Doctoral Thesis

Genetic engineering of Staphylococcus gallinarum for overproduction of the lantibiotic gallidermin

Author(s):
Valsesia, Giorgia

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Genetic engineering of *Staphylococcus gallinarum*
for overproduction of the lantibiotic
gallidermin

A dissertation submitted to the
ETH Zurich

for the degree of
Doctor of Sciences

presented by
Giorgia Valsesia
Dipl. Natw., ETH Zurich

born on January 18th, 1977
from Contone (TI)

accepted on the recommendation of
Prof. Dr. Sven Panke (ETH Zurich, Switzerland), examiner
Prof. Dr. Brigitte Berger-Bächi (University of Zurich, Switzerland), co-examiner
PD Dr. Wolfgang Minas (Biotech Concepts GmbH, Switzerland), co-examiner

2008
Abstract

Antibiotic resistance has spread dramatically throughout the last two decades, particularly in hospitals, and it is threatening to undermine the ability to treat infections, as even drugs that once served as a last resort are losing their potency. As the number of resistant pathogens continues to grow, the number of new antibiotics to fight them is steeply declining. Therefore, the development of new antimicrobial agents is essential.

Class I lantibiotics are cationic, amphiphilic peptides produced by a wide range of Gram-positive bacteria and show bactericidal activity against other Gram-positive bacteria. They show a dual mode of action at nanomolar concentrations, which involves pore formation and inhibition of peptidoglycan biosynthesis by specific interaction with cell wall precursor lipid II. Lantibiotics are ribosomally synthesized and undergo posttranslational modifications resulting in the formation of unusual residues like the thioether amino acids lanthionine and methyllantionine, which are crucial for a proper functional structure. So far, no lantibiotic has been developed into a clinical product, even though nisin has proven its potential to treat peptic ulcers caused by *Clostridium difficile* and *Helicobacter pylori*, gallidermin is effective against acne caused by *Propionibacterium acnes* and bovine mastitis caused by *Staphylococcus aureus*, and mersacidine can treat MRSA infections in mice. The major obstacle to the clinical development of class I lantibiotics is their generally very low production titer, which is a consequence of their toxicity for the producing strains. Attempts to improve volumetric productivity have been carried out, but generally failed to provide significant increase in titer.

Gallidermin is a tetracyclic, 21 amino acids class I lantibiotic peptide, which is produced by the non-pathogenic *S. gallinarum* Tü3928, and shows broad-spectrum activity against important pathogens like *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus faecalis*, and *P. acnes*. Gallidermin (6L-epidermin) is closely related to epidermin, which is synthesized by the class II strain *S. epidermidis* Tü3298. Biosynthesis, modification, secretion, and activation of epidermin have been broadly studied. Since gallidermin and epidermin have a high degree of identity on DNA and protein level, and several genes of the two gene clusters have been shown to be interchangeable, many
assumptions on gallidermin biosynthesis and function have been made based on previous research on epidermin.

In this study, various genetic engineering strategies have been followed in order to enhance the production of gallidermin and to develop a production strain suitable for an industrial-scale production process. These strategies include circumventing the effect of gallidermin toxicity by producing an inactive precursor molecule, increasing mRNA template concentration for gallidermin overproduction, and heterologous production of gallidermin. The first strategy lead to a mutant that produced pregallidermin to a 50% higher molar titer than the wild-type *S. gallinarum*, suggesting that the absence of self-toxicity has a beneficial effect on gallidermin production. The second approach, which resulted to be very difficult due instability and recombination of plasmid vectors, did not lead to increased pregallidermin titers. Finally, among the many strains tested for pregallidermin heterologous production, only *S. aureus* produced pregallidermin in detectable quantities.

Extensive efforts are still required for the implementation of an industrial-scale gallidermin production process. Nevertheless, this study has contributed with the development of a suitable strategy for overproduction, which might be adequate to an industrial context, and elucidated the problems and drawbacks of several other genetic strategies for gallidermin overproduction.
Riassunto

Il fenomeno della resistenza agli antibiotici si è diffuso sempre più ampiamente negli ultimi decenni, soprattutto negli ospedali, e oggi minaccia di compromettere l’abilità dei medici nel trattare con successo importanti infezioni batteriche, poiché anche l’efficacia degli antibiotici, usati come ultima risorsa, sta scemando. E, mentre il numero di microorganismi patogeni continua a crescere, il numero di nuovi antibiotici per combatterli diminuisce costantemente. Per questo motivo è essenziale e necessario sviluppare nuovi agenti antimicrobici.

I lantibiotici di classe I sono peptidi cationici e anfifilici, prodotti da una vasta gamma di batteri Gram-positivi, che mostrano un’azione battericida contro altri batteri Gram-positivi. A concentrazioni nanomolari mostrano un duplice meccanismo di azione, che implica la formazione di pori nelle membrane cellulari e l’inibizione della biosintesi dello strato peptidoglicanico, grazie a un’interazione specifica con il lipide II, un precursore della parete cellulare. I lantibiotici sono prodotti della sintesi ribosomale e sono sottoposti a modificazioni post-traslazionali, che si manifestano nella formazione di amminoacidi inusuali, quali la lantionina e la metillantionina, contenenti gruppi tioeteri che sono essenziali per l’assunzione di una struttura funzionale adatta. Finora nessun lantibiotico è stato sviluppato in un prodotto clinico, nonostante siano stati dimostrati l’efficacia della gallidermina contro l’acne, causata da Propionibacterium acnes, della mersacidina contro le infezioni causate da MRSA in un modello murino e il grande potenziale della nisina nella cura delle ulcere peptiche causate da Clostridium difficile e Helicobacter pylori. Il maggior ostacolo per lo sviluppo clinico dei lantibiotici di classe I è il basso titer di produzione, dovuto alla loro tossicità verso i ceppi produttori. In passato, sono stati fatti vari tentativi per migliorarne la produttività volumetrica, senza mai sfociare in un aumento significativo della loro concentrazione finale.

La gallidermina è un rappresentante dei lantibiotici di classe I, è formata da 21 amminoacidi e presenta una struttura tetraciclica. È prodotta da S. gallinarum Tü3928, un batterio apatogeno (classe I), e mostra un’attività a largo spettro contro importanti patogeni, quali S. aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus faecalis, e P. acnes. La gallidermina (6L-epidermina) è molto simile all’epidermina sintetizzata dal ceppo di classe II S. epidermidis Tü3298. Nel caso dell’epidermina, la biosintesi, la modificazione, la
secrezione e l’attivazione sono state dettagliatamente studiate. Dato l’alto grado di identità sia a livello di DNA che proteico tra la gallidermina e l’epidermina e, essendo stato dimostrato che diversi geni dei due cluster genetici sono intercambiabili, sono state fatte molte supposizioni sulla biosintesi e sulla funzione della gallidermina, basate su precedenti ricerche sull’epidermina.

In questo studio sono state seguite diverse strategie di ingegneria genetica al fine di aumentare la produzione di gallidermina e di sviluppare un ceppo adeguato a un processo di produzione su scala industriale. Queste includono la produzione di un precursores inattivo della gallidermina così da evitare il suo effetto tossico, l’aumento della concentrazione dei trascritti dei geni del suo cluster e la sua produzione eterologa. La prima strategia ha portato alla generazione di un mutante che produce pregallidermina a concentrazioni molari del 50% più alte rispetto al wild-type S. gallinarum, suggerendo che l’assenza di tossicità ha effettivamente un effetto benefico sulla produzione di gallidermina. Il secondo approccio è risultato essere difficoltoso a causa dell’instabilità e della ricombinazione dei vettori di produzione e non ha portato a un miglioramento dei titer di produzione della pregallidermina. Infine, tra i vari microorganismi testati per la produzione eterologa di pregallidermina, solo S. aureus è risultato produrla in concentrazioni rilevabili.

Ampi studi sono dunque tuttora necessari per l’implementazione di un processo di produzione di gallidermina su scala industriale. Ciò nonostante questo studio rappresenta un importante contributo, poiché fornisce una strategia adeguata per la sovrapproduzione di gallidermina, che potrebbe essere rilevante anche in un contesto industriale, e elucida problemi e svantaggi di altre strategie di ingegneria genetica.
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Chapter 1

Lantibiotics: Biosynthesis, mode of action, applications and production

1. Abstract

Lantibiotics are small peptides with sizes ranging from 1’800 to 5’000 Da, which are ribosomally synthesized and post-translationally modified to include non-proteinogenic amino acids – lanthionines and methyllanthionines – which are responsible for their polycyclic structure. The broad, unspecific activity of lantibiotics against Gram-positive bacteria warrants their consideration as a powerful novel class of antibiotics in a context of increasing antibiotic resistance.

Increasing efforts are currently devoted to the elucidation of the mechanisms underlying the biosynthesis, mode of action, and the production of this class of antimicrobial peptides.

Here we discuss their principal characteristics and review novel developments, focusing on the application of lantibiotics and their industrial production.

2. Introduction

Gram-positive bacteria are capable of producing a wide range of antimicrobial substances, including organic acids, hydrogen peroxide, inhibitory enzymes, and bacteriocins (371, 444, 469). Bacteriocins include a plethora of antimicrobial proteins and peptides that play a major role in antagonistic interactions between the producing strains and other closely related bacteria in a ecosystem (146, 435). Four classes of bacteriocins from Gram-positive bacteria were defined by Klaenhammer (224): class I, modified membrane-active peptides (lantibiotics); class II, heat-stable, non-modified membrane-active peptides; class III, large (> 30 kDa), heat-labile proteins; and class IV, complex bacteriocins that contain essential lipids or carbohydrate moieties in addition to protein.

As a characteristic feature, lantibiotics contain unique amino acid residues from post-translational modifications that lead to intramolecular thioether bridges (383, 384). Until now, approximately 50 different lantibiotics have been isolated from Gram-
positive bacteria. The prototype lantibiotic, nisin, was discovered in 1928, when Rogers and Whittier (371) observed inhibition of lactic acid bacteria by metabolites of *Lactococcus lactis*. Nisin is being applied for the preservation of processed foods for more than 40 years, and has gained the generally recognized as safe (GRAS) status by the US Food and Drug administration (FDA) for its non-toxicity in 1988.

Lantibiotics constitute a complex set of molecules that have been intensely investigated in recent years, as they represent a valuable alternative to conventional antibiotics, which are becoming increasingly obsolete for treatment of infections caused by antibiotic resistant-pathogens. This review summarizes the principal features of lantibiotics and focuses on recent findings in their biosynthesis, mode of action, and potential applications. Moreover, various strategies for lantibiotic overproduction will be discussed.

3. Classification of lantibiotics

While the majority of the bacteriocins are linear, unmodified peptides, which may contain disulphide bonds (198), lantibiotics are small, complex post-translationally modified peptides with multiple rings and a number of dehydrated amino acids resulting in unique structural features. Their intramolecular rings are formed by the non-canonical thioether amino acid lanthionine (Lan) or 3-methyllanthionine (MeLan), which led to the designation lantibiotics (*lanthionine-containing antibiotics*). These unusual amino acids are introduced by non-ribosomal synthesis mechanisms, thereby enabling Gram-positive bacteria to overcome restrictions of the genetic code in order to establish structural diversity (383). Two lantibiotics subgroups were defined on the basis of their distinctive ring structures (204). Following this proposal, type-A lantibiotics are elongated, flexible, amphipathic molecules that are positively charged and act by disrupting bacterial membranes. In contrast, type-B lantibiotics have globular structures, carry a negative or no net charge and act by interfering with various enzyme functions (410) and as immunomodulators (115). As type A included lantibiotics with different structures, they were further subdivided into three subgroups: A (I) lantibiotics which are elongated and flexible, e.g. nisin; A (II) peptides that display a linear N-terminus and a globular C-terminal part, e.g. lacticin 481; and A (III) lactocin S and lantibiotics that are composed of two peptides (two-component bacteriocins) (384, 447). However, this classification was not generally accepted (293,
Chapter 1- Introduction

454). Moreover, recent characterization of new lantibiotics with intermediate characteristics or novel features (e.g. the two-component lantibiotics requiring the synergistic interaction of two structurally different peptides) (126, 314, 377), and the revelation that activity of type A lantibiotics is partly due to interference of enzymatic reactions such as peptidoglycan formation, have made their classification into type A and type B categories difficult (146). Therefore, lantibiotics classification schemes are currently under discussion. Guder separated lantibiotics into eight groups (the nisin, epidermin, Pep5, lactacin 481, mersacidin and cinnamycin groups, and two groups of lantibiotics with incompletely known structures) (146), whereas Twomey identified six groups of lantibiotics: nisin, lactacin 481, mersacidin, LtnA2, cytolysin and lactocin S groups (444). In a recent review, Willey (474) chose an alternative approach first suggested by Pag and Sahl (331), who proposed that two different classes of lantibiotics should be distinguished based on their biosynthetic pathways. Class I lantibiotics are modified by two separate enzymes LanB and LanC, processed by a dedicated serine protease LanP and exported by the ABC-transporter LanT (Tab.1, Fig. 1). In contrast, class II lantibiotics are modified by the modification enzyme LanM and simultaneously exported and activated by an ABC-transporter LanT(P) with an associated N-terminal protease activity (Tab.1, Fig.1). This classification accounts for differences in leader peptide sequence, biosynthetic operon structure, peptide function, and, to a lesser extent, structural characteristics (474).

