Bacteriocins of Streptococcus pneumoniae and its response to challenges by antimicrobial peptides
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Chapter 6

General Discussion
To treat infections, ancient Egyptians, Chinese and Greeks were using molds and plants that contained antimicrobial substances, although they were probably unaware of the working mechanism. However, as early as in 1877 the history of antimicrobials did begin with an observation made by Louis Pasteur and Robert Koch of an air-borne bacillus that inhibited the growth of *Bacillus anthracis* (299). Subsequently, in 1928, Alexander Fleming discovered penicillin produced by fungi of *Penicillium* spp. Nevertheless, it took more than ten years before penicillin and another antibiotic, namely gramicidin, were isolated by Ernst Chain and Howard Florey, and used commercially to treat infections (140). Since then, the search for antibiotic compounds with similar capabilities and produced by microorganisms has led to the discovery of various antibiotics and antimicrobial peptides (AMPs). Antimicrobial substances *e.g.* antibiotics and AMPs play a major role in the lives of almost all living organisms. AMPs are small proteins produced by many living organisms in order to inhibit the growth or kill microorganisms in their vicinity, while producers stay immune themselves. They contribute to the survival of the organism, protection of their ecological niche and safeguarding essential nutrients by elimination of competitors. AMPs are mostly cationic peptides and those produced by bacteria are named bacteriocins. According to their structural features, bacteriocins are divided into four classes, namely *i)* posttranslationally modified bacteriocins named lantibiotics, *ii)* unmodified peptides, *iii)* large proteins and *iv)* cyclic peptides (203). In 1925, the first antimicrobial activity due to bacteriocin was described for an antibiotic-like substance “prinicipe V” produced by a bacterium and active against bacteria (134,135). Later the substance was named “colicin”. Subsequently, in 1928, a bacteriocin that is now widely used as a food preservative was discovered and was named nisin in 1947 (336,444,544). From then on, a variety of bacteriocins have been reported to be produced by a wide range of bacterial genera.

Bacteriocins have been the subject of intense research for the last two decades because of their potential applications in food preservation and medical treatments. Bacteriocins used in food industry should meet several criteria, *i.e.* the bacteriocin producing strain preferably should be recognized as a safe one, the bacteriocin should not cause any health problems, the bacteriocin should have a broad spectrum of inhibition or have specific activity, the bacteriocin should be stable during the manufacture process and soluble, and the bacteriocin should not change the flavor of food. Food lactic acid bacteria (LAB), *i.e.* natural bacteria of fermented food products, produce a great number of bacteriocins. Currently, only two products of class I and class II bacteriocins, namely nisin and pediocin PA-1, respectively, have been used safely as a preservative for *e.g.* meat, dairy products, canned food, alcoholic drinks, salads and bakery products (90).

Since some lantibiotics are antimicrobially active at low-nanomolar concentrations against antibiotic resistant pathogens, they are considered to have an excellent potential in medical applications, see Table 1. Besides that, the unusual features of lantibiotics, *i.e.* lanthionine rings, protect them from protease activity and render them stable in a broad range of pH and heat. Nisin may have a therapeutic potential in *e.g.* treatment of
*Helicobacter pylori*, a pathogen of the human gastric mucosa, causing gastric diseases (106) and in curing *Staphylococcus aureus*, *Streptococcus pneumoniae* or *Clostridium difficile* infections, see Table 1 (106,156,470). Another lantibiotic, mersacidin, is active against *S. aureus* (MRSA) strains and vancomycin-resistant enterococci, and is already in the preclinical stage of development, (Table 1) (180). For two other lantibiotics, *i.e.* epidermin and gallidermin, preliminary clinical tests have demonstrated their potential in topical treatment of acne, a skin infection caused by *Propionibacterium acnes*, (Table 1) (252). For lacticin 3147 various clinical applications have been considered, including use in veterinary medicine and as a food preservative, (Table 1) (450-453). Cinnamycin, ancoevenin and duramycin may have medical applications in blood pressure regulation, treatment of inflammations and viral infections, see Table 1, (143). Currently, to our knowledge, only three lantibiotics have been licensed for use in clinical applications, namely nisin, lacticin 3147 and salivaricin (90). Nisin and lacticin 3147 are allowed to be used in curing animal diseases (90). Producer strains of two related lantibiotics, *i.e.* salivaricin A2 and B, are used in New Zealand as a probiotic treatment of throat infections and chronic bad breath (123). Although there are many studies concerning the successful biomedical use of lantibiotics (Table 1), government drug industrial regulators are not yet convinced of the suitability of bacteriocins as antimicrobial agents in medicine (41,89).

