiotics

The representatives of type IC are two-component lantibiotics, e.g. plantaricin W (214), staphylococcin C55 (364), cytolysin (154), BHT-A (223), haloduracin (342) and lacticin 3147 (453). The two-component lantibiotics require both peptides for their full antimicrobial activity. This group of lantibiotics generally uses the LanM type of modification enzyme and often each of the prepeptides has its own LanM protein. The prepeptides are usually designed as LanA1 and LanA2, and the mature versions are named Lanα and Lanβ. Generally, all α peptides described here have three rings and β peptides have two, three or four (325). Of the multi-component lantibiotics, cytolysin is atypical since it is also active against eukaryotic cells including erythrocytes and the prepeptides are processed twice in order to establish their activity (91). It seems that plantaricin W and haloduracin also may require double processing (214,342).

Modifications, processing and transport of lantibiotics

The LanBCT/LanMT proteins form the lantibiotic synthetase complex. The Lan and/or MeLan are formed, in two steps, by either LanB and LanC or LanM (Fig. 2). First, the hydroxyl of serine and threonine residues is dehydrated forming the α, β-unsaturated amino acids, Dha and Dhb, respectively. Second, a thioether bridge might be formed by joining a sulfhydryl group of a cysteine residue with a double bond of either Dha or Dhb (Fig. 2). The LanB dehydratases do not show sequence similarity to other proteins. They demonstrate rather low sequence identity, of approximately 30%, to each other, when the prepeptides are not analogous. The LanC cyclases are zinc metalloproteins, of which the C-terminus shows roughly 27% sequence identity to LanM. The LanB, LanC and LanM
proteins have low substrate specificity. In other words, the Lan enzymes can modify the C-terminus of any lantibiotic or nonlantibiotic, the only requirement is that propeptide is fused to a leader sequence of a dedicated Lan enzyme (66,67,123,163,265,393,553).

Generally, lantibiotics are transported out of the cell by a dedicated LanT ABC transporter (Fig. 3). Nevertheless, some lantibiotics such as Pep5 and epicidin 280 likely do not require LanT for secretion (67,197,346). The LanT of nisin (NisT) has low substrate specificity because it can transport modified and unmodified bacteriocins and non-bacteriocin peptides (282). LanP is a subtilisin-like serine protease that cleaves the leader sequence from the prepeptide and unlike LanT, LanP exhibits high substrate specificity. The LanP enzyme of nisin (NisP) removes the leader peptide only from the prenisin with already formed thioether rings (Fig. 2 and 3).

Figure 3. Schematic overview of the regulation and production of class I (on the left) and class II (on the right) bacteriocins. HK, histidine kinase; RR, response regulator; ABC, ABC transporter; AP, accessory protein; IM, immunity protein; LanB, dehydrogenase; LanC, cyclase; LanP, protease; LanT, transporter; LanFEG, immunity proteins. The numbers next to the arrows indicate processes as follow: (1) regulation by TCS, i.e. RR and HK, as it is marked in this figure or by a non-TCS (single) regulator; (2) expression of the bacteriocin locus genes; (3) synthesis; (4) processing and export; (4a) modifications and export (by LanBCT as shown in this figure or by LanMT), and processing; (5) immunity.
An exceptional processing occurs for cytolysin, the two-peptide lantibiotic. Here, each of the peptides is processed twice, namely the first time by a LanT protein (CylT) at the transport stage and the second time by LanP (CylP) outside the cell. Lantibiotics such as lacticin 481 and mutacin II do not have a dedicated LanP enzyme but instead they are processed by a LanT protein, which combines function of an ABC transporter and protease because it contains an N-terminal protease domain (67,123,163,393,553).

The LanD proteins introduce unusual amino acids, namely AviCys or AviMeCys, to the C-terminus of some lantibiotics, *e.g.* epidermin, gallidermin, mersacidin and mutacin 1140. The LanD enzymes contain a noncovalently bound cofactor, either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). The LanD of epidermin (EpiD) has low substrate specificity in contrast to LanD of mersacidin (MrsD), which is able to modify only mersacidin.

A number of other modifications have been shown to occur in lantibiotics, *e.g.* lacticin 3147 and lacticin S have γ-Ala, which is introduced by the LanJ enzyme. Less common modifications include erythro-3-hydroxy-L-aspartic acid in cinnamycin and duramycin, head-to-tail lysinoalanine bridge in cinnamycin, and in cypemycin bis-methyletion, and allo-isoleucine (67,123,163,393,553).