In this review, we adopt this last classification scheme, as it is straightforward, comprehensive, and might show enough flexibility to allow assimilation of novel lantibiotics. Moreover, Willey proposed a novel lantibiotic class, class III, which include lanthionine-containing peptides without antibiotic activity as SapB produced by Streptomyces coelicolor (232, 412, 475), SapT from Streptomyces tendae (233), and AmfS produced by Streptomyces griseus (445). These peptides have morphogenetic activity during formation of aerial hyphae, and will not be discussed in this review.
Tab.1: Classification of representative lantibiotics

<table>
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<tr>
<th>Lantibiotic</th>
<th>Molecular mass (Da)</th>
<th>Producing strain</th>
<th>Ref.</th>
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<tbody>
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<tr>
<td>Nisin A</td>
<td>3353</td>
<td><em>Lactococcus lactis</em> NIZOR5, 6F3, NCFB894, ATCC11454</td>
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<td><em>L. lactis</em> N8, NIZO22186</td>
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<td>Subtilin</td>
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<td><em>Bacillus subtilis</em> ATCC6633</td>
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<td><strong>Class II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinnamycin</td>
<td>2042</td>
<td><em>Streptomyces cinnmoneus</em></td>
<td>(19)</td>
</tr>
<tr>
<td>Mersacidin</td>
<td>1825</td>
<td><em>Bacillus</em> spp.</td>
<td>(234)</td>
</tr>
<tr>
<td>Sublancin 168</td>
<td>3877</td>
<td><em>B. subtilis.</em> 168</td>
<td>(332)</td>
</tr>
<tr>
<td>Staphylococcin C55α/β</td>
<td>3339, 2993</td>
<td><em>Staphylococcus aureus</em> C55</td>
<td>(314)</td>
</tr>
<tr>
<td>Lacticin 3147α/β</td>
<td>3306, 2847</td>
<td><em>L. lactis</em> DPC2147</td>
<td>(377)</td>
</tr>
<tr>
<td>Cytolysin L/Ls</td>
<td>4164</td>
<td><em>Enterococcus faecalis</em> DS16</td>
<td>(126)</td>
</tr>
<tr>
<td>Haloduracin Haloβ</td>
<td>3164, 2334</td>
<td><em>Bacillus halodurans</em> C-125.</td>
<td>(294)</td>
</tr>
<tr>
<td>Mutacin II</td>
<td>3245</td>
<td><em>Streptococcus mutans</em> T8</td>
<td>(61)</td>
</tr>
<tr>
<td>Lactocin S</td>
<td>3764</td>
<td><em>Lactobacillus sakei</em> L45</td>
<td>(305)</td>
</tr>
<tr>
<td>Lacticin 481</td>
<td>2901</td>
<td><em>L. lactis</em> CNRZ481</td>
<td>(348)</td>
</tr>
<tr>
<td>Nukacin ISK-1</td>
<td>2960</td>
<td><em>Staphylococcus warneri</em> ISK-1</td>
<td>(389)</td>
</tr>
</tbody>
</table>
Chapter 1- Introduction

Class I

Nisin A

Subtilin

Epidermin

Gallidermin

Pep5
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Class II

Lacticin 481

Mersacidin

Lactocin S

Lacticin 3147 A1 (Ltn )

Lacticin 3147 A2 (Ltn )

Haloduracin

Haloduracin

Fig. 1: Representative examples of lantibiotics, modified from Willey (474).
4. Biosynthesis of lantibiotics

In contrast to many peptide antibiotics, such as the gramicidins, polymyxins, bacitracins, glycopeptides, etc., which are synthesized non-ribosomally by condensation of amino acids by peptide synthetases, lantibiotics (and all the bacteriocins) are ribosomally synthesized.

Lantibiotic production is a complex process that includes formation of a lantibiotic precursor peptide, its post-translational modification, cleavage of the N-terminal leader peptide from the precursor and secretion. In some cases, proteolytic cleavage occurs prior to or concomitantly with secretion. Moreover, the producing cells have to be immune to the lantibiotic that they produce (293).

4.1. Post-translational modifications

Lantibiotics are highly modified peptides. The percentage of residues involved in posttranslational modifications ranges from 24% in lactocin S to 58% in the recently discovered compound 107891 (262). Post-translational modifications are introduced enzymatically and mostly involve the hydroxyl amino acids Ser and Thr and the sulphydryl Cys. First, Ser and Thr are selectively dehydrated to generate 2,3-didehydroalanine (Dha, from Ser) or \((\text{Z})-2,3\)-didehydrobutyrine (Dhb, from Thr).

The \(\alpha,\beta\)-unsaturated amino acids are then targets for a nucleophilic attack of the thiol group of a Cys residue to form the acid-stable thioethers of the characteristic lanthionine (Lan), and 3-methylanthionine (MeLan) bridges, respectively (Fig. 2, 3). However, as the number of Cys residues is usually smaller than the number of didehydroamino acids, almost all lantibiotics contain one or more didehydro-residues that have not been processed. This biosynthesis model was first proposed by Ingram (193, 194), who demonstrated that when purified nisin from \textit{Lactococcus lactis} is incubated in the presence of \[^{3}\text{H}\]-Thr and \(^{35}\text{S}\)-Cys the radioactivity is incorporated into 3-methylanthionine. This model is the basis of all published lantibiotic biosynthesis models (255).

Some lantibiotics from different classes show an additional modification: enzymatic cyclization occurs at the C-terminus where the terminal Cys residue is oxidized and decarboxylated before undertaking nucleophilic attack on Dha or Dhb, resulting in either 2-aminovinyl-D-cysteine (AviCys), as in epidermin and gallidermin
(256), or in 2-aminovinyl-3-methyl-D-cysteine (AviMeCys), as in mersacidin (234) (Fig. 2, 3).

In lactocin S, a conversion of L-Ser to D-Ala was discovered (422). The presence of D-Ala residues was also confirmed for the two-component lantibiotics lacticin 3147 (377) and haloduracin (294). Lactocin S, produced by *Lactobacillus sake*, as well as Pep5, epicidin 280, and epilancin K7 produced by *S. epidermidis* are N-terminally blocked by 2-oxo-propionyl (from Dha) or –oxobutyryl groups (from Dhb) (159, 207, 213, 422). The Dha and DhaB residues that are N-terminally exposed after leader processing spontaneously hydrolyze to generate 2-oxopropionyl and 2-oxobutyryl groups. The former can be further modified by reduction to 2-hydroxypropionyl group (474). Additionally, a variety of other modified amino acids like 2S,8R-lysinoalanine (116), chlorinated Trp and mono- or dihydroxylated Pro (262), *erythro*-3-hydroxyl-L-aspartic acid (205), and hydroxypyruvate have been identified in other lantibiotics.

The role of these post-translationally modified residues remains unclear in most cases. Nevertheless, some have been shown to contribute to enhanced stability under extreme temperatures (188) and oxidizing conditions (384), to increased acid tolerance, as well as to resistance against proteolytic degradation (23). Generally, removal of dehydro-amino acids from lantibiotics reduces their biological activity. As an example, the exchange of Dha5 for Ala in subtilin and nisin lead to the loss of inhibition of outgrowth of *Bacillus subtilis* spores (52, 270). In particular, the bridging pattern formed by Lan and MeLan was shown to be required for structural stability, resistance against proteolytic degradation, and activity of these cyclic peptides (55). When a fourth thioether bridge (MeLan) was introduced into Pep5, proteolytic stability of the peptide against chymotrypsin and Lys-C was greatly enhanced (248). However, it also resulted in a significant decrease in antimicrobial activity. Other Pep5-derived peptides in which thioether crosslinking had been removed, displayed a pronounced susceptibility towards proteolytic degradation (55). (Me)Lan residues stabilize lantibiotics against proteases by restricting the conformational freedom of potential cleavage sites (23). Also the presence of D-amino acids in some lantibiotics is thought to contribute to their stability against proteases (377, 422). In nisin Z, replacement of Thr13 with Cys produced a disulfide bridge instead of MeLan resulting in reduced activity (248).
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The final post-translational modification reaction involved in lantibiotic synthesis is the enzymatic cleavage of the N-terminal leader peptide, yielding the active lantibiotic moiety. The activation will be discussed in detail later in this chapter.

![Chemical structures of lantibiotic residues](image)

Fig. 2: Most common post-translational modifications involved in the creation of non-proteinogenic amino acid residues observed in lantibiotics.
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4.2. Primary structures

4.2.1. Class I lantibiotics

Class I lantibiotics are elongated, flexible peptides, which consist of a minimum of 21 and a maximum of 38 amino acids. They have between no and 7 net positive charges. They are relatively unstructured in aqueous solutions, whereas in more lipophilic environments they tend to adopt corkscrew-like shapes with charged residues and hydrophobic side chains aligning on opposite sides of the molecule (197).

Their leader peptides are required for secretion of the peptides (94, 354, 427). Leader peptides are conserved (55), but share no homology with signals of sec-dependent or twin-arginine transported proteins. They are also required to ensure the complete maturation of the lantibiotics (264) and to keep the peptides inactive while they are in the cytoplasm, as the active peptides are membrane-active and can also target the producing cells (17, 33, 93, 158, 264, 336, 451, 473).
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As discussed above, nisin A is the prototype class I lantibiotic and is produced by the lactic acid Gram-positive microorganism *L. lactis*. Nisin contains five intramolecular ring structures formed by a single Lan and four MeLan residues (Fig. 1). Until now, four nisin variants were discovered: nisin Z and nisin Q are produced by *L. lactis* and differ from nisin A in one and four residues, respectively (307, 488). Recently, *Streptococcus uberis* strain 42 was shown to produce nisin U, which is 78% identical (82% similar) to nisin A from *L. lactis* (476), whereas nisin F, which differs from nisin A in two residues was shown to be produced by *L. lactis* F10, isolated from freshwater catfish (260).

Other well-studied class I lantibiotics include subtilin produced by *B. subtilis* during sporulation, epidermin and Pep5 from *Saphylococcus epidermidis*, and gallidermin produced by *S. gallinarum*, which differs from epidermin only in a Leu/Ile exchange (402) (Fig. 1). Subtilin is similar to nisin in its structural bridging pattern, even though the amino acid sequences of the two peptides vary significantly (Fig. 1) (331). The tetracyclic lantibiotic epidermin is produced by *S. epidermidis* Tü3298 and shows high similarity to nisin and subtilin in its N-terminus, whereas the C-terminus is completely different and has an AviCys residue (Fig. 1, 2). Gallidermin, mutacin I, and mutacin III are variants of epidermin produced by *S. gallinarum* and *S. mutans* respectively. On the contrary, Pep5, epicidin 280 and epilancin K produced by different *S. epidermidis* strains are strongly charged tricyclic peptides, which contain oxopropionyl or –oxobutyryl groups (159, 213).

4.2.2. Class II lantibiotics

This class includes very diverse structural peptides, which however share the characteristic of being post-translationally modified by one single enzyme LanM and of being simultaneously exported and activated by LanT(P). The lacticin 481 group consists of at least 16 members, including the well-studied lacticin 481 (348, 349, 366), mutacin II (320, 477), nukacin ISK-1 (389), and salivaricin A (373) (Fig. 1). They are hydrophobic peptides with no net charges at neutral pH (474) and show a linear N-terminus and a globular C-terminal domain, due to the presence of three intertwined rings. Therefore, they do not fit into classification schemes that are based on structural properties. All or most of the lantibiotics of the lacticin 481 group share the same bridging pattern, where the overlapping thioether bridges result in a
globular structure that involves two thirds of the molecules, whereas the N-terminus remains linear (100). Four of the seven modified residues (one Dhb, two Lan, one MeLan) are conserved in all propeptide sequences (100). However, mature salivaricin A contains three MeLan and has a shorter C-terminus than the other members of the lactacin 481 group (373). They are believed to form pores into the membranes of susceptible microorganisms (100), although their mechanism of action has not yet been elucidated.

Mersacidin has been classically classified as a type-B lantibiotic, which have characteristic compact, globular structures and carry no net charge or a net negative charge at neutral pH. Mersacidin is the smallest lantibiotic known so far and contains four thioether rings, formed by three MeLan and one C-terminal AviMeCys (Fig. 1) (22, 56). Its bactericidal action is due to inhibition of peptidoglycan biosynthesis by binding to lipid II (41), and not to pore formation.

Cinnamycins and duramycins have also been classified as type-B lantibiotics, and are produced by *Streptomyces* spp. They contain one Lan and two MeLan and have an identical bridging pattern including a characteristic lysinoalanine bridge which connects the C-terminus with Dha in position 6 and contain a single residue of hydroxy-aspartic acid (115, 331). This group of lanthionine-containing peptides potently inhibit phospholipase A$_2$ indirectly by specifically sequestering its substrate phosphatidylethanolamine (PE) (281, 284). Moreover, cinnamycin induces transbilayer movement of phospholipids in target cells that leads to the exposure of PE of the inner leaflet to the toxin (281).