For several years, production of bacteriocins by a human pathogen, namely *S. pneumoniae*, has been investigated and in recent times, bacteriocin-like activities of *S. pneumoniae* proteinaceous substances have finally been elucidated (97,168,307). The activity belongs to two individual AMPs, namely Blp (also known as Pnc) and CibAB (97,168,307). The ability to produce a variety of AMPs and the activity spectrum of these AMPs may vary considerably among different *S. pneumoniae* strains (97,307). This can be explained by the genetic variability of the *blp* (*pnc*) cluster and of other bacteriocin-like clusters among *S. pneumoniae* strains described in chapter 2 of the thesis (172). Chapter 2 describes data obtained by a bioinformatic study of the putative bacteriocin-like genomic regions in *S. pneumoniae* and their comparisons in streptococci. This chapter reflects the genetic variation in those genomic regions, which is observed for at least six out of nine described bacteriocin-like clusters. It is remarkable that except Blp and CibAB no bacteriocin-like activity has been found for at least one of the described clusters. The comparative analysis of the variety and significant numbers of potentially encoding bacteriocin clusters demonstrates that bacteriocins play an important role in the lifestyle of *S. pneumoniae*. Additionally it seems that the species *S. pneumoniae* could potentially produce a wide variety of bacteriocins and the nine bacteriocin-like clusters described in chapter 2 are probably just the start of the description of the amount and diversity of the AMPs that *S. pneumoniae* strains could produce. We speculate that in order to find antimicrobial activity that is mediated by novel bacteriocins of *S. pneumoniae*, broad screening of many of the *S. pneumoniae* strains grown under a wide variety of conditions is required.
<table>
<thead>
<tr>
<th>Lantibiotic</th>
<th>Producing strain</th>
<th>Inhibitory activity of commercial interest</th>
<th>Potential Biomedical Applications</th>
<th>Clinical development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin A</td>
<td><em>Lactococcus lactis</em></td>
<td>Gram-positive Gram-negative</td>
<td>Bacterial mastitis, oral hygiene, cosmetic deodorants and topical formulations; treatment of methicillin-resistant <em>S. aureus</em> (MRSA) and enterococcal infections, peptic ulcer, enterocolitis, and lung mucus clearing (89,156,470)</td>
<td>(Pre)Clinical trials</td>
</tr>
<tr>
<td>Lacticin 3147</td>
<td><em>L. lactis</em></td>
<td>Gram-positive</td>
<td>Bacterial mastitis, oral hygiene treatment of MRSA and enterococcal infections, and acne (89,148,415)</td>
<td>ND</td>
</tr>
<tr>
<td>Gallidermin/Epidermin</td>
<td>*Staphylococcus gallinarum/<em>Staphylococcus epidermidis</em></td>
<td><em>P. acnes</em>, staphylococci, streptococci</td>
<td>Acne, eczema, folliculits, impetigo (39,89)</td>
<td>ND</td>
</tr>
<tr>
<td>Mutacin 1140</td>
<td><em>Streptococcus mutans</em></td>
<td><em>S. mutans</em></td>
<td>Prevention of dental caries, treatment of streptococcal throat infection (206,483)</td>
<td>Preclinical trials</td>
</tr>
<tr>
<td>Mersacidin/Actagardine</td>
<td><em>Bacillus subsp.</em>/<em>Actinoplanes subsp.</em></td>
<td>Staphylococci including methicillin-resistant strains, streptococci</td>
<td>Treatment of MRSA and streptococcal infections (39)</td>
<td>Preclinical trials</td>
</tr>
<tr>
<td>Duramycin</td>
<td><em>Streptomyces subsp.</em> and <em>Streptoverteillium subsp.</em></td>
<td>Inhibitor of phospholipase A2</td>
<td>Treatment of MRSA and streptococcal infections, and dry eyes syndrome and reduced mucociliary clearance (39,89)</td>
<td>Phase II clinical trials</td>
</tr>
<tr>
<td>Cinnamycin</td>
<td><em>Streptomyces cinnamoneus</em></td>
<td>Inhibitor of herpes simplex virus, phospholipase A2, angiotension converting enzyme (ACE)</td>
<td>Inflammation, blood pressure regulation, treatment of viral infection (89)</td>
<td>ND</td>
</tr>
<tr>
<td>Ancovenin</td>
<td><em>S. cinnamoneus</em></td>
<td>Inhibitor of ACE</td>
<td>Blood pressure regulation (89)</td>
<td>ND</td>
</tr>
<tr>
<td>NVB302 (modified type-B lantibiotic)</td>
<td>ND</td>
<td><em>C. difficile</em></td>
<td>Treatment of <em>C. difficile</em> Associated Diarrhoea (CDAD) (39)</td>
<td>Preclinical trials</td>
</tr>
</tbody>
</table>
Most of the time, the initiation and duration of bacteriocin production may be associated with growth conditions that resemble the natural niche of the microorganism. There are many growth condition factors that can influence bacteriocins production e.g. nitrogen, buffer, sugar, temperature, pH and/or other factors (167,398). Therefore, it might be difficult to induce biosynthesis of some AMPs, e.g. those of S. pneumoniae. In agreement with this, the influence of environmental factors, such as temperature, on the Blp bacteriocins production has been shown (307). Interestingly, some S. pneumoniae strains produce the Blp bacteriocins at 37°C, whereas others produce them at 35°C (97,307), which is the temperature of the upper nasopharynx. This niche can be colonized by S. pneumoniae and the temperature regulation of bacteriocins production might aid in intra- and interspecies competition. The growth conditions affecting bacteriocin production have also been described for other streptococci. For instance production of streptococcin AFF-22 by Streptococcus pyogenes has been shown to be affected by temperature, pH and medium composition (232). Similarly, production of some S. mutans and Streptococcus thermophilus bacteriocins depends on the type of medium (230,443). Therefore, production of novel bacteriocins by S. pneumoniae could be strictly influenced by environmental conditions and more research is needed to find the AMPs production conditions. Accordingly, many growth conditions were screened to find the one, which stimulated expression of one of the bacteriocin-like gene clusters, namely ppu, described in chapter 3. However, the ppu cluster seemed not to be involved in bacteriocin-like peptide production (chapter 3). Nevertheless, we showed that the function of the ppuRABCDE cluster is related in some way to general nitrogen metabolism in S. pneumoniae and that the cluster is under negative control of CodY, a branched-chain amino acid responsive regulator (199,484). CodY is one of the bacterial regulators, which is able to adjust globally bacterial cell metabolism to environmental changes, and additionally influences the expression of genes involved in virulence. In S. pneumoniae, CodY contributes to colonization of the nasopharynx and it regulates the expression of a broad range of genes encoding proteins involved in amino acid uptake, metabolism and biosynthesis, as well as the ppu cluster (199). We have shown that PpuR is a positive regulator of ppuABCDE and that CodY, likely by repression of the ppuR transcription, inhibits the expression of the whole ppu cluster. However, a putative operator region(s) of PpuR has not yet been identified in PppuA. Moreover, we do not know whether CodY additionally represses expression of ppuABCDE. Hence, to prove a direct regulatory effect of PpuR and CodY on the PppuA, direct binding of these proteins to the promoters needs to be performed. Additionally in chapter 3, we identified two novel clusters, i.e. prcRABCD and taaBC, which might as well be involved in nitrogen metabolism and we hypothesize that together with ppu they form a novel regulon in S. pneumoniae. Nevertheless, we do not know how the ppu cluster influences the expression of prcRABCD and taaBC, and whether the link between the three clusters is functional, regulatory or both. In a study of Hendriksen et al., expression of the whole CodY regulon, including the ppu cluster, but not prc and taa, was
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changed in a *S. pneumoniae* D39 ΔglnAP mutant (*glnAP*), genes encoding glutamine synthetase and glutamine ABC transporter (201). The data suggests that CodY does not regulate the *prc* and *taa* cluster directly, but only the *ppu* cluster. The adjustment of *ppu*, *prc* and *taa* expression in response to nitrogen sources in a specific medium is probably important for *S. pneumoniae* in order to survive in three different niches, namely the nasopharynx, lungs and/or blood stream. Nevertheless, the regulation of the *ppu*, *prc* and *taa* cluster expression and their exact roles in nitrogen metabolism is not yet well understood and will require more study.