**Class II, unmodified peptides**

Bacteriocins of the class II are non-lanthionine containing peptides and, unlike lantibiotics, they do not require posttranslational modifications in order to be antimicrobially active. Commonly, the gene cluster of the class II bacteriocins is composed of a structural gene(s) encoding a precursor peptide, one or two dedicated ABC transporters often containing a protease domain, optionally a protease, an immunity gene(s) and an accessory protein. The unmodified bacteriocins become active through the following process: the peptide is secreted and then the precursor peptide’s N-terminus leader is cleaved off behind the GG cleavage site by the protease, although not always in this order. The accessory proteins are thought to be important in bacteriocin translocation and/or processing (Fig. 3). However, their exact role is still being investigated. The class II of bacteriocins is divided into three subclasses, *i.e.* pediocin-like peptides (IIa), miscellaneous peptides (IIb) and multi-component peptides (IIc) (114,121,131,203,368).

**Class IIa, the pediocin-like peptides**

The class IIa consists of more than 20 bacteriocins and includes *e.g.* leucocin A (187,397), sakacin A and P (14,211,222,511), curvacin A (510), mesentericin Y105 (194,195), pediocin PA-1 (198,332), enterocin A and P (15,59,75), divergicin (212), carnobacteriocin B2 and BM1 (425), acidocin A (248), listriocin 743A (245), bacteriocin 31 (514), and enterocin SE-K4 (126). The characteristic feature of this subclass is a “pediocin box”, YGNGV/L(x)C(x)₄C(x) (x, stands for any amino acid), in the N-terminal part of the propeptide. Each of two cysteine residues of the pediocin box forms, with a
dedicated residue of the C-terminus, a disulfide bridge. Moreover, all of the bacteriocins from this class display strong antilisterial activity (121,131,368).

Most bacteriocins are secreted by a dedicated ABC transporter, though some of the class IIa bacteriocins e.g. enterocin P (75), mesentericin Y105 (36), carnobacteriocin B2 (343) and divergicin (558) are transported by the Sec-dependent translocation system. Accordingly, these four peptides have the N-terminal leader sequence of the Sec-system and not the common leader peptide with a GG cleavage site (121,131,374).

Class IIb, miscellaneous peptides

This subclass combines all peptides other than the pediocin-like and the two-peptide bacteriocins. There are more than 30 miscellaneous peptides e.g. lactococcin A (216,528), lactococcin B (527), lactococcin 972 (327,328), enterocin B (59), enterocin EJ97 (147), lacticin Q (144), lacticin Z (231), BHT-B (424), aureocin A70 (369), carnobacteriocin A (557) and bovicin 255 (545) and S. pneumoniae bacteriocins, BlpM and BlpN (97,307). Some of the enterocins produced by Enterococcus species, i.e. L50A, L50B, Q and EJ97, and aureocin A70, BHT-B, lacticin Q and Z are produced without an N-terminal leader sequence. In addition, the three latter peptides have formylated N-terminal methionine residues and they show rather high 46% sequence identity. Aureocin A70 is an atypical bacteriocin because it is composed of four peptides encoded by four genes located in the same operon. The aureocin peptides have high sequence similarity to each other. It was shown that three of these peptides have antimicrobial activity. However, it is not known whether the antimicrobial activity of aureocin 70 is due to a synergistic work of four of them (369,374). Lactococcin 972 is a unique peptide in this subclass since the active form of this peptide is a homodimer, it has another mode of action than most of the class II bacteriocins and it is secreted by the Sec-dependent pathway (326-328).

Class IIc, multi-component peptides

The multi-component class IIc bacteriocins (two-peptide bacteriocins) are those that consist of two very different peptides, designed as the α and β, and both peptides need to act synergistically for full antimicrobial activity. More than 15 two-peptide bacteriocins have been isolated and described. Examples of the class IIc include lactococcin M (355), lactococcin Q and lactococcin G (373,569), mutacin IV (424), plantaricin E/F and plantaricin J/K (188,353), plantaricin S (237), lactacin F (360,361), leucocin H (37), enterocin 1071 (18), enterocin L50 (76), brochocin-C (479), acidocin J1132 (497,498), termophilin 13 (316) and S. pneumoniae CibAB (80,168). In almost all cases the two peptides of each bacteriocin have one or two GxxxG motifs in their C-terminus. These motifs are commonly involved in helix-helix interactions in membrane proteins. It has been shown that because of the GxxxxG motif, peptide α interacts with peptide β forming a helix-helix structure (149,368,374). Generally, the α and β peptides of the same bacteriocin do not show amino acid sequence similarity to each other or to other two-peptide bacteriocins.
Nevertheless, the α and β peptide of enterocin L50 share more than 70% identity to each other; and the α and β peptide of enterocin 1071 and lactococcin G show significant similarity of above 60%. Interestingly, the β peptides of mutacin IV, termophilin 13 and lacticin F show sequence homology to each other and to the α peptides of termophilin 13 and mutacin IV. Notably, acidocin J1132 is an atypical bacteriocin because the α and β peptide are transcribed from one gene and thus, it is unclear whether acidocin J1132 should be qualified as a two-peptide or a one-peptide bacteriocin (149,368,374).