Two-component lantibiotics consist of two prepeptides (LanA1/A2) that are each post-translationally modified (Lanα/β) and act in synergy to provide antibacterial activity (121). To date, seven two-component lantibiotics have been identified: cytolysin (126), staphylococcin C55 (314), lactacin 3147 (377), plantaricin W (176), Smb (483), BHT-A (189), and haloduracin (294). Cytolysin was the first reported example of a two-peptide lantibiotic and shows unusual characteristics, as it exerts activity against a broad spectrum of cell types including a wide range of Gram-positive bacteria, eukaryotic cells such as human erythrocytes, polymorphonuclear leukocytes, and human intestinal epithelial cells (79).

In most cases, the sequence similarity of the two prepeptides is rather low (< 25%). The exception is cytolysin, whose two peptides have a sequence homology higher than 90%. Staphylococcin C55 shows significant similarities to lactacin 3147
(314), and Plwα and Plwβ of plantaricin W share 31% and 32% homology to staphylococcin C55, as well as 40% and 26% similarity to lacticin 3147 (176). The NMR structure of the best-studied two-component lantibiotic, lacticin 3147, has been recently elucidated and shows similarity of the lanthionine bridging pattern of Ltnα peptide to that of mersacidin, whereas the more flexible and elongated Ltnβ has similarity to lactocin S (286). Structural information combined with sequence homology suggests that three C-terminal rings are conserved in all α-peptides. According to Willey (474), the N-terminal MeLan ring identified in haloduracin seems to be conserved in all β-peptides except lacticin 3147 and staphylococcin C55, and the two C-terminal Lan/MeLan rings are conserved in all β-peptides except cytolysin.

4.3. Organization of the gene clusters

Sequencing of the genes for lantibiotic biosynthesis has shown a high level of similarity in the genetic organization for production of lantibiotics (417), and the organization of representative members is shown in Fig. 4. Hence, lantibiotic biosynthesis genes have been given the generic locus symbol lan (454). However, each lantibiotic member has a more specific genetic designation, e.g. nis for nisin, gdm for gallidermin, spa for subtilin, or epi for epidermin. Conserved genes in the clusters were proposed to encode similar functions. Following the generic nomenclature, these include the precursor peptide (LanA, or SpaS for subtilin), the enzymes responsible for post-translational modifications (LanB,C/LanM), the protease responsible for cleavage of the leader peptide (LanP/LanT), the ABC transporters involved in peptide translocation (LanT, LanH), regulatory proteins (LanK/LanR, LanQ), and immunity proteins (LanFEG). Moreover, besides being responsible for translocation, the LanT transporter of class II lantibiotics are also involved in proteolytic activation of the precursor peptides.

Non-homologous genes are also found in some lantibiotic gene clusters. As an example, nisl and spal encode for lipoproteins that are involved in immunity (229, 247). Moreover, epiD, as well as gdmD and mrsD encode enzymes involved in special post-translational modification found in the C-termini of epiderminm gallidermin, and mersacidin (165, 258, 280).

Biosynthetic genes have been shown to be clustered on conjugative transposable elements (e.g. nisin), on the chromosome of the host (e.g. subtilin (227)
and gallidermin (165)), or on large plasmids (e.g. epidermin (402), Pep5 (207), cytolysin (126), and lacticin 3147 (251)). There seems to be no order or uniform orientation of the genes in different gene clusters, as rearrangements have occurred during evolution (417). However, there are also cases of strong similarity in genetic organization, for example the nis and spa gene clusters, where the organization appears highly conserved, and the epi and gdm clusters, where it is identical.

At least parts of the gene clusters of some lantibiotics are organized as operons. Many consist of several transcriptional units (12, 24, 97, 250, 339), and weak terminator sequences are often found in intergenic sequences between the structural genes and the downstream biosynthetic genes (293), enabling high transcription of prepeptide mRNA in comparison to the mRNA of the latter genes.

Fig. 4: Representative biosynthetic gene clusters of the lantibiotics nisin, subtilin, epidermin, Pep5, lacticin 481, lacticin 3147, and mersacidin. Promoters for the transcriptional units in these clusters (where known) are indicated by red wedges.
4.4. Lantibiotic biosynthetic pathway

4.4.1. Prepeptides

The \textit{lanA} genes are a common feature to all lantibiotic biosynthetic gene clusters, and they encode the prepeptide LanA. In comparison to the mature lantibiotic, the lantibiotic precursor peptides are biologically inactive \cite{93, 451}, as they carry an N-terminal extension, called leader peptide, followed by the C-terminal propeptide moiety that will undergo post-translational modifications \cite{463}. After modification, the C-terminal moiety is activated upon removal of the leader peptide by proteolytic cleavage. Leader peptides are usually between 23 and 30 amino acids-long, they are in general hydrophilic, as revealed by hydropathy profiles, and they adopt $\alpha$-helical conformations in lipophilic environments, as shown by secondary structure predictions \cite{47}.

Class I leaders are generally hydrophilic, possess a high proportion of charged amino acids, and in contrast to the fully matured lantibiotic can have a net negative or slightly positive charge. Processing sites of the class I lantibiotics prenisin A \cite{46, 206}, prenisin Z \cite{307}, presubtilin \cite{14}, prepep5 \cite{207}, preepidermin \cite{402}, and pregallidermin \cite{401} all show a charged or polar amino acid (Arg, Gln) in the P$^{-1}$ position relative to the cleavage site and a hydrophobic residue in P$^{+1}$. Their leader peptides contain a conserved motif (Phe/Asn/Asp-Leu-Asn/Asp/Glu-Ile/Leu/Val) \cite{384}, which was shown to have an impact on the production titers of Pep5. These were drastically reduced if this consensus was modified \cite{316}. Moreover, van der Meer and coworkers demonstrated that mutations in the conserved motif did not lead to secretion or accumulation of nisin or of its precursors at all, indicating that changes in this highly conserved region lead to a block in nisin production \cite{94}. This region might also be essential for recognition by modification enzymes such as NisC and NisB \cite{104, 247}. Mutations in the leader peptide of nisin in the vicinity of the cleavage site affected processing of the leader sequence and resulted in the extracellular accumulation of a biologically inactive precursor peptide, demonstrating that cleavage of the leader peptide is the last step in class I lantibiotic processing and that cleavage is not necessary for secretion.

Typical class II leaders possess highly negative net charges and have different consensus sequences than class I leaders as they contain “double glycine” Gly-Gly, Gly-Ser, or Gly-Ala at P$^{-2}$ and P$^{-1}$ relative to the cleavage site and either a charged or
neutral residue (Lys or Gly) in P$^{+1}$ (195). In addition, they are characterized by an excess of Glu and Asp residues and are processed concomitantly with export (125, 348).

Independently from the lantibiotic class, the role of the leader peptides may include stabilization of the prepeptide during translation, guaranteeing its biological inactivity, preservation of its specific conformation during processing, and assisting with its translocation by specific transport systems (177, 458). Secretion of nisin seems to be directed by the leader peptide, as a series of non-lantibiotic peptides attached to the C-terminus of the NisA leader sequence have been shown to be transported by NisT (245). Moreover, the leader peptide of nisin has been shown to play an essential role for the binding and recognition by NisC, as cleavage of the leader peptide prior to the in vitro modification by NisC resulted in impaired cyclization (264). NMR studies comparing prenisin and mature nisin suggested that a different interaction between the membrane and the N-terminal region of the modified propeptide in both compounds is responsible for loss of antimicrobial activity in prenisin (91).

4.4.2. Enzymes involved in post-translational modifications

4.4.2.1. Dehydration and cyclization reactions

A function has been assigned to most of the lantibiotic biosynthetic genes as a result of homology with known genes. However, the gene products of $lanB$ and $lanC$ in class I lantibiotic systems, and $lanM$ in class II systems do not share sequence similarity with any known proteins. Disruption of these genes in various lantibiotics has been shown to inhibit production of active compounds (8, 447).

Hence, it was first proposed that they play a role in the dehydration of Ser and Thr residues and in the formation of thioether bridges, which are the unique features of the lantibiotics (208, 399).

The LanB proteins are large hydrophilic proteins, with molecular masses of approximately 120 kDa, which also possess some hydrophobic domains, suggesting a membrane association that has been demonstrated for NisB of the nisin system (104) and SpaB of the subtilin system (150). EpiB, on the other hand, was found to be only loosely associated with the cytoplasmic membrane (343). The function of LanB enzymes, which are responsible for selective dehydration of Ser and Thr
residues in the LanA propeptide region, has been suggested in various \textit{in vivo} studies. A first indirect evidence was provided by the isolation of a dehydrated Pep5 peptide after inactivation of \textit{pepC} in a Pep5-producing \textit{S. epidermidis} strain (297). Sen and coworkers proved that NisB is involved in dehydration of nisin precursor by showing that overexpressing the \textit{nisB} gene increased the efficiency of dehydration of Ser at position 33 of nisin, which had partly escaped dehydration in engineered nisin variants ([Trp30]nisin A and [Lys27, Lys31]nisin A) (405). More recently, a study reported the isolation of unmodified nisin in strains lacking \textit{nisB}, whereas dehydrated nisin prepeptide was isolated in \textit{L. lactis} strains with a \textit{nisC} deletion (236). Similar results were achieved in a study where \textit{nisABT} were expressed in a \textit{L. lactis} strain that did not produce nisin. Expression lead to the production of a dehydrated nisin without thioether bridges, further corroborating the dehydratase function of NisB (245). Moreover, NisB has also been shown to have low substrate specificity, as it was able to dehydrate nonlantibiotic peptides fused to the leader peptide of NisA in \textit{L. lactis} (230, 368). The putative dehydratase EpiB, involved in epidermin biosynthesis, was produced in \textit{Staphylococcus carnosus}, however \textit{in vitro} dehydration activity could not be demonstrated (343). Similarly, SpaB was produced in \textit{E. coli} but efforts to detect dehydratase activity in the presence of subtilin prepeptide SpaS were unsuccessful (478). Only recently, a study presented the first experimental evidence of LanB activity \textit{in vitro} and the first complete \textit{in vitro} lantibiotic biosynthesis. The nisin prepeptide was synthesized with an \textit{in vitro} transcription and translation system and was successfully modified \textit{in vitro} by NisB and NisC (60).

LanC enzymes are half the size of LanB proteins and have alternating hydrophobic and hydrophilic domains (104). Although \textit{in vivo} experiments involving \textit{lanC} disruption showed the impairment of the cyclization reactions leading to accumulation of dehydrated peptides (236, 245, 297), efforts to detect their properties \textit{in vitro} were unsuccessful for a long time (311). Only recently \textit{in vitro} reconstitution of NisC was reported, which post-translationally cyclized dehydrated prenisin (264). NisC resulted to be a zinc metalloprotein with two histidines and two cysteines, which are conserved in all LanC proteins and are involved in catalytic reactions. They have been shown to function as ligands for zinc, which may activate cysteine thiols towards intramolecular addition to Dha and Dhb in the prepeptide (311, 312, 325). Heterologously produced NisC was shown to catalyze cyclization within the dehydrated nisin prepeptide \textit{in vitro}. Moreover, modified prepeptides were biologically
active following tryptic cleavage of the leader sequence (264). Recently, Rink and coworkers demonstrated that also NisC has low substrate specificity, as it was able to cyclize \textit{in vivo} a wide array of unrelated and designed peptides that were fused to the nisin leader peptide in \textit{L. lactis} cells containing NisBT (367). By taking advantage of the low substrate specificity of NisC as well as NisB and EpiD (398), such \textit{in vitro} biosynthesis systems might be used for the cyclization and stabilization of nonlantibiotic synthetic peptides.

In the case of nisin and subtilin, the presence of a multimeric synthetase complex consisting of two molecules of Nis/SpaC, one molecule of Nis/SpaB, and two molecules of Nis/SpaT was detected by using a yeast two-hybrid-system and co-immunoprecipitation (220, 415, 478). However, a recent study contradicts these findings, as fully modified prenisin was produced using \textit{nisA}, \textit{nisB}, and \textit{nisC} as templates for \textit{in vitro} transcription and translation, strongly suggesting that all modifications of prenisin can take place \textit{in vitro} without membrane association and the transporter protein NisT (60).