Despite characteristic features of lantibiotics *e.g.* i) a wide variety of structures, ii) thioether rings, which stabilize the structure of lantibiotics and make them less sensitive to heat, proteases and reducing agents, iii) activity in nanomolar quantity, and iv) activity against multi-resistant microorganisms; lantibiotics are not generally approved for medical applications. There are many possible reasons for this, for instance poor solubility, a relatively narrow activity spectrum, and the possibility of resistance development and lack of suitable and cheap technology to produce lantibiotics for commercial use. However, by use of peptide engineering, many of the drawbacks can be overcome. What is more, the engineering of lantibiotics via genetic and/or chemical modifications, gives the opportunity to study structure-function relationship of lantibiotics and to develop novel, and improved peptides with *e.g.* medical potential. Diverse strategies have been described for these purposes for instance: peptide sequence modification by amino acids substitutions/deletion/insertion, the chemical modifications, the backbone cyclization and engineering of the modification enzymes. An example of peptide sequence modification is, in nisin Z, substitution of a residue in position 27 or 31 to lysine, by which the bacteriocin solubility was improved without diminishing the activity, which is of importance if the peptide is going to be commercially used (446). The effects of amino acids substitutions in a peptide sequence are difficult to predict. Although, it has been shown that mutation of amino acids involved in thioether formation usually results in a substantial decrease of activity (35,66,71,287).