**Mode of action of AMPs**

All characteristic features of bacteriocins such as their small size, amino acid sequence structure, cationic charge, hydrophobicity and amphipathicity, determine their mode of action. In general, the antimicrobial activity of AMPs is due to their action towards either the bacterial cell membrane (pore formation) or synthesis of peptidoglycan, or other mode of actions.

**Pore formation**

The majority of cAMPs, *e.g.* LL-37, most of the lantibiotics and class II bacteriocins, forms pores in the cytoplasmic membrane of sensitive cells. The attraction of bacteriocins to bacterial membranes is enhanced by the fact that both have opposite charge, *i.e.* bacteriocins are cationic and membranes are anionic. Through these pores, which can be up to three nm wide, the efflux of ions and small molecules occurs. Additionally depletion of ATP and dissipation of pH and/or membrane potential might take place. The mechanism that causes membrane permeation can be divided into three models namely the barrel-stave, carpet or toroidal-pore (47,196,352). Each of the models starts with the attraction of a bacteriocin to the membrane, followed by attachment of a bacteriocin and interaction with lipid bilayers. Once a threshold amount of the bacteriocin is reached, the peptides begin with their insertion and membrane permeabilization. In the barrel-stave model, the attached peptides aggregate and install themselves into the membrane bilayer in such a way that the hydrophobic peptide regions are aligned with the lipid core region and the hydrophilic peptide regions form the interior region of the pore. In the carpet model, bacteriocins disrupt the membrane by orienting themselves parallel to the surface of the lipid bilayer forming an extensive layer of a carpet. Hydrophilic regions of the peptides are on the side of the pore and hydrophobic ones are directed in the lipid region. In the toroidal-pore model, bacteriocins enter the membrane and induce the lipid monolayers to bend so that both bacteriocins and the lipid head groups line the water core. Here also the hydrophilic regions of bacteriocins face the pore (47,562). Lacticin Q, a class II bacteriocin produced by *L. lactis* species (144), exhibits an unique pore formation model named “huge toroidal-pore”, which occurs as follows. Briefly, lacticin Q binds to the negatively charged bacterial membrane, after which it forms the largest pore described so far of 4.6 up to 6.6 nm. The large pores cause protein leakage from the susceptible cell and a lipid transbilayer
movement named flip-flop, during which lacticin Q migrates to the inner side of the bacterial membrane. This bacteriocin does not require a target or docking molecule and it is active in nanomolar amounts. Until now, lacticin Q is the only example of a bacteriocin produced by Gram-positive bacteria, which in nanomolar concentration range is able to form huge pores, protein leakage, lipid flip-flop and the bacteriocin translocation to inner side of the membrane (563-565).

Interestingly, some cAMPs, e.g. nisin, may require a docking molecule for their full antimicrobial activity. Nisin primarily uses lipid II as a docking molecule. This is the most known and common target for bacteriocins with this mode of action (533). Lipid II is a precursor for peptidoglycan, and thus the bacterial cell wall synthesis, because it carries the subunit components of the cell wall across the bacterial membrane, i.e. N-acetylglucosamine (GlcNAc)-N-acetylmuramic acid (MurNAc)-pentapeptide. The single molecule of lipid II is composed of one GlcNAc-MurNAc-pentapeptide subunit linked to a polyiosoprenoid anchor of 11 subunits long, via a three pyrophosphate moieties (45,46,49). Nisin binds to the pyrophosphate molecules of lipid II forming the so-called pyrophosphate cage (220,550). Briefly, one molecule of nisin first binds to the lipid II, which generates docking sites for other nisin molecules. Nisin’s first two lanthionine rings (A and B) interact with the pyrophosphate of lipid II (220). Subsequently, the pore complex in the cell membrane is formed and it constitutes of eight nisin and four lipid II molecules. Additionally to membrane permeabilization, nisin inhibits cell wall synthesis by binding to lipid II (45,46,49,185,549,552).

Some, if not all, two-component lantibiotics, e.g. lacticin 3147, use a mode of action similar to nisin with lipid II as a docking molecule. Shortly, first the A1 peptide of lacticin 3147 binds to lipid II, which is followed by binding of the A2 peptide and subsequent pore formation. In this case, the pore complex is composed of four molecules of each: A1, A2 and lipid II. This complex is analogous to that of nisin, in which there are also eight bacteriocin molecules binding to four lipid II molecules (43,551). Interestingly, the bactericidal activity of the two-peptide bacteriocins is higher, when both components are involved in generating lipid II binding and permeabilization.