Whereas the \textit{lanB} and \textit{lanC} genes are missing in the biosynthetic clusters of class II lantibiotics, another gene, \textit{lanM}, is present that encodes a bifunctional dehydratase-cyclase (LanM) responsible for their modification. The C-terminus of LanM shows 20-27\% sequence identity to LanC, with seven conserved motifs including the possible metal ligands discussed above (479), but no identity to the dehydratase LanB. Interaction of LctM with the prepeptide of lacticin 481, LctA, was demonstrated by means of the yeast-two-hybrid system, supporting the role of LanM in the modification process (447). In the case of the two-component lantibiotic lacticin 3147, the presence of two independent \textit{lanM} genes (\textit{ltnM1}, \textit{ltnM2}) each necessary for modification of one of the prepeptides LctA1 and LctA2 was demonstrated by disruption experiments (291). Recently, a similar organization was revealed for haloduracin (294). Moreover, in this study, the biosynthesis of haloduracin was reconstituted \textit{in vitro}, showing that the modification enzymes HalM1 and HalM2 successfully processed the two precursor peptides HalA1 and HalA2. Evidence for the role of LanM as a bifunctional enzyme catalyzing both dehydration and cyclization reactions has been first obtained with the \textit{in vivo} modification of NukA by coexpression of \textit{nukA} and \textit{nukM} in \textit{E. coli} (312), and with the \textit{in vitro} reconstitution of LctM in lacticin 481 biosynthesis (480).
LanB, LanC, and LanM are common features of all class I and class II lantibiotics, respectively, but more enzymes play an important role in post-translational modifications of some lantibiotics. As an example, the LanD proteins are involved in the formation of the C-terminal aminovinyl cystein residues AviCys and AviMeCys, in epidermin and mersacidin, respectively (Fig. 1). Both EpiD and MrsD have been shown to induce the in vitro oxidative decarboxylation of a C-terminal Cys residue to form AviCys and AviMeCys (280, 398). Crystal structure elucidation of EpiD and MrsD revealed characteristic flavin-containing decarboxylases, which utilize FMN and FAD as cofactors, respectively (29, 30). The GdmD protein of the gallidermin biosynthetic cluster shows 87% identity to EpiD, suggesting that it encodes the enzyme responsible for the oxidative decarboxylation of the C-terminal Cys residue, leading to AviCys (165, 451).

More unusual amino acids are observed among lantibiotics: lactocin S and the two-component lantibiotic lacticin 3147 possess the unusual amino acid D-Ala (286, 377, 422), which has been shown to result from the enzymatic conversion of L-Ser. Skaugen and coworkers speculated that a two-step process might be responsible for D-Ala formation in lactocin S, involving conversion of L-Ser to Dha by LasM followed by a stereospecific hydrogenation to form D-Ala (422). In the case of lacticin 3147 biosynthesis, the enzyme responsible for this hydrogenation step – LtnJ – has been identified. Production of intermediates containing Dha but no D-Ala upon deletion of the ltnJ gene strongly suggested a role for LtnJ in the formation of D-Ala in lacticin 3147 (78).

4.4.2.2. Processing and export

After modification of the precursor peptide, the N-terminal leader sequence is removed by the serine protease LanP (class I lantibiotics) or by the proteolytic domain of LanT, an ATP-binding cassette transporter protein (class II lantibiotics) (336).

Deduced amino acid sequences of the LanP proteins show homology with the serine protease subtilisin, especially in the sequence encompassing the residues involved in the catalytic triad (Asp, His, and Ser) and the Asn involved in oxyanion hole formation (55, 419). LanP proteins vary among lantibiotics with respect to the presence of an N-terminal sec-dependent signal sequence defining an extracellular
presence, as in the case of gallidermin and epidermin, or an intracellular one, as for lactocin S and Pep5. NisP, which is responsible for the processing of prenisin, has a predicted molecular mass of 75 kDa, but its active form is a 54 kDa protein (93). This mass loss is due to peptidase cleavage of the N-terminal sec-dependent secretion signal sequence. However, NisP possesses a consensus C-terminal cell wall anchoring sequence, Leu-Pro-X-Thr-Gly, and is thus coupled to the peptidoglycan of the cell wall (93). Specificity of NisP has been investigated by using a chimeric substrate consisting of the nisin Z propeptide region attached to the leader peptide of subtilin, which differs from the leader of nisin for the presence of a Gln residue instead of Arg in position P\(^{-1}\) (249). A fully modified chimeric substrate was successfully produced in *L. lactis*, but proteolytic cleavage did not take place, suggesting a high specificity of NisP. The importance of the Arg residue for processing was also demonstrated by homology modelling studies on the interaction of NisP with prenisin, showing that the most dominant is an electrostatic interaction due to the Arg residue in position P\(^{-1}\) of the processing site Ala-Ser-Pro-Arg↓Ile-DhB (418). The leader peptidase EpiP of *S. epidermidis* has 42% sequence identity to NisP, and is responsible for the cleavage of the leader peptide of preepidermin. This processing sequence is very similar to that of prenisin (Ala\(^{-4}\)-Glu-Pro-Arg↓Ile-Ala\(^{+2}\)) (122). Thus, on the basis of homology modelling, it has been proposed that also the binding of EpiP to preepidermin is dominated by electrostatic interactions of the Arg and Glu residues in position P\(^{-1}\) and P\(^{-3}\) of the processing site (418). This indicates that modification of preepidermin might not to be necessary for binding of preepidermin to EpiP (122). However, Kuipers and co-workers have shown that *in vivo* removal of the leader by NisP occurs only in the presence of a fully modified nisin prepeptide (245). The C-terminal anchor sequence found in NisP is absent in EpiP, suggesting the protein is not covalently bound to the cell surface, and treatment of preepidermin with supernatants of cultures of *S. camosus* expressing the protease gene *epiP* resulted in the specific cleavage of the leader peptide, indicating an extracellular location for EpiP (122). The protease PepP involved in maturation of Pep5 lacks the N-terminal sec-signal sequence which is common to other serine proteases (419), suggesting that it is not exported but localized in the cytoplasm (297). Such a sequence is also missing in LasP, responsible for leader processing of lactocin S, which indicates an intracellular location of this protease. Thus, prelactocin S and prepep5 are cleaved prior to their translocation across the cytoplasmic
membrane or, alternatively, LasP is exported across the membrane by a sec-independent mechanism (419, 421). Interestingly, a lanP gene encoding the processing protease is absent in the subtilin biosynthesis cluster. Three intrinsic B. subtilis extracellular serine proteases have been shown to activate subtilin precursors instead of a dedicated protease: subtilisin (AprE), WprA, and Vpr (73).

The LanT proteins are responsible for translocation of lantibiotics to the extracellular space and show high homology to hemolysin B-like ATP-dependent transport proteins designated bacterial ABC (ATP binding cassette) exporters (108). These proteins, which are found in both Gram-negative and Gram-positive organisms, facilitate the ATP-dependent translocation of a wide range of molecules across the cytoplasmic membrane and often require additional factors for the formation of an active transport complex (108). Based on sequence homology, potential transporters of this type has been found in every lantibiotic gene clusters studied to date (293).

LanT transporters of class I lantibiotics (NisT, SpaT, GdmT) contain an N-terminal hydrophobic domain, a six-helix membrane spanning domain, and a C-terminal ATP-binding domain where ATP hydrolysis probably takes place, which conveys the energy required for export. Translocation complexes are formed by dimerization of LanT proteins at the cytoplasmic membrane. Their function has been verified by gene disruption experiments. As an example, NisT has been shown to be necessary for export of nisin, as no nisin was found in culture supernatants of ΔnisT mutants and intracellular accumulation was observed (354). NisT was able to transport unmodified or partially modified prenisin and fusions of NisA leader peptide with non-lantibiotic prepeptides and therefore specifies a broad spectrum transporter (245). In contrast to nisin, investigations on Pep5 and epicidin 280 biosynthesis showed that LanT is not required for extracellular transport (159, 297), which is likely to be carried out by host-encoded transporters. Also epidermin can be secreted in the absence of a functional EpiT, as the epiT gene in the epidermin biosynthesis cluster has been found to be defective, and would require a frameshift to yield a functional protein (122). In contrast, the gdmT gene of the closely related S. gallinarum was found to be intact and mediated an increase of epidermin production when introduced in epidermin-producing strains (166). In both epidermin and gallidermin biosynthetic clusters two additional genes were discovered, epiH and gdmH, coding for membrane proteins without similarity to other proteins of known function (166, 346).
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GdmH and EpiH are thought to be accessory factors for the ABC transporters EpiT and GdmT (166, 346).

LanT proteins found in class II lantibiotics contain an extra N-terminal cysteine peptidase domain (55). As demonstrated for lacticin 481 (446), these transporters seem to display the dual function of cleaving the leader peptide while exporting the mature lantibiotics. Members of this family include also LntT from lacticin 3147 (98), CylT (CylB) from cytolysin (126), and MrsT from mersacidin (5). An exception among class II lantibiotics is represented by lactocin S, which is processed and translocated by the action of two separate proteins LasP and LasT (421). Interestingly, cytolysin has been shown to require a second round of proteolysis by the extracellular protease CylA for complete peptide maturation. CylA has been shown to be responsible for the removal of an N-terminal six-residue sequence (Gly-Asp-Val-Gln-Ala-Glu) from the peptides CylL′ and CylL″ to yield the synergistically active CylL‴ and CylL‴′ (79). Accordingly, also the β-peptides of haloduracin and plantaricin W seem to require a second proteolytic step for activation (176, 294).

4.5. Self-immunity

Any lantibiotic-producing bacterial strain must have protection mechanisms by which it protects itself against the active compound. Immunity is provided by specific proteins (LanI) and/or by dedicated ABC transporters (LanFEG).

According to Willey, one of the simplest protection mechanisms is displayed by Pep5-producing S. epidermidis 5 (474). In this strain the immunity peptide PepI is produced, which has been shown to accumulate at the membrane-cell wall interface, thereby shielding the Pep5 target molecule (174). In contrast, the membrane-bound lipoprotein NisI of nisin-producing L. lactis strains interacts with nisin itself, thereby reducing its local concentration (429, 437). A similar mechanism was reported for immunity to subtilin in B. subtilis (428). Moreover, L. lactis strains produce a lipid-free NisI (LF-NisI) variant that is secreted in the extracellular space and protects the cell by binding to nisin and reducing its local concentration (235). In addition to these examples, immunity to other lantibiotics rely on the presence of single proteins, e.g. CylI for immunity to cytolysin (68), and Epil for immunity to epicidin 280 (159). However, in many cases, as for nisin (414) and lacticin 3147 (291), immunity conferred by LanI is further enhanced by the action of the ABC transporter LanFEG.
The relative contributions of the two systems to nisin immunity were estimated by a knockout approach, showing that each system alone only accounts for 5-20% of the full immunity level provided by the two entities together, indicating strong synergistic action (355). Sequence similarities and hydrophobicity profiles suggest that LanFEG proteins form an ABC transporter complex consisting of two transmembrane subunits forming a heterodimer (LanEG) and two nucleotide-binding subunits forming a homodimer (LanF), as firstly described for the epidermin ABC transporter EpiFEG of *S. epidermidis* (341). The mechanism of action of EpiFEG was shown to rely on the specific ATP-driven expulsion of epidermin molecules from the cytoplasmic membrane to the surrounding space (329). The same mechanism was later reported for the action of NisEFG (429).

Immunity of *B. subtilis* sp. HILY-85 to mersacidin relies solely on the presence of ABC transporter MrsFEG, which has been shown to confer immunity towards exogenous mersacidin and inhibited binding of mersacidin to whole *Bacillus* cells (145). Homologous ABC transporters confers immunity for the producing strains to the lantibiotics mutacin II (59), and lacticin 481 (366).

Like the subtilin and nisin gene clusters, also the gallidermin and epidermin determinants encode a second specific immunity system, which is nevertheless different from the ones previously discussed. *S. epidermidis* and *S. gallinarum* synthesize the membrane proteins EpiH and GdmH, respectively (166). GdmH was shown to contribute independently from GdmT to the immunity towards gallidermin, as well as EpiH conferred resistance against epidermin (166). However, the mode of action of these proteins has not yet been elucidated. Immunity to nukacin ISK-1 in *Staphylococcus warneri* ISK-1 is based on the cooperative action of the membrane-bound immunity protein NukH and the NukFEG transporter (8, 9). The underlying mechanism of this synergistic action was recently elucidated: NukH was shown to capture nukacin ISK-1 in an energy-independent manner by recognizing its C-terminal ring region and NukFEG transported nukacin ISK-1 to the extracellular space in an energy-dependent manner (326). NukH shows no sequence homology with EpiH and GdmH, and its hypothesized function is similar to that of substrate-binding proteins (SBPs) found in general ABC transporter systems (164), i.e. to confer high affinity and specificity to the transport systems for efficient transport (326).
4.6. Regulation of lantibiotic biosynthesis

All lantibiotic-producing bacteria have regulatory mechanisms for their production, which usually starts at mid- to end-exponential growth phase and increases to maximum production level in the early stationary phase (150, 225, 376). Many of these mechanisms rely on quorum-sensing systems, which enable a bacterial population to act in a coordinated manner (151, 246, 427). Quorum-sensing involves production of peptide pheromones that accumulate during bacterial growth and, after reaching a specific threshold concentration, trigger a signal transduction pathway leading to target gene expression. Signal transduction is based on two-component regulatory systems (226, 237), and is mediated by the interaction of a peptide pheromone with its cognate receptor, a membrane-bound histidine kinase sensor protein (in case of the lantibiotics: LanK), resulting in phosphorylation of an Asp residue of a response regulator (LanR), which then activates transcription of target genes (146).