Since it has been established that the specificity of the lantibiotics’ modification enzymes, *i.e.* LanBC and LanM, is relaxed and that they can modify peptides, which are fused to a dedicated leader sequence, various studies have been conducted with use of this information (66,265,439). Production of novel AMPs, or those already known, with the heterologous expression systems could be beneficial for commercial use and especially in medicine, where the engineered peptides could be used as *e.g.* a substitute of, or next to, antibiotics. However, until recently, many peptides produced with the lantibiotics’ heterologous expression systems were modified, but only closely related peptides still had an antimicrobial activity. To our knowledge we presented for the first time (chapter 4) the successful expression, modification, secretion and biological activity of novel unknown, not closely related to nisin, class IC lantibiotics of *S. pneumoniae* (pneumococcins; PneA1 and PneA2) by the nisin synthetases, *i.e.* LanBTC, which normally produce nisin, a member of
the class IA lantibiotics (Fig. 1). The PneA1 and PneA2 peptides were antimicrobially active but only against *Micrococcus flavus* (Fig. 1), which is sensitive to most known AMPs. The approach used in chapter 4 to ‘awaken’ otherwise difficult to obtain novel lantibiotic could be successfully used for other unknown AMPs. Moreover, in chapter 2, screens through the *S. pneumoniae* genomes in search for putative, novel, bacteriocin encoding genes showed that there is a great variety of them. Consequently, these many unknown AMPs could reveal new modes of action, a broader spectrum of activity and/or unusual structures. What is more, production of those AMPs with the use of the lantibiotics’ heterologous expression systems, could enable selection of “improved” peptides that could putatively be used in medicine as therapeutic agents e.g. used as substitutes of, or next to, antibiotics (87,348).

![Figure 1. Antimicrobial activity of trypsinated chimeric peptides (pneumococcins, i.e. PneA1 and PneA2) and the controls (BSA, buffer and nisin) in the agar diffusion assay against *M. flavus*. A culture of *M. flavus* was mixed with medium and poured into plates to solidify. Subsequently, in the solidified medium holes were made and filled with fourfold dilution of various substances. Continuing fourfold dilution for each substance is shown in six holes divided over two rows. The directions of the dilutions, for each row, are marked below the name of the tested substance.](image)

The resistance of some bacteria to commonly used antibiotics is on the rise. Therefore, it is not only important to find alternatives for antibiotics but it is also of great interest to better understand the resistance mechanisms of bacteria, which would possibly help to develop new or modify existing surrogates for antimicrobials e.g. AMPs. Following this idea, in chapter 5 we investigated the response of *S. pneumoniae* to challenges by three distinct AMPs, i.e. bacitracin, nisin and LL-37. A few transporters, namely SP0912-0913, SP0785-0787 and SP1715, and some putative immunity proteins of the Blp bacteriocin cluster were identified as those involved in resistance of *S. pneumoniae* to the examined antimicrobial substances such as bacitracin, nisin, LL-37, Hoechst 33342, gramicidin
and/or lincomycin. Surprisingly, we found out that in *S. pneumoniae* D39, SP1715 was involved in sensitivity to LL-37 on one hand and on the other hand in resistance to bacitracin and Hoechst 33342. The reason for that is not known but we speculate that the ABC transporter, SP1715, might serve as a kind of receptor for LL-37, as it is done by some bacteriocins, which use membrane-bound proteins as a docking molecule. It has been shown that LL-37 is a pore-forming molecule, but whether it binds to a receptor has still to be established. Interestingly, a novel regulator, *i.e.* SP1714, was identified and associated with negative regulation of its own promoter and two ABC transporters, namely SP0785-0787 and SP1715. The exact function of SP1714 remains to be determined. However, the transcriptome data indicated that the expression of the regulator depends on external stimuli and that SP1714 might regulate the response to a wide variety of toxic components, most likely via an additional regulatory mechanism. The findings in **chapter 5** extend the understanding of defense mechanisms of this important human pathogen against antimicrobial compounds and points toward novel ABC transporters, which can be used as targets for the development of new antimicrobials.

In conclusion, the ability of *S. pneumoniae* to produce a whole set of bacteriocins could improve the colonization by killing competing bacteria and could increase the availability of foreign DNA for genetic exchange. Thus, bacteriocins might be considered as one of the triggers for the evolution of *S. pneumoniae* pathogenesis. A great challenge however, is still to find out under which conditions they are being produced. What is more, bacteriocins, being used as additives important for food production, have also a great potential to be applied in medicine.