Class IIa and IIb pore forming bacteriocins, e.g. lactococcin A and lactococcin B, enterocin P, mesentericin Y105 and sakacin A, use another docking molecule, namely the mannose phosphotransferase system (man-PTS). It was shown that lactococcin A employs the membrane-located proteins IIC and IID of man-PTS system to recognize a sensitive cell and subsequently forms pores in the membrane (95,115,195,429,430,561).

Inhibition of cell wall synthesis

Lantibiotics such as mersacidin, actagardine, epidermin, gallidermin, staphylococcin T and mutacin 1140 trigger bactericidal effects by inhibition of peptidoglycan synthesis via binding to the lipid II but do not form pores. Epidermin, gallidermin, staphylococcin T and mutacin 1140 have similar ring structures as nisin and
they bind in a similar manner to lipid II. However, they are too short to be able to form pores in the bacterial cell membrane. Consequently, by binding to lipid II these lantibiotics remove lipid II from the site of peptidoglycan synthesis, *i.e.* the cell division site, and segregate lipid II in a process named sequestration (186). Mersacidin, actagardine, plantaricin C and lacticin 3147 are lantibiotics, which block the transglycosylation step of peptidoglycan synthesis by binding to the lipid II. These peptides bind to all three subunits of lipid II, *i.e.* GlcNAc, MurNAc and pyrophosphate (48). Thereby, the binding is different from that of nisin, epidermin, gallidermin, staphylococcin T and mutacin 1140, which all bind to the pyrophosphates of lipid II (220,550). Importantly, Ca\(^{2+}\) ions improve the bactericidal activity of mersacidin, plantaricin C and lacticin 3147 by facilitating the interaction of the peptides with the cell membrane and with lipid II (40,551).

Lactococcin 972 is a non-lantibiotic that has an unusual mode of action. It inhibits cell division by blocking septum formation. Thus, lactococcin 972 is active only against dividing cells and causes cell elongation and broadening. This bacteriocin binds to lipid II and additionally inhibits the activity of two enzymes that use lipid II as a substrate namely PBP2 and FemX thereby lactococcin 972 inhibits polymerization of the peptidoglycan. It has been shown that lactococcin 972 most likely has a different lipid II binding site than that of nisin and mersacidin meaning that a novel, third binding motif may be used by bacteriocins (326-328).

### Other mode of actions

It is worth mentioning that nisin, subtilin and sublancin have additional modes of action. These lantibiotics are able to inhibit germination of spores from *Bacillus* and *Clostridium* species (394,433). Other lantibiotics, *i.e.* cinnamycin and duramycin, induce hemolysis of erythrocytes, inhibit phospholipase A2 or interfere with leucotriene and prostaglandin synthesis in addition to their bactericidal activity. Furthermore, cinnamycin can inhibit bacterial ATP-dependent protein translocation and calcium uptake, and duramycin inhibits chloride transport and sodium and potassium ATPase (363,365,490,560). All together, the mode of action of bacteriocins may be also other than bactericidal, indicating an important role for these small peptides in the lifestyle of bacteria.

### Self-immunity to produced bacteriocins

All AMPs producers are resistant to their own product. Although the structure, production, modification and mode of action of bacteriocins are relatively well studied, the self-protection mechanism to cognate bacteriocins is still not well understood.

For most of bacteriocins, the immunity gene(s) is located either in the same operon as the structural bacteriocin gene or in close vicinity. Generally, self-immunity to bacteriocins of class I consists of a single protein LanI, *e.g.* for cytolysin and Pep5 (82,346), and/or an ABC transporter composed of LanFEG, *e.g.* in case of mersacidin (162). The LanI and LanFEG proteins can act together or separately. A fourth uncommon
immunity protein namely LanH is an accessory molecule of the LanFEG ABC transporter and it is found in the cluster of nukacin ISK-1, epidermin and gallidermin (6,7,136,384-386,405). Usually, gene clusters of class II bacteriocins have one gene encoding an immunity protein.

Nisin producers use two membrane bound, autonomous immunity systems, \textit{i.e.} LanFEG (NisFEG for nisin) and LanI (NisI associated with nisin) (173,426,475,488). However, both systems are necessary for complete immunity towards nisin. It was shown that NisI is important to interact with nisin and that 21 amino acids of the C-terminus of NisI are involved in this process (500). Accordingly, it has been proposed that NisI recognizes nisin and that NisFEG exports the peptide (120,272,489,500). The exact protective action of lantibiotics’ immunity proteins is not yet well understood. It was suggested that LanI-type proteins either aggregate lantibiotics to prevent pore formation or, for the bacteriocins that bind to lipid II, LanI might compete for lantibiotic-lipid II interaction. Additionally the ABC transporter, LanFEG, of lantibiotics targeting lipid II could possibly separate a peptide from its target and export it outside the cell (120).