An autoregulation mechanism was demonstrated for nisin production by *L. lactis* and subtilin production by *B. subtilis*, in which both lantibiotics have been shown to act as peptide pheromones (246, 427). In the case of nisin, this phenomenon was demonstrated with the construction of a mutant with a 4-base pair deletion in the *nisA* gene (\(\Delta nisA\)). In this mutant, not only nisin production was blocked, but also transcription of the \(\Delta nisA\) gene was completely abolished. Transcription could be restored by the addition of subinhibitory nisin concentrations, strongly suggesting a peptide pheromone role for nisin (246). The promoter regions of *nisABTCPRK* and *nisFEG* are subject to transcriptional activation by NisR, and their activity was shown to be proportional to the extracellular nisin concentration (228, 376). In contrast, the promoter of the *nisRK* operon was shown to be independent of nisin regulation and the genes *nisRK* to be constitutively expressed (228, 246, 376). Recently, it has been reported that intracellular prenisin produced by a translocator deficient strain could induce NisK and initiate a signal transduction pathway. It has been suggested that prenisin might be cleaved by unidentified intracellular protease(s) and that intracellular active nisin might insert into the membrane and activate the signal-recognition domain of the histidine kinase NisK (169).
A more complex regulatory network has been shown to underlie subtilin biosynthesis by *B. subtilis*, as the *spaRK* two-component regulatory system has been shown to be under dual control of two independent regulatory systems: autoinduction via subtilin and transcriptional regulation via the alternative sigma factor $\sigma^H$. Transcription of *sigH* is under negative control of the general transition state regulator AbrB (432, 464), whose synthesis is repressed by the key regulator of late-growth processes Spo0A (431). Therefore, an abrB deletion mutant lead to increased subtilin production, which started already during the logarithmic growth phase (427). Two heterologous gene expression systems were constructed, which were based on the nisin and subtilin autoregulatory systems: the NICE (nisin-controlled gene expression) system and the SURE (subtilin-regulated gene expression) system (225, 376).

Another cell-density dependent regulation mechanism has been described for cytolysin, which reflects its role as a virulence factor of enterococci. In the absence of a target cell, CylR1 represses high-level expression of cytolysin genes, so that CylLL and CylLS are expressed at basal levels. Together, they form an insoluble complex that does not show cytolytic or regulatory activity. As CylLS, in addition to possessing toxin and bacteriocin activity, also induces high-level expression of the cytolysin structural genes, CylLL titrates the level of free CylLS below the threshold for high-level cytolysin production. In the presence of a target cell, CylLL preferentially binds to target phosphatidylcholine:cholesterol in lipid bilayers, allowing free CylLS to accumulate and induce expression of the cytolysin genes (69).

Regulation of production of the class II lantibiotic mersacidin has been shown to be $\sigma^H$-independent and to occur in the stationary phase. A two-component regulatory system MrsR2/K2 as well as a single regulator protein, MrsR1 are encoded in the mersacidin biosynthesis gene cluster. While MrsR2 has been shown to be responsible for activation of transcription of the *mrsFGE* immunity operon, MrsR1 regulates biosynthesis of mersacidin (145). MrsR1 very likely does not depend on phosphorylation for activity, since a dedicated kinase is missing and phosphorylation by MrsK2 has been excluded by experiments showing that production of mersacidin was retained in a MrsR2/K2 deletion mutant (145).

Single regulatory proteins without corresponding histidine kinase (HPK), also called orphan response regulators, have been also described for several lantibiotic gene clusters: EpiQ in the epidermin cluster (339), MutR in mutacin II (352), and LtnR in lacticin 3147 (292). The C-terminus of EpiQ shows some homology to response
regulator proteins, but lacks the highly conserved phosphoryl-acceptor Asp residue (400). As no corresponding HPK has been identified in the epidermin gene cluster, it has been proposed that EpiQ might be activated by an intrinsic HPK (12). Anyhow, EpiQ has been shown to activate the promoters of the biosynthesis genes epiABCD, of the immunity genes epiFEG, and of the transporter genes epiT and epiH by binding to an inverted repeat (ANAATTAC-N_6-GTAATTNT) upstream of the -35 promoter region (339, 341, 346). Sequence homology suggests that the same mechanism underlies regulation of the gallidermin genes by GdmQ (165, 451).

Besides a regulation on the transcriptional level by EpiQ, epidermin maturation has also been shown to be regulated by the only quorum sensing system known in staphylococci: the agr (accessory gene regulator) system (31, 323). This system down-regulates the production of surface proteins and up-regulates synthesis of exoproteins in S. aureus and S. epidermidis (322, 456), and has been shown to interfere with proteolytic processing during epidermin maturation via the agr-regulated EpiP protease (219). A different regulation mechanism has been described for lacticin 3147: the regulator LntR has been shown to act as a transcriptional repressor of the ltnRIFE operon encoding the immunity genes, and to regulate its own synthesis, so that a steady-state level of immunity within the cell is maintained (292). Interestingly, no impact of the LntR regulator on lacticin 3147 biosynthesis has been observed (291).

Production of lantibiotics may also be regulated by environmental factors, such as extracellular pH. As an example, transcription of the lacticin 481 biosynthesis genes was shown to be activated upon acidification of the medium by lactic acid (170).
5. Mode of action and activity spectrum

5.1. Mode of action

Class I lantibiotic show two distinct mechanisms of action: At nanomolar concentrations, they bind to specific docking molecules in the cell wall, thereby blocking peptidoglycan biosynthesis. Additionally, at nanomolar concentrations, some class I lantibiotics are also able to form high-affinity, target-mediated pores. At micromolar concentrations they disrupt the cytoplasmic membrane by formation of non-targeted pores. In contrast, class II lantibiotics only act by specifically binding to lipid or lipid-bound targets and inhibit subsequent enzymatic reactions. Additional antibiotic effects, such as induction of autolysis, might also play a role in the action of several lantibiotics, further increasing the complexity of the mode-of-action picture.

5.1.1. Class I lantibiotics

5.1.1.1. Formation of target-independent pores

In 1960, Ramseier observed leakage of UV-absorbing intracellular compounds from nisin treated cells and suggested that the peptide may exert a detergent effect (358). This was the first report on the mechanism of action of class I lantibiotics. Subsequent studies with nisin and other class I lantibiotics demonstrated rapid efflux of ions and small metabolites such as amino acids and ATP from susceptible cells (471), as well as inhibition of DNA, RNA, protein and polysaccharide biosynthesis (386). Furthermore, lantibiotic-treated bacterial cells were shown to be unable to actively take-up amino acids and to become leaky for inorganic ions and small metabolites (375). The action of class I lantibiotics was shown to be based on a voltage-dependent membrane-depolarizing effect, and the driving force for this effect is provided by a membrane potential of at least 50-100 mV (20, 385). Membrane depolarization results in dissipation of the transmembrane potential ($\Delta \psi$) and the pH gradient ($\Delta p\text{H}$), which are essential components of the proton motive force, and in an instant termination of all biosynthetic processes (17, 375, 385). Studies using artificial bilayer membranes (black lipid membranes) revealed that class I lantibiotics form small (nisin: 1 nm in diameter, subtilin: 2 nm) transient pores with a lifetime in the millisecond to second range when an external voltage is applied (20, 385).

Nisin is characterized by two rigid domains, the first formed by the rings A to C and the second by the rings D and E, which are interconnected by a flexible hinge
region of three amino acids, and a flexible, positively charged C-terminus (452). These ring structures separate hydrophilic and hydrophobic residues at opposite sides of the molecule (452), while the central hinge region is indispensable for the pore-forming process, as it provides nisin with enough conformational freedom to bend the lipid surface (99). The positive charges in the C-terminal region and the amount of anionic lipids in the membrane, which are responsible for its overall negative surface charge, are important for binding and pore formation (36, 88, 241).

Initially, a “barrel-stave” model for the action of class I lantibiotics was proposed, in which the α-helical amphipathic peptides bind by electrostatic interaction to the outer leaflet of the cytosolic membrane in a parallel orientation, then monomers aggregate laterally and, after insertion into the membrane, non-polar side chains of the peptides interact with the lipid core and the hydrophilic residues point inward, resulting in the formation of a water-filled pore (385).

The alternative “wedge” model resulted from NMR studies with nisin in the presence of membrane-mimicking micelles, and takes into account the high flexibility of class I lantibiotics in aqueous solution (90, 92, 99). However, in both models, several peptides are supposed to associate with the membrane in order to form a pore, as lantibiotics are too short to span the membrane repeatedly (44). According to the wedge model, pore formation by class I lantibiotics involves local perturbation of the bilayer structure and a \( \Delta \psi \)-dependent reorientation of the peptides from a surface-bound into a transmembrane orientation (Fig. 5) (99). The peptides associate tightly with the anionic membrane surface leading to a high local concentration and, upon contact with the membrane, they adopt a helical amphiphilic structure. The hydrophilic side chains interact with the head groups of the phospholipids by ionic forces causing a locally disturbed bilayer structure, while the hydrophobic residues insert into the outer leaflet of the bilayer. In the presence of a transmembrane electrical potential above the threshold level, several molecules can then build a wedge-like, nonspecific, water-filled pore by moving through the membrane while remaining surface bound and carrying the lipids across without changing their orientation to the lipid head groups (Fig. 5) (301). Therefore, in contrast to the barrel-stave model, the peptides do not have contact with the hydrophobic core of the membrane (17, 158, 452).
5.1.1.2. Formation of high affinity target-mediated pores

The barrel stave and wedge models describe the behavior of micromolar concentrations of lantibiotics on pure lipid bilayers, and might be representative for the killing mechanisms of those bacteria which have MICs in the µM ranges (158). However, the very efficient killing of bacteria already at nanomolar concentrations of nisin and other class I lantibiotics cannot be explained with these models. The latter was elucidated when it became clear that high-affinity pore formation is determined by the presence of a specific docking molecule in the bacterial membranes (44).

Linnet and Strominger observed in 1973 interference of nisin with peptidoglycan biosynthesis (269). Later, inhibition was reported to be due to the binding of nisin and epidermin to peptidoglycan precursors (43, 362). Moreover, an inactive N-terminal nisin fragment was shown to antagonize the membrane-activity of nisin, indicating that a specific binding site may be blocked by the fragment (52). Then the involved molecule was purified, and resulted to be the membrane-bound peptidoglycan precursor MurNAc(pentapeptide)-GlcNAc, or lipid II (Fig. 6 A) (41).

The Gram-positive bacterial cell wall is a 3D network comprising a polymer of alternating amino sugars, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) (155). The glycan polymer chains are cross-linked by a pentapeptide, often L-alanyl-γ-D-glutamyl-diaminopimelyl (or L-lysyl-D-alanyl-D-alanine), that is attached to the carboxyl group of MurNAc sugars (34). This cross-linking gives the cell wall its rigidity and mechanical strength (37). Assembly of the cell-wall subunits begins in the cytosol, where UDP-MurNAc-pentapeptide is coupled to a special lipid carrier - bactoprenol phosphate - by the integral membrane protein MraY, to yield lipid I (Fig. 6 B). Next, lipid II is produced by coupling of GlcNAc by the peripherally membrane-associated MurG (Fig. 6 B). This is then transported across the plasma membrane via a yet unknown mechanism, and provides the cell wall synthesis machinery with subunits for the peptidoglycan polymer. The subunits are polymerized and inserted into the pre-existing cell wall by the action of penicillin-binding proteins (PBPs), and the remaining bactoprenol pyrophosphate flips back to the cytosolic side of the membrane where it is dephosphorylated for the next synthesis cycle (Fig. 6 B) (34). Gram-positive cell walls usually have up to 20 peptidoglycan layers, but only a limited amount of lipid II can be synthesized, due to the small amount of bactoprenol phosphate molecules (37). Therefore, the lipid II molecules have a high turnover rate...
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and represent a bottleneck of bacterial cell wall synthesis, and are thus an ideal target for antibiotics (37). The most prominent example of an antibiotic targeting lipid II is vancomycin, used as a last resort to treat MRSA infections (62). Vancomycin forms a complex with the C-terminal D-alanyl–D-alanine residue of the pentapeptide of lipid II, thus exerting a steric inhibition on the PBPs and inhibiting cell wall synthesis, which ultimately leads to cell death (156). Other examples are bacitracin, which acts by inhibiting the enzymatic dephosphorylation of C₅₅-isoprenyl pyrophosphate (430), and ramoplanin, which blocks the conversion of lipid I to lipid II by forming a complex with lipid I and also inhibits polymerization of lipid II (272).

Peptidoglycan synthesis has been shown to take place at various defined positions on the membrane at different moments: During cell division synthesis occurs at the septum, while during cell elongation (in case of rod-shaped cells) it is organized in helical threads along the longitudinal axis of the cell (81, 156). Moreover, localization of lipid II coincides with the positions where peptidoglycan synthesis occurs, and if lipid II cannot co-localize in these positions, cell wall formation is inhibited and bacteria are killed (81).

To confirm the interaction of nisin and epidermin with lipid II, the latter was incorporated into artificial phospholipid vesicles liposomes (43). This led to a reduction in the nisin concentration necessary to observe pore formation from micromolar to nanomolar concentrations. Two lipid II molecules per 10⁵ phospholipid molecules were sufficient to yield this effect (38, 43).

In addition, vesicles made of the cytoplasmic membrane of Micrococcus flavus were more sensitive to nisin when their lipid II content was increased (34). The highly specific interaction of nisin and epidermin with lipid II was presumed to involve parts of the lipid moiety and of the disaccharide-pyrophosphate headgroup of the lipid intermediate (41). Site-directed mutagenesis experiments with nisin indicated that variations in the N-terminal ring system have strong effects on the interaction with lipid II. An exchange of Lan in the first ring for MeLan drastically reduced the binding constant for the peptidoglycan precursor, which correlated with a parallel decrease in pore-forming activity in lipid II-containing liposomes. On the contrary, variations in the C-terminal region of nisin had relatively minor influence on the binding to lipid II (473). Although nisin variants with a mutated hinge region were defective in pore formation and had reduced antibacterial activity, their minimal inhibitory concentrations (MIC)
were still in the same range as those of mersacidin, which only blocks peptidoglycan biosynthesis (40, 44, 473).

These results demonstrated the dual-function of nisin, which displays two bactericidal activities: It efficiently blocks lipid II from incorporation into the peptidoglycan and simultaneously uses lipid II for targeted pore formation (Fig. 5). Pores formed by nisin in the presence of lipid II have been shown to be more stable and larger than the ones formed in absence of this docking molecule (35, 163, 471). Moreover, lipid II has been demonstrated to be a constituent of the pore complex, with a stoichiometry of four lipid II molecules and eight nisin peptides (33, 34).

Fig. 5: Molecular activities of class I lantibiotics: (A) At micromolar concentrations, these peptides from wedge-like, target-independent pores. (B) At nanomolar concentrations, nisin forms target-mediated pores using lipid II as a docking molecule. Others class I lantibiotics, as epidermin and gallidermin, bind to lipid II and inhibit peptidoglycan biosynthesis but their ability to form target-mediated pores is restricted to membranes containing short acyl chains phospholipids. Modified from (158).
Fig. 6: (A) The structure of lipid II. (B) Peptidoglycan biosynthesis and the function of the precursors lipid I and lipid II. Positioning of the bactoprenyl moiety is schematic; its actual location in a bacterial membrane is not known. M, N-acetylmuramic acid (MurNAc); G, N-acetylglucosamine (GlcNAc); P, phosphate; shaded circles represent the pentapeptide chain. Antibiotics that act on the peptidoglycan synthesis (mersacidin, vancomycin, ramoplanin, bacitracin) and their sites of action are depicted. Picture from (43).
NMR resolution of the structure of the nisin-lipid II complex revealed the presence of a pyrophosphate cage in nisin, formed by the rings A and B, which binds lipid II via hydrogen bonding between the amide groups of the nisin backbone and the pyrophosphate moiety of lipid II (185). Also, the two N-terminal amino acids that precede the A ring have been shown to be essential for the cage feature, as the first is required for formation of a side-chain interaction with ring B and the NH backbone of the second is part of the pyrophosphate cage (37, 185).

Many class I lantibiotics feature the same N-terminal ring pattern as nisin, suggesting an interaction with lipid II. Indeed, for subtilin, epidermin, gallidermin and mutacin 1140 this interaction has been demonstrated (43, 185, 335). However, beyond the N-terminal double-ring system there is hardly any structural similarity between epidermin-like peptides and nisin. Ring C is completely missing in epidermin and gallidermin peptides, and their flexible hinge region is longer. Besides, the C-terminal tail, which is essential for nisin pore formation, is not flexible as in nisin, but very compact because of the presence of AviCys. In addition, the solution structures of epidermin and gallidermin show amphiphilic screw-shaped molecules with an overall length of 30 Å (117), which is considerably shorter than the length of nisin (50 Å) (129). In a recent study, Bonelli and coworkers showed that lipid II-mediated pore formation capacity of gallidermin in model membranes was reduced in comparison to nisin, and depended on membrane thickness (32). Pore formation was observed only in liposome membranes containing phospholipids with C14 or shorter acyl chains, and was facilitated by the presence of lipid II in the bilayer. Pore formation in intact cells was possible only in staphylococcal and micrococcal strains, in good agreement with the observation that several staphylococcal species (261, 462) and Micrococcus luteus (465) show high amounts of C15 and only little C17 or longer acyl chains. On the contrary, L. lactis membranes have been reported to contain considerable proportions of C16, C18, and C19 phospholipids (191). Possibly therefore, gallidermin was not able to form pores into the membrane of Lactococcus lactis subsp. cremoris HP, while [A12L]gallidermin, a mutant peptide unable to form pores, was as potent as wild-type gallidermin, suggesting that pore formation does not contribute to killing of Lactococcus lactis by gallidermin (32). Remarkably, the authors of this study also observed that epidermin and gallidermin were 10 to 20 times more potent against L. lactis than nisin, despite the missing pore formation capacity (32).
Recently, it was proposed that the high level of antibacterial activity of lantibiotics similar to mutacin 1140 (such as epidermin, and gallidermin), for which pore formation could be observed only in short-chain phospholipids-containing membranes, can be explained by lipid II sequestration from the cell division site (septum), leading to inhibition of cell wall formation and cell death (156). Hasper and coworkers demonstrated that, when giant unilamellar vesicles (GUV) doped with fluorescent lipid II were treated with nisin, an extracellular dye filled up the vesicles as a result of pore formation, and the random-distributed fluorescence originating from lipid II segregated in the membrane. The addition of mutacin 1140 to identical GUVs resulted only in the formation of hot spots of lipid II fluorescence, demonstrating the lipid II sequestration capacity of mutacin 1140. Sequestration was also observed in vivo with B. subtilis and L. lactis cells, where fluorescent patches were observed upon treatment with nisin (156).

For others class I lantibiotics such as Pep5, epicidin 280, and epilancin K, target-mediated pore-formation is likely to occur, although the docking molecule remains to be identified.

5.1.1.3. Additional modes of action

In addition to pore formation and inhibition of the cell wall biosynthesis, nisin and Pep5 have been shown to induce autolysis of susceptible staphylococcal cells, resulting in massive cell wall degradation, predominantly in the area of the septa between dividing daughter cells. Through interaction with the negatively charged teichoic and lipoteichoic acids in the cell wall, these peptides competitively displace by a cation exchange-like process and thereby activate two cell wall hydrolysing enzymes, N-acetylmuramoyl-L-alanine amidase and N-acetylglucosaminidase, resulting in enzyme activation and rapid cell lysis (26, 27). Furthermore, nisin and subtilin inhibit the germination of bacterial spores. The underlying mechanism relies on the presence of a Dha residue in position 5, whose double bond is presumed to provide a reactive group for interaction with a spore-associated factor that is essential for outgrowth of spores (158, 270). Therefore, nisin has at least five different antimicrobial activities based on both high-affinity targets and low-affinity membrane interactions (345).
5.1.2. Class II lantibiotics

5.1.2.1. Mersacidin

Mersacidin has been shown to interfere with the cell wall biosynthesis of *S. simulans* 22 by inhibiting the incorporation of glucose and D-alanine into macromolecules, whereas DNA, RNA and protein synthesis were not affected (40). Brötz and coworkers showed that adsorption of [14C]mersacidin to growing cells was dependent on the availability of lipid II. Moreover, [14C]-mersacidin associated tightly with [14C]-lipid II micelles, and the addition of isolated lipid II to the culture broth efficiently antagonized the bactericidal activity of mersacidin (41). In contrast to nisin, the molecular target site of mersacidin on the lipid II molecule was proposed to be the disaccharide-pyrophosphate moiety of lipid II, as mersacidin seems to have a significantly higher affinity for lipid II than for lipid I, which lacks the GlcNAc residue (41, 42). Therefore mersacidin, like vancomycin, blocks access of the transglycosylase to its substrate (Fig. 7) (41).

The structure of mersacidin was elucidated by NMR analysis and changes in its charge distribution were proposed to play a crucial role in the mersacidin-lipid II interaction (184). In dodecylphosphocholine micelles without lipid II, mersacidin assumed a ‘closed’ conformation, whereas upon addition of lipid II the molecule appeared to ‘open’. A minute flexible hinge was shown to enable these conformational changes and to allow the molecule to adjust the exposure of charges when binding to its "docking molecule," lipid II (184).

![Fig. 7: Class II lantibiotic mersacidin acts by binding to lipid II and blocking its incorporation into peptidoglycan layers without forming pores. Modified from (158).](image-url)
5.1.2.2. Two-peptide lantibiotics

Examples of two-peptide lantibiotics are limited so far, and only for lacticin 3147 substantial efforts have been reported to determine its mode of action. A recent study has revealed a synergistic interaction between Ltnα and Ltnβ which results in antibiotic activities in the nanomolar concentration range, while the individual peptides only possess marginal (Ltnα) or no activity (Ltnβ) (472). The presence of D-amino acids in these peptides has also been shown to contribute to its effectiveness as an antimicrobial agent (286, 377). Lipid II has been shown to be the target of lacticin 3147 on the membrane, similarly to nisin and other class I lantibiotics. Lipid II was shown to be bound by Ltnα, which is structurally similar to mersacidin and, akin to mersacidin but unlike most lantibiotics, it is not positively charged. However, Ltnα was not able to significantly inhibit peptidoglycan biosynthesis in vitro; for full inhibition the presence of Ltnβ was necessary. A model for the mode of action of lacticin 3147 has been proposed, in which Ltnα first interacts specifically with lipid II in the outer leaflet of the bacterial cytoplasmic membrane, and through binding to lipid II acquires a conformation that facilitates the interaction with the more hydrophobic Ltnβ. With the recruiting of Ltnβ, a high-affinity, three-component complex is formed that inhibits cell wall biosynthesis and allows pore formation by Ltnβ (472). As Ltnβ contains three positively charged residues, it may insert in the membrane in response to the trans-negative polarization of the energized bacterial membrane, while its hydrophobic N-terminal helical domain (286), might be located in the core of the membrane and keep contact with the lipid II:Ltnα complex (472). In contrast to nisin, which forms pores of 2 mm in diameter, lacticin 3147 pores were shown to be smaller (0.6 nm), suggesting for a monomeric or dimeric complex with a 1:1:1 stoichiometry (lipid II: Ltnα: Ltnβ). Similarly to mersacidin, lipid II: Ltnα interaction was shown to be restricted to the MurNAc-PP moiety of lipid II and ring B in Ltnα (472).

Given the structural relatedness of plantaricin W and staphylococcin C55 to lactacin 3147, the proposed model is likely to apply to these systems as well.

5.1.2.3. Cinnamycin-like lantibiotics

According to Héchal (158), the cinnamycin-like class II lantibiotics act by increasing membrane permeability (356), by impairing ATP-dependent protein translocation (58)
and calcium uptake (315). Moreover, duramycin and cinnamycin have been shown to inhibit various metabolic processes in eukaryotic cells (115), such as induction of haemolysis of erythrocytes (63) or inhibition of phospholipase A2, leading to interference of prostaglandin and leucotriene biosynthesis (284). Most of these effects described in the literature can be explained by the specific binding of these lantibiotics to phosphatidylethanolamine (PE). Indeed, PE is the substrate of phospholipase A2 and is sequestered by duramycins (115, 331). Moreover, PE, which resides in the inner layer of the plasma membrane, was shown to be bound by cinnamycin (281). A recent study showed that cinnamycin induces transbilayer phospholipid movement in target cells that leads to the exposure of inner leaflet PE to the toxin and to membrane re-organizations with a cytotoxic effect (281).

5.1.2.4. Cytolysin

Cytolysin is unique among the lantibiotics for its ability of forming pores in eukaryotic cell membranes, in addition to bacterial membranes. This activity requires the synergistic action of its two peptides, CylL_{L}''/L_{S}'''. As cytolysin has been shown to form pores in the membrane of human erythrocytes, which have a low transmembrane potential (8 mV) (382), the minimum membrane potential required for pore formation by cytolysin is supposed to be lower than in other lantibiotics. However, as for other lantibiotics, the membrane potential of the target cell may not be the only factor that influences pore formation by cytolysin. It was shown that sheep and goat erythrocytes were not susceptible to cytolysin-mediated haemolysis, in contrast to human, horse, cow and rabbit erythrocytes (231). This susceptibility was shown to correlate to the higher phosphatidylcholine levels in the outer leaflet of the latter erythrocyte membranes (370), suggesting that phosphatidylcholine may be the target for cytolysin. A recent study aiming at identification of functional domains of cytolysin, reported that CylL_{S}''' mostly contributes to the lytic activity against eukaryotic cells and bacteriocin activities (67). When tested with complementing, wild-type CylL_{L}'', activities could be assigned to three distinct domains of CylL_{S}''': The N-terminal domain appeared to be critical for haemolysin and bacteriocin activity, while a central hydrophobic region was found to be critical for haemolysin- but not for bacteriocin activity. Moreover, several residues found near the C-terminus were necessary for either haemolysin or bacteriocin activities, but not for both activities (67). As haemolytic and bacteriocidal activities seem to involve distinct regions of cytolysin, it
is likely that different mechanisms mediate disruption of prokaryotic and eukaryotic membranes (66).

5.2. Biological activity

Bacteriocins were originally defined as bacterially produced peptides which inhibit only closely related species (435). In agreement with this notion, cationic antimicrobial peptides produced by Gram-positive bacteria, such as lantibiotics, exert high antibacterial activity against Gram-positive but not against Gram-negative bacteria and mammalian cells. Gram-negative bacteria differ from Gram-positive for having a smaller cell wall/peptidoglycan layer, and for the presence of an outer membrane in addition to the cytoplasmic membrane that functions as a size-selective permeability barrier (153). Both the outer leaflet of the plasma membrane as well as the outer membrane contain anionic molecules (105), therefore, the insensitivity of Gram-negative bacteria to nisin and other lantibiotics is likely to be due to their size (1.8-4.6 kDa), which restricts their passage across their outer membrane (161, 383). On the contrary, lantibiotic show no action against mammalian cells, as the extracellular monolayer of their plasma membranes contain zwitterionic amphiphiles, for which cationic antimicrobial peptides have low affinity (105).