Nukacin ISK-1 requires the LanFEG (NukFEG) and LanH (NukH) proteins for full immunity. The LanH protein of nukacin ISK-1 (NukH) is membrane located and is able to bind to nukacin ISK-1 and bacteriocins structurally similar to nukacin. It was shown that NukH captures nukacin molecules and transfers them to the ABC transporter (NukFEG) in an energy-dependent manner. NukH recognizes unusual amino acids in the C-terminus of nukacin and binds to the bacteriocin by a disulfide bridge. Importantly, nukacin ISK-1 related lantibiotics of class IA can be recognized by NukH indicating that the immunity protein recognizes the ring pattern on the lantibiotics (384-386).

The immunity protein of class IIa bacteriocins is located in the cytosolic part of the bacterial cell and does not interact considerably with the membrane, which is in contrast to the immunity protein of bacteriocins from class IIb and IIc, which is associated with the bacterial membrane (139,239,372,536). The specificity of the immunity proteins of class IIa bacteriocins is similar to that of LanI and is determined by the C-terminal part of these proteins (239). It is not yet well understood how the process of self-protection for class IIa bacteriocins is determined since the immunity protein does not interact specifically with the bacteriocin (139). It is speculated that the immunity protein might either block pores in the bacterial membrane formed by a bacteriocin, or interact with the putative receptor for a bacteriocin, as is the case for other class IIa members namely lactococcin A (139,536). Lactococcin A uses the IIC and IID proteins of man-PTS system as a target. The immunity protein of lactococcin A (LciA) binds to the targets forming a strong complex and thereby preventing bactericidal action of the bacteriocin. The complex is formed only in the presence of lactococcin A or during the bacteriocin production. This mechanism of self-immunity was proposed also for other class II bacteriocins including some of class IIa such as enterocin P and sakacin A (95,115,195).
Introduction

The immunity protein of the two-component non-lantibiotic bacteriocins, e.g. lactococcin G and enterocin 1071, requires an unknown cytosolic compound in order to protect the bacterial cell. Importantly, it was shown that the immunity protein of lactococcin G, namely LagC, is able to recognize each peptide, i.e. α and β, of the two-component bacteriocin (387).

Some of the members of a CAAX amino-terminal protease family, also known as the Abi (an abortive infection) family, confer a novel self-immunity mechanism to bacteriocins of class II, e.g. plantaricin EF and JK, Blp-bacteriocins, streptolysin S and sakacin 23K (96,112,257,307,377,379). Because the Abi family proteins are not yet well studied in prokaryotes, in contrast to eukaryotes, little is known about their function and mechanism of protection in these organisms. The Abi group consists of putative membrane-bound metallopeptases that share three conserved motifs in their amino acid sequence. It is thought that the motifs are the active site of the proteases (112,119,257,401). Notably, the Abi immunity proteins of plantaricin EF and JK, and sakacin 23K conferred cross-immunity against each other’s bacteriocins. It is suggested that the CAAX proteases recognise and protect, most likely by a proteolytic cleavage, a common receptor(s) or pathway(s) in these bacteriocins producer strains (257).

In general, immunity proteins are very specific to their corresponding bacteriocin and they do not show amino acid similarity to other immunity proteins even when the bacteriocin peptides are alike, which makes it difficult to identify them. However, there are exceptions: the amino acid sequence of the sakacin A and curvacin A peptide is different but their immunity proteins are similar (211,510). Additionally the immunity proteins do not confer cross-immunity to other bacteriocins from the same or other class of bacteriocins. However, the immunity proteins of some lantibiotics, e.g. Pep5 or epidermin, give cross-immunity to other related lantibiotics, namely epicidin 280 or gallidermin, respectively (209,391). Moreover, there are a few class IIa bacteriocins, of which immunity proteins may provide some protection against bacteriocins from the same class (368).