Nisin, produced by L. lactis subsp. lactis, is highly active against lactococci, which emphasizes its bacteriocinic nature. It is mostly inhibitory for Gram-positive bacteria, and shows remarkable activity against Staphylococcus spp. (MIC 4.2 µg mL⁻¹), Streptococcus spp. (MIC 8.4 µg mL⁻¹), Bacillus spp. (MIC 4.2 µg mL⁻¹), Micrococcus spp. (MIC 1.1 µg mL⁻¹), Clostridium spp. (MIC 1.1 µg mL⁻¹), Enterococcus spp. (MIC 16.7 µg mL⁻¹), Listeria spp. (MIC 4.2 µg mL⁻¹), and Propionibacterium acnes (MIC 2.1 µg mL⁻¹) (306, 357, 407). Besides, it is also active against some Gram-negative strains of Neisseria and Flavobacter spp, as well as against Helicobacter pylori (86, 288, 324). In addition, it has been shown to be active against Mycobacterium tuberculosis, the causal agent of tuberculosis, both in vitro and in vivo (171, 288). Moreover, nisin and subtilin inhibit the germination (sporostatic) of spores from Bacillus and Clostridium species, with spores being more sensitive to nisin than vegetative cells (86). Yeasts, fungi, and viruses are not inhibited by low concentrations of nisin (51, 324, 484).
Interestingly, a recent study reported that purified nisin Z was able to cause permeabilisation of the cytoplasmic membranes of both *S. aureus* and *E. coli*, if the antimicrobial assay was performed in the presence of a low NaCl concentration (259). In contrast, the presence of 100mM NaCl in the assay reduced the antimicrobial activity of nisin Z against *E. coli* but not against *S. aureus*. Moreover, purified nisin Z was shown to be able to permeabilize the outer membrane of *E. coli*. Minimum inhibitory concentrations of purified nisin Z against *S. aureus* and *E. coli* were reported to be 75 nM and 600 nM, respectively. The authors proposed nisin to have different antimicrobial mechanisms against Gram-positive and Gram-negative bacteria (438).

In a mouse infection model, nisin has been shown to be 8-16 times more active than vancomycin against *Streptococcus pneumoniae*, although they both had similar MICs. 100% protection of the mice was achieved with a two-dose intravenous regimen of ≥ 0.16 mg kg⁻¹ treatment⁻¹ (128).

Gallidermin and epidermin have been reported to have good antimicrobial activity against *S. aureus* (MIC 4-8 µg mL⁻¹), *S. epidermidis* (MIC 4 µg mL⁻¹), *S. pneumoniae* (MIC 4 µg mL⁻¹), *Streptococcus pyogenes* (MIC 1 µg mL⁻¹), *Corynebacterium xerosis* (MIC 1 µg mL⁻¹), *M. luteus* (MIC 0.25 µg mL⁻¹), and *Propionibacterium acnes* (MIC 0.125-0.25 µg mL⁻¹) (212).

Pep5 has been shown to have similar MICs as gallidermin and epidermin against *S. aureus* (MIC 4 µg mL⁻¹), *S. epidermidis* (MIC 2 µg mL⁻¹), *C. xerosis* (MIC 8 µg mL⁻¹), but also to be significantly less active against *M. luteus* (MIC 128 µg mL⁻¹), *S. pyogenes* (MIC 128 µg mL⁻¹), and *P. acnes* (MIC 128 µg mL⁻¹) (180).

Unfortunately, comparison with MICs of nisin A is not possible, as different methods were used for determination of the minimal inhibitory concentrations. Epidermin has also been shown to have strong activity against both *Streptococcus mutans* and *Streptococcus sobrinus*, the principal causative agents of caries (13). Recently, the activity of gallidermin, nisin A, and vancomycin, as well as their cytotoxicity against intestinal epithelial cells have been investigated in vitro (277). Toxicity of nisin in vaginal, colonic and kidney epithelial cells had been already reported (6, 309). Nisin A and gallidermin were more potent than vancomycin against both *E. coli* and *M. luteus* (277). Gallidermin had the lowest level of cytotoxicity against intestinal epithelial cell lines HT29 and Caco-2, with two-fold lower toxicity than FDA approved nisin A, and, in contrast to it, gallidermin did not result in
erythrocyte haemolysis (277). Therefore, gallidermin has great therapeutic potential for treatment of infections of the gastrointestinal tract.

*Lactococcus lactis* DPC3147 produces the two-component lantibiotic lacticin 3147, and its supernatants were shown to have a broad spectrum of inhibition against *Lactococcus* spp., *Lactobacillus* spp., *S. aureus*, *Streptococcus thermophilus*, *Bacillus* spp., *Clostridium* spp., *Enterococcus* spp, and *Listeria* spp. (380). Lacticin 3147 was also recently shown to be active against a range of genetically distinct *Clostridium difficile* (MIC 3.6 µg mL⁻¹) (361). Another two-component lantibiotic, antibiotic 107891, which was newly reported by Vicuron Pharmaceuticals and shares structural similarities with the epidermin class (262), was shown to have good MICs against a broad range of bacteria, from *S. aureus* (MIC < 2 µg mL⁻¹), *S. pneumoniae* (MIC < 0.1 µg mL⁻¹), *P. acnes* (MIC < 0.25 µg mL⁻¹), *Enterococcus* spp (MIC 0.5-4 µg mL⁻¹), to the Gram-negative pathogens *Moraxella catharralis* (MIC < 1 µg mL⁻¹) and *Neisseria gonorrhoeae* (MIC < 0.5 µg mL⁻¹) (262). Moreover, it was reported to be active against MRSA, VISA and VRE (84).

Mersacidin exhibits only moderate *in vitro* MIC values against staphylococci, streptococci, bacilli, clostridia, corynebacteria, peptostreptococci, and *P. acnes* (268, 318). However, it is significantly active *in vivo*: It effectively cured systemic staphylococcal infections in mice including those caused by methicillin-resistant *S. aureus*, as well as subcutaneous staphylococcal abscesses in rats (244, 318). In both cases, the lantibiotic had the same or more activity than vancomycin.

The cinnamycin-like class II lantibiotics show a relatively restricted bactericidal activity, affecting only a few bacterial strains, in particular, specific strains of *Bacillus* spp. (331).

Cytolysin is lethal for a wide range of Gram-positive bacteria (*Enterococcus* spp., *Streptococcus* spp., *Staphylococcus* spp., *Lactobacillus* spp., *Bacillus* spp., *Micrococcus* spp., *Clostridium* spp., *Corynebacterium* spp.) (79) and is also toxic to higher organisms (66). By interacting with and perturbing normal host cell function, cytolysin is presumed to enhance tissue invasion by *Enterococcus faecium* and *Enterococcus faecalis*, which have the ability to break the normal commensal mode in the human gastrointestinal tract and to invade extra-intestinal niches resulting in disease (365). *E. faecium* and *E. faecalis* rank among leading causes of nosocomial surgical site, bloodstream and urinary tract infections. Cytolysin is thus a virulence factor, whose toxicity in murine lethality tests was first reported by Ike and coworkers.
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(190), and which has later been shown to be an important determinant of lethality in endocarditis (64).

6. Mechanisms of lantibiotic resistance

Microbial resistance to antibiotics appears as a result of the evolutionary pressure exerted by the exposure of bacteria to these compounds, and is eventually unavoidable (461). This problem is particularly acute in clinical settings, where the selective pressure for the evolution of antibiotic resistance is higher because of the intense use of antibiotics (37). There are several mechanisms of antibiotic resistance: The first involves enzymatic inactivation of the active molecule, e.g. degradation of the β-lactam ring of penicillins by β-lactamases. The second mechanism relies on shielding of the target such that the antibiotic cannot get access to it, for example by cell-surface alteration or by removing the antibiotics by efflux pumps. The third mechanism involves the replacement or modification of antibiotic targets (461). The latter mechanism is responsible for high-level bacterial resistance to vancomycin, where five enzymes act together to effect a change in the sequence of the pentapeptide of lipid II. Conversion of the terminal D-alanine to a D-lactate or D-serine leads to a considerable reduction in the binding affinity of vancomycin for lipid II, rendering the antibiotic inactive (37, 461).

Often, the development of clinically applicable conventional antibiotics has been biased towards the concept of each antibiotic having a single primary target and a single mode of action (154). In contrast, in the case of lantibiotics, several antibiotic activities are combined in one molecule, and their dual mechanism of action might attenuate the emergence of bacterial resistance (55, 154).

However, even though the immunity/resistance systems of lantibiotic producing strains seem to be highly specific for the corresponding antimicrobial peptides, and although no examples of lantibiotic immunity/resistance genes occurring independently of the genetic information for lantibiotic synthesis were reported to date, nisin resistance in Gram-positive bacteria has often been generated in sensitive strains by repeated exposure to increasing amounts of cationic peptides. Moreover, resistant S. gallinarum and S. epidermidis strains that tolerated gallidemin concentrations up to 600 mg L⁻¹ and epidermin concentrations up to 1 g L⁻¹ were isolated by repeated selection of wild-type strains (181, 448, 449). The authors
suggested that this increase in tolerance was probably due to the presence of immunity genes or to modifications of the cell membrane. To our knowledge, the nature of this tolerance has never been investigated. However, as immunity genes were later discovered in the clusters of the wild-type producers, this effect is likely to be based on membrane modifications, although it might be also caused by amplification of the immunity genetic determinants.

Most of the data published so far indicate that the main mechanism leading to lantibiotic resistance is a shielding mechanism involving incorporation of positive charges in the cell wall and thereby avoiding interaction with their targets molecules, i.e. lipid II for nisin and the majority of lantibiotics.

6.1. Alteration of composition of the cell wall or membrane.

Gram-positive cell walls contain polymers of alternating phosphate and alditol groups called teichoic acids (112). These highly charged polymer chains are either covalently connected to peptidoglycan (wall teichoic acids, WTA) (103) or to membrane glycolipids (lipoteichoic acids, LTA) (113). Both LTA and WTA polymers are substituted with D-alanine (D-Ala) esters and hexose or N-acetyl hexosamine residues (112, 113). Peptidoglycan (394) and teichoic acids (103) bear net negative charges, as does the cytoplasmic membrane surface (413).

Induced resistance to lantibiotics is a complex phenotype relying on changes in cell wall and/or cell membrane composition, which has been largely investigated for the prototype class I lantibiotic nisin (80, 266, 290, 299, 453). In a resistance-induced *Listeria monocytogenes* Scott A strain, dissipation of the proton motif force (formed by $\Delta\text{pH}$ and $\Delta\psi$) was shown to be significantly reduced (299). The authors reported an increase in phosphatidylglycerol/diphosphatidylglycerol ratio from 5 (in the wild-type strain) to 7 (in the resistance-induced strain). As diphosphatidylglycerol shows a higher negative charge density than phosphatidylglycerol, and therefore contributes to stronger binding of the cationic nisin, a greater ratio may therefore increase resistance by reducing binding affinity (55, 299). Other changes in cytoplasmic membrane composition that might result in reduced binding of nisin have been reported for the resistance-induced *L. monocytogenes* Scott A: A lower ratio of C15/C17 long-chain fatty acids, an increased amount of PE, and lower amounts of anionic phospholipids cardiolipin and phosphatidylglycerol (80). Moreover, these
changes might also inhibit pore formation by increasing membrane rigidity. In addition, nisin resistance in *L. monocytogenes* and *C. botulinum* has been related to increased membrane rigidity due to the presence of more straight-chained, monounsaturated, and saturated fatty acids and less branched, polyunsaturated fatty acids (289, 290, 299).

Recent studies have also reported a highly regulated antimicrobial resistance mechanisms that involves cytoplasmic membrane modification in Gram-positive bacterial pathogens (340). Enhanced *S. aureus* resistance to defensins and protegrins, two antimicrobial peptides that are components of the human and porcine innate immune defense, has been linked to lysine modification of phosphatidylglycerol present in the cytoplasmic membrane (342). Lysyl-phosphatidylglycerol reduces net electronegativity of the cytoplasmic membrane and presumably diminishes affinity for or increases repulsion of cationic peptides. The production of lysyl-phosphatidylglycerol was dependent on the presence and function of *mprF* in *S. aureus* (426). Interestingly, *mprF* is a gene believed to have close analogs in other human pathogens, including *M. tuberculosis*, *P. aeruginosa*, as well as *E. faecalis*.