Regulation of bacteriocins production

Mostly, bacteriocins are produced either under specific environmental condition(s) or in a defined bacterial growth stage, often from mid exponential to stationary growth phase, or as response to an extracellular signal. Regulation of some bacteriocins, e.g. lacticin 481 (207), nukacin ISK-1 (5,8,459,460), mutacin II (421), sakacin A and P (55,111) etc., is strictly under control of an environmental signal(s), such as pH, osmotic stress, temperature and nutrition composition. Given this, it might be difficult to find expression condition(s) for putative bacteriocins. Expression of bacteriocins is under the control of either a single specific regulator or a two-component response regulatory system. Additionally transcription of bacteriocins is regulated co-ordinately with their dedicated biosynthetic and/or immunity operon(s).
Generally, the two-component response regulatory system is composed of an intracellular response regulator (RR; for lantibiotics named LanR) and a membrane bound histidine kinase (HK; for lantibiotics named LanK). In response to a specific extracellular signal, the HK protein becomes autophosphorylated and subsequently activates, by phosphorylation, its cognate RR, which after some conformational changes is able to activate or repress transcription of a target gene cluster (Fig. 3). For nisin, subtilin and salivaricin A the extracellular signal is the bacteriocin peptide itself, which acts as a peptide pheromone (284, 427, 487, 524). Thus, transcription of the bacteriocin cluster is autoregulated by its own bacteriocin-peptide pheromone, a process which is known as a quorum-sensing (284, 487). Quorum-sensing in bacteria is a type of coordinated gene expression in response to the local density of its own population. Uninduced bacteriocin producer cells make a small amount of the pheromone peptide often in an early exponential growth phase or earlier, which at a certain threshold concentration, mostly reached during exponential stage, is able to induce the HK protein. Thereby, the quorum-sensing system functions for bacteria as a cell density sensor (145, 308, 416). Generally, bacteriocin pheromones activate their own gene cluster by inducing the HK (Fig. 3). Nisin A and nisin Z induce the nisin cluster, boosting also the rr and hk genes (for nisin the genes are designed as nisR and nisK, respectively) (100). Making use of the fact that nisin is required for transcription of its own cluster and that very small amounts of the peptide are sufficient to induce transcription, a heterologous-controlled protein expression system was developed: the NICE system, which stands for nisin-controlled expression (284).

Production of the lantibiotic epidermin is likely under control of two different regulatory systems, namely the Agr (accessory gene regulator) two-component system that controls the stress response, production of many surface proteins and biofilm formation, and a single regulator, EpiQ. The Agr system controls the extracellular processing of the N-terminal leader peptide of epidermin by the LanP protease (EpiP) (255, 404, 405, 408). The EpiQ protein regulates transcription of genes involved in epidermin production, modification and immunity. This unusual regulatory system of epidermin, involving one dedicated regulator and one general two-component system, might be found for other bacteriocins, for which regulation is not yet well studied and/or understood. Comparable to epidermin, transcription of the lantibiotic mersacidin is controlled by two regulators, the single MrsR1 regulator and the two-component system MrsR2 and MrsK2. Nevertheless, in contrast to the Agr system the two regulators only control expression of the mersacidin locus. MrsR2 activates transcription of the immunity genes and MrsR1 controls biosynthesis of mersacidin (162). Regulation by only one orphan cognate regulator was shown for e.g. lactacin 3147 (340) and mutacin II (421). Transcription activation of the mutacin II operon is dependent on a dedicated regulator, MutR, from the Rgg family of (regulator gene of glucosyltransferase). In addition, transcription of the mutacin II cluster is affected by yet unknown component(s) of the medium (421, 496).
Interestingly, the lantibiotics salivaricin A, A1, A2, A3 and A4 produced by *Streptococcus salivarius* and *Streptococcus pyogenes* strains, activate their own production by interaction with a cognate two-component system. These structurally related lantibiotics can act also as the pheromone peptides and thereby, they are able to induce each other’s expression. (524,543). Notably, for bacteria, the recognition of the peptide pheromone of another or the same species enables inter- as well as intraspecies communication and apparently, salivaricins are involved in the cross-talk between the producers of these lantibiotics.

Only a few dedicated repression systems for bacteriocin production have been described and among them are those for cytolysin and plantaricin A. Production of the lantibiotic cytolysin depends on the presence of target cells, *i.e.* microbes or erythrocytes. Briefly, the cytolysin specific regulators, namely CylR1 and CylR2, repress expression of the bacteriocin’s biosynthesis cluster in the absence of target cells. However, despite this repression, the peptides of the two-component cytolysin, *i.e.* CylLs and CylLr, are produced at a low-level and both peptides form an inactive complex. Once the target cells are present, CylLr binds to phosphatidylcholine in the membrane of erythrocytes, which causes accumulation of free CylLs in the medium. Subsequently, when CylLs reaches a threshold concentration in the medium, derepression of cytolysin biosynthesis genes takes place (83,171). Expression of plantaricin A is controlled by two response regulators, *i.e.* PlnC and PlnD, and one histidine kinase, PlnB. However, contrary to cytolysin, PlnC acts as an activator and PlnD as a repressor of plantaricin transcription, and both regulators are phosphorylated by PlnB (113,440).

Production of bacteriocins in coordination with competence development might bring benefits to a bacterial competent cells, namely the uptake of DNA from non-competent cells of the same or diverse species through lysis (278). Competence is a stage for bacteria for natural genetic transformation, an ability to take up extracellular DNA from the environment of the same strain or of foreign origin. Competence, an example of the quorum-sensing system mentioned before, is a highly coordinated process. Shortly, competence in the *Streptococcus* genus is mediated by the extracellular concentration of a competence-stimulation peptide (CSP), which is sensed by a dedicated two-component system (ComDE). In response to the CSP concentration, a certain amount of genes (in *S. pneumoniae* more than 120 genes) is expressed. These genes are involved in processes such as binding, uptake of DNA and recombination, and production of bacteriocins (80,81,127,238,250,357). Consequently, production of AMPs from *Streptococcus* species, *i.e.* SmbAB (409,566), mutacin IV (276,279,531), mutacin N (174), mutacin V (403), termophilin 9 (141,142), Blp (97,307), CibAB (80,168), is coordinated by the competence development since these bacteriocin clusters belong to the competence regulon.
Regulation of the Blp bacteriocins in *S. pneumoniae* is rather complex (Fig. 4). The BlpMN bacteriocins are a part of a *blp* locus, which consists of the two-component regulatory system (BlpRH), pheromone peptide (BlpC), dedicated ABC transporter (BlpAB), bacteriocin-like peptides and immunity proteins. Regulation of the *blp* regulon might be mediated by three independent mechanisms. The first mechanism concerns a cognate two-component regulator system, *i.e.* BlpRH. Briefly, addition of synthetic BlpC to the growth medium stimulates BlpH, which after autophosphorylation, phosphorylates BlpR. Subsequently, activated BlpR induces expression of the entire *blp* locus (102,436). The second mechanism, namely the competence two-component regulatory system, ComDE, is able to upregulate, likely indirectly, only some of the *blp* genes, *i.e.* *blpZYA* involved in a production of a transport and immunity proteins for BlpMN bacteriocins (410). The third mechanism consists of the global two-component regulatory system CiaRH that regulates the *blp* locus via HtrA, which is directly controlled by CiaR, at the posttranscriptional level (98,176,334). It is thought that HtrA influences BlpMN production at the signalling level, *i.e.* by affecting BlpC peptide pheromone (98) (Fig. 4).

**Bacteriocins of the genus Streptococcus**

Species of the genus *Streptococcus* such as *S. mutans*, *S. pyogenes*, *Streptococcus rattus*, *S. salivarius*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus*...
**Introduction**

*Dysgalactiae, Streptococcus gordonii, S. thermophilus* and *Streptococcus mascedonicus*, produce a great number and diversity of bacteriocins. *S. mutans* produces a great variety of class I bacteriocins, namely mutacin I (423,424), II (280,371,421), III (mutacin 1140) (206,422), mutacin K8 (441), SmbA and SmbB (566), and B-Ny266 (358), and one class II *i.e.* mutacin IV (424). Interestingly, production of some of these bacteriocins depends on the environmental conditions since mutacin IV is biosynthesized only in planktonic cultures and mutacin I only in a biofilm-resembling conditions (424). *S. pyogenes* produces a lantibiotic such as streptococcin A-FF22 (225). *S. rattus* produces two lantibiotics, *i.e.* streptin (542) and BHT-A (223), a variant of Smb from *S. mutans* (566), and bacteriocin of class II, BHT-B (223). The streptin encoding gene was detected in 40 out of 58 strains, however, only 10% were able to produce this bacteriocin (542). Salivaricin A, B and A2 are the lantibiotics of *S. salivarius* (224,543). Salivaricin A (SalA) is active *e.g.* against most of *S. pyogenes* strains, and although 90% of these strains carry a variant of the *salA* gene, namely *salA*, still they are sensitive to SalA. Other derivatives of salivaricin A, *i.e.* salivaricin A2 to A5, are produced by *S. pyogenes, S. salivarius, S. agalactiae* and *S. dysgalactiae* (543).

The rumen dwelling *Streptococcus bovis* produces two types of class I bacteriocins namely bovicin HJ50 and bovicin 255, and bovicin-like bacteriocins were found among majority of rumen streptococci (86,545). *Streptococcus uberis*, another rumen bacterium, is a producer of a nisin variant, nisin U, which shows 78% identity to nisin (555).

Food streptococci such as *S. mascedonicus* produces the class I macedocin (151) and *S. thermophilus* produces the class II termophilins and Blp peptides (141,142,316).

In contrast to other strains of the *Streptococcus* genus, biologically active bacteriocins of *S. pneumoniae*, namely the BlpMN and the CibAB peptides, were identified only recently (97,307). However, purification of these AMPs was not successful and thus little is known about their structure, antimicrobial mode of action and mechanism of immunity. Interestingly, both bacteriocins *i.e.* the Cib and the Blp peptides show inraspecies antimicrobial activity and BlpMN additionally demonstrate interspecies activity (97,168,307). The *blp* locus demonstrates some genetical variations among different *S. pneumoniae* strains (97,307), which would result in a production of various Blp peptides. With agreement to the statement, this likely might aid the inraspecies competition among *S. pneumoniae* strains. In contrast, the CibAB bacteriocins are produced only by competent cells and they are active against those cells of the *S. pneumoniae* strain, which are non-competent, the fratricide phenomenon (168). It is known that isogenic bacteria growing under the same *in vitro* condition might demonstrate a different gene expression pattern, which is named bistability (122). Therefore, some cells might become competent and others not. However, the CibAB peptides could hypothetically be involved in inraspecies competition also between two different *S. pneumoniae* strains. Briefly, when *in vivo* two *S. pneumoniae* strains of another competence stimulating peptide type would meet (54) the strain, which first develops competence would be able to kill a non-competent
population of the other strain by means of CibAB. A similar process was described for \textit{S. gordonii} (202) and it was proposed that this phenomenon occurs generally among streptococcal species (80).

\textit{The scope of this thesis}

Bacteriocins are well described among the Gram-positive bacteria including a variety of AMPs produced by the genus \textit{Streptococcus}. Nevertheless, little is known about bacteriocins produced by \textit{S. pneumoniae}. Therefore, the main aim of the thesis was to find and characterize bacteriocin(s) produced by \textit{S. pneumoniae}. The thesis contributes to the complex story of bacteriocins in \textit{S. pneumoniae}. Moreover, it presents information about three new clusters likely involved in nitrogen metabolism in this bacterium. It adds data on the subject of \textit{S. pneumoniae} resistance to selected AMPs. Additionally, it contributes to development of novel lantibiotics that once might find use in food industry or in medicine.

\textbf{Chapter 2} presents the analysis of a variety of bacteriocin-like gene clusters of class I and II that occur in the genome of \textit{S. pneumoniae} strains, namely R6, TIGR4, D39, G54, CGSP14, Hungary 19A-6, Taiwan19F-14, P1031, JJA, ATCC 700669 and 70585. In total, nine bacteriocin-like clusters were described, of which two were introduced before, \textit{i.e.} the Blp (Pnc) and CibAB cluster. Among the \textit{S. pneumoniae} strains, some of the clusters are genetically identical and some show deletion/insertion mutations. Two bacteriocin-like gene clusters, \textit{i.e.} a pneumococcal peptide of unknown function (\textit{ppu}) cluster and a pneumococcin cluster, were selected for further study. \textbf{Chapter 3} describes experiments aiming to show that the \textit{ppu} cluster produces active bacteriocin, but no active bacteriocin was found to be produced by the cluster. Further, \textbf{chapter 3} describes that the expression of the \textit{ppu} cluster is reduced in a presence of a nitrogen compounds and that a negative regulator, \textit{i.e.} CodY - a branched-chain amino acid responsive regulator, controls its expression. This suggests that the cluster is involved in nitrogen metabolism. In addition, PpuR, a regulator encoded by the first gene in the \textit{ppu} cluster, has been shown to be an activator of the cluster. What is more, \textbf{chapter 3} indicates that two other clusters, for which the same function is suggested, are functionally linked to the \textit{ppu} cluster and that they might form a regulon.

\textbf{Chapter 4} describes for the first time that it is possible to produce and modify, otherwise difficult to obtain, antimicrobially active lantibiotics of \textit{S. pneumoniae}. Here, the class IA nisin production machinery, NisBTC, was used to generate, modify and secrete biologically active, previously not yet isolated and characterized pneumococcin bacteriocins of class IC, which have no sequence homology to nisin.

\textbf{Chapter 5} focuses on the response of \textit{S. pneumoniae} towards AMPs such as nisin, LL-37, and bacitracin and elucidates some resistance mechanisms to these AMPs. By use of genome-wide transcriptome analysis (a DNA microarray), the response of \textit{S. pneumoniae} to
Introduction

non-bactericidal concentrations of three AMPs, we demonstrated that for a limited number of genes, expression was changed in all conditions. Consequently, several novel ABC transporters, *i.e.* namely SP0785-0787, SP0912-0913 and SP1715, were associated with the resistance of *S. pneumoniae* to these three different AMPs. In addition, a GntR-like regulator, SP1714, was shown to regulate two of these ABC transporters. Notably, the chapter describes involvement of the *blp* locus in determining the resistance of *S. pneumoniae* D39 to LL-37.

In **chapter 6**, a summary of the thesis is provided. In addition, the most important findings and the possibility to use novel and genetically manipulated bacteriocins in medicine is discussed.