A further study with a nisin resistance-induced strain of *Listeria innocua* reported a thickening of the cell wall, which was also shown to be less hydrophobic and to be resistant to hydrolysis by murein enzymes (lysozyme and mutanolysin) (279). Bierbaum and Sahl demonstrated that cationic peptides (including nisin) adsorb to the teichoic acids of the cell wall of Gram-positive bacteria, thereby activating its autolytic enzymes (25, 26). However, high concentrations of the cationic peptides inhibited autolysin activity (25). Therefore, both activation of murein synthesis and autolysin inhibition were suggested to have contributed to the cell wall thickening of the nisin resistance-induced strain of *L. innocua* (279). Moreover, the development of resistance to nisin was accompanied by altered sensitivities to cell wall-acting antibiotics, resulting in increased resistance against carbenicillin and vancomycin (279). A similar thickening of the cell wall was also observed by transmission electron microscopy in a nisin-induced resistant *Streptococcus thermophilus* INIA 463 (120). However, in contrast to most other cases in which resistance persists even under non-selective conditions (282, 289, 290), this strain lost resistance after a single transfer to nisin-free medium (120).

The frequency of emergence of spontaneously resistant mutants of *L. lactis* ssp. *lactis* IL1403 to lacticin 3147 was recently investigated (148). Exposure to
increasing concentrations of lacticin 3147 resulted in the isolation of spontaneous mutants which showed a cross-resistance to other lantibiotics such as nisin and lacticin 481. As reduced adsorption of the bacteriocin in to the cell was observed, resistance is likely to be acquired by adaptation of the cell wall or membrane to reduce access of the bacteriocin to the cell. The same feature has been reported for certain nisin-resistant mutants of *L. monocytogenes* (83) and for a lacticin 3147 resistant mutant of *Lactobacillus paracasei* spp. *paracasei* (381).

As discussed before, the net charge of the bacterial envelope, which is modulated during the cell cycle and under varying physiological conditions, is an important contributor to the susceptibility of bacteria towards cationic peptides. A nisin-resistant strain of the rumen bacterium *Streptococcus bovis* was shown to have a decreased negative surface charge (282). Besides, reduction of the net negative charge of the cell wall has been suggested to confer increased resistance to nisin and gallidermin in *S. aureus* Sa113 and *S. xylosus* C2a (344). Peschel and coworkers demonstrated the direct involvement of the *dlt* operon in resistance of *S. xylosus* and *S. aureus* to both lantibiotics (344). The gene products of the *dlt* operon are involved in D-alanylation of teichoic acids: DltA activates D-Ala in the cytoplasm by via ATP hydrolysis and couples it to the phosphopantetheine prosthetic group of the D-Ala carrier protein DltC (157). Moreover, the hydrophobic protein DltB is likely to be involved in the transfer of D-Ala across the cytoplasmic membrane, and DltD is assumed to catalyze the esterification of teichoic acid alditol groups with D-Ala resulting in the introduction of positive charges into the otherwise negatively charged teichoic acids (344).

Sensitivity toward a variety of membrane-active antimicrobial peptides was significantly increased in *S. aureus* and *S. xylosus* *dlt* mutants, providing evidence for a role of D-Ala residues in reduction of the negative cell wall charge and thus in the protection of the bacteria against these substances. The same mechanism was shown to be true for nisin resistance in *B. subtilis* (50). Cao and Helmann showed that inducible protection against antimicrobial peptides in *B. subtilis* is likely to be achieved by the action of the extracytoplasmic-function σ^X^ factor, which partly controls the enzymes involved in biosynthesis of PE as well as the D-alanylation of LTA and WTA. Thus, esterification of the glycerol moieties of teichoic acids with D-alanine (D-Ala) introduces free amine (-NH2) groups into the cell wall and leads to a reduction in negative charge. Similarly, the increased content of the zwitterionic PE
molecule in place of anionic phospholipids lowers the overall negative charge (55). The $\sigma^X$ regulon is composed by $\text{pbpX}$ (penicillin-binding protein), $\text{wynJ}$, the $\text{dlt}$ operon, and the $\text{pss ybfM psd}$ operon (PE biosynthesis). Analysis of $\text{sigX}$ (encoding $\sigma^X$), $\text{pssA}$ (encoding a phosphatidyl serine synthase) and $\text{dltA}$ deletion mutants showed an increased sensitivity to nisin. Therefore, in $\text{B. subtilis}$, $\sigma^X$ may serve to regulate the net charge in the cell envelope by affecting the expression of both the $\text{dlt}$ and $\text{pssA}$ operons, and this, in turn may affect sensitivity to cationic antimicrobial peptides (50). Recently, the role of $\text{dltA}$ operon in conferring resistance to cationic antimicrobial peptides has been also demonstrated in the case of $\text{S. pneumoniae}$ (239): Phenotypic analysis of $\text{dltA}$-proficient and $\text{dltA}$-deficient $\text{S. pneumoniae}$ strains showed that $\text{dltA}$ inactivation resulted in enhanced sensitivity to nisin and gallidermin, in full agreement with results with $\text{dltA}$ mutants of other low G+C Gram-positive bacteria. Moreover, $\text{dltA}$ was deleted in a clinical $\text{E. faecalis}$ isolate, which was significantly impaired in biofilm formation, and sensitivity to several cationic antimicrobial peptides (polymyxin B, colistin, and nisin) was considerably increased, underlining the important role of D-Ala esters of teichoic acids in $\text{E. faecalis}$ virulence (106). From these results, it can be assumed that D-Ala esters are part of a system that reduces bacterial susceptibility to a broad range of cationic antimicrobial molecules.

In conclusion, evidence is accumulating to support the hypothesis that microbial pathogens modify their envelope to reduce the accessibility of lipid II and subvert antimicrobial peptide susceptibility.

6.2. Resistance mechanisms involving the lantibiotic target

Several lantibiotics have been reported to act by binding with high affinity to lipid II and thereby inhibiting peptidoglycan formation and, in some cases, by successively forming pores in the Gram-positive cell membrane. Therefore, high selective pressure for target modification is likely to be present. However, in contrast to recent reports on resistance or tolerance to vancomycin (321), no resistance to nisin has been reported, despite its prolonged use as a preservative for almost 50 years (38).

Kramer and coworkers recently investigated the relation between resistance to nisin and the amounts of membrane associated lipid II in $\text{M. flavus}$, $\text{L. monocytogenes}$ and their isogenic nisin resistance-induced variants (242). No
significant differences were observed in the maximum amount of lipid II in the membranes of nisin resistance-induced variants and the wild-type strains, indicating that resistance to nisin is not related to lipid II levels (242).

Gravesen and coworkers have studied nisin resistance in a spontaneous mutant of *L. monocytogenes* 412 by analyzing changes in gene expression by restriction fragment differential display, and reported increased expression of *pbp2229*, encoding a homologous protein to the glycosyltransferase domains of high molecular-weight penicillin binding proteins (PBPs), and *hpk1021*, coding for a histidine protein kinase (HPK) (134). As PBPs catalyze the incorporation of the disaccharide-pentapeptide moiety of lipid II into the growing peptidoglycan chain, PBP2229 was suggested to mediate enhanced nisin resistance by shielding lipid II and possibly also by reducing the extracellular lipid II concentration (133). The production of PBP2229 was dependent on the production of the histidine protein kinase, and may be thus controlled by a two-component signal transduction system that includes HPK1021 (133). Expression of *dltA* was also increased, and thus resistance might also rely on the same mechanism reported for *S. aureus* by Peschel and coworkers (344). Another study elucidated the role as a virulence factor of a *L. monocytogenes* LO28 two-component signal transduction system, LisRK (75). The authors showed that LisRK is involved in the ability of the cell to tolerate antimicrobials used in food and in medicine, e.g., nisin and cephalosporin. Indeed, a *lisK* deletion mutant displayed significantly enhanced resistance to nisin and increased sensitivity to the cephalosporins, as well as a significant reduction in the expression of genes encoding a PBP and a HPK (other than LisK). Interestingly, in this case transcription of the genes encoding a putative PBP and HPK is reduced while nisin resistance increased, which is in contrast to the observations of Gravesen. This difference has been reported to be attributable to the different experimental conditions of the two studies.
6.3. Enzymatic inactivation

Already in 1967, several *Bacillus* spp. strains were shown to express a protein that specifically modified lantibiotics, including nisin (201). Although this protein has never been characterized, it was proposed to inactivate the peptide by modification of the dehydrated residues (202).

One obvious method for bacteria to inactivate cationic antimicrobial peptides is to produce peptidases and proteases that degrade these peptides (345). Simple linear structure or α-helical structure of some antimicrobial peptides, as LL-37, a human antimicrobial peptide, are particularly susceptible to proteolysis and several microbial proteases have been shown to cleave such peptides. For instance, the aureolysin protease from *S. aureus* was shown to inactivate LL-37 and contribute to bacterial survival during exposure to this antimicrobial peptide (416). However, the thioether-crosslinks of lantibiotics create a rigid bridged structure, which renders them considerably more resistant to proteolysis (154). However, proteolytic degradation is suspected to be the cause for nisin resistance in the nonproducer *L. lactis* subsp. *diacetylactis* DRC3 (55, 118). This strain bears a 60 kb plasmid, containing a nisin resistance (*nsr*) gene, without significant sequence homology with genes of the nisin producer *L. lactis* 11454 (118). Recent BLAST analysis revealed the presence in Nsr of a C-terminal conserved tail-specific endopeptidase domain (55). However, the role that would be played by such a protease is disputable, as nisin activity is tolerant to C-terminal cleavage as the N-terminus is responsible for binding to lipid II (37).

6.4. Lantibiotic efflux

It is of general concern that resistance based on lantibiotic efflux systems might arise once these peptides would be clinically used. As producer self-immunity is based on ATP-binding cassette transporters, there is a theoretical possibility for this resistance to be transferred to pathogenic strains. Moreover, recently, enhanced resistance levels to nisin and polymyxin B were generated in a *L. lactis* strain in which two genes were expressed, encoding hypothetical membrane proteins with a high homology with members of the ATP-binding cassette (ABC) family of multidrug efflux transporters (283). These genes were discovered in a *Bifidobacterium breve* strain, an important beneficial member of the human gut microbiota (283).
Another study showed that the plasmid-encoded gene qacA mediates staphylococcal resistance to the human antimicrobial peptide tPMP-1 (thrombin-induced platelet microbicidal protein-1) (252). However, the expression of qacA did not appear to impart cross-resistance to other structurally distinct cationic peptides, including defensins or the lantibiotics Pep5 or nisin (252).

Taken together, the above findings indicate that bacteria have evolved structure-specific and energy-dependent mechanisms to subvert actions of antimicrobial peptides, and these mechanisms might also lead to resistance of lantibiotics.

7. Application of lantibiotics

A number of features of lantibiotics make them particularly attractive for both food and biomedical applications. Many of these post-translationally modified peptides have a broad spectrum of activity against Gram-positive food spoilage and pathogenic bacteria and are relatively thermostable and resistant to proteolytic breakdown. Moreover, it can be assumed that these molecules are relatively safe, at least when taken orally, because bacteriocins of lactic acid bacteria, in particular nisin, have a long history in food preservation (77). According to Cotter, nisin was first marketed in England in 1953 and has since been approved for use in over 60 countries. In 1969, nisin was assessed to be safe for food use by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives. In 1983, this bacteriocin was added to the European food additive list as number E234 and, in 1988, it was approved by the US Food and Drug Agency (FDA) for use in pasteurized, processed cheese spreads (76).

7.1. Pharmaceutical applications

The potential of lantibiotics for clinical applications has been apparent ever since 1928, when activity of nisin against tubercle bacilli was first reported (371). Since then, various clinical applications of lantibiotics have been envisaged, including the topical treatment of infections, the treatment of bovine mastitis, of systemic infections due to staphylococci, streptococci, enterococci, and C. difficile gastrointestinal infections (84, 154).
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However, the most significant potential application of lantibiotics may be in the treatment of antibiotic resistant pathogens (76). Mersacidin is promising for the treatment of MRSA nasal colonization, and was shown to be able to eradicate colonization in a mouse-rhinitis model (244). Improved systems for production of mersacidin variants in a non-producing mrsA-deficient strain of Bacillus sp. HIL Y-85,54728 have been disclosed by Novacta Biosystems Ltd. (85). While vancomycin is still used to treat MRSA and enterococcal infections, vancomycin-intermediate S. aureus (VISA), vancomycin-resistant S. aureus (VRSA) and vancomycin-resistant enterococci (VRE) have emerged. Notably, it has been shown that vancomycin-resistant strains remain susceptible to mersacidin, and that similar mersacidin concentrations to those of vancomycin are effective in treating these infections (40-42). Moreover, mersacidin is remarkably resistant to proteolytic digestion and antibodies against it are difficult to obtain (268).

Also nisin might be a good candidate for the treatment of systemic infections, as it has been reported to be 8-16 times more active than vancomycin in the treatment of S. pneumoniae in a mouse infection model (128). Moreover, nisin was active against VRE, multidrug-resistant S. pneumoniae and S. aureus, and reduced the viable titer about 10^3 -10^4-fold (406). However, MRSA and VRE had to be treated with concentrations of nisin as high as 10-20 mg L^{-1}, and serial exposure with nisin resulted in the appearance of stable nisin-resistant S. pneumoniae mutants (406). Lacticin 3147 is also effective against MRSA, VRE, and penicillin-resistant pneumococci (119), and, together with nisin, has great potential as a treatment of C. difficile-associated diarrhoea (CDAD), the most common hospital-acquired enterocolitis (361). Management of diarrhoea caused by infection with C. difficile is frequently complicated by relapses after apparently successful therapy (217). Nisin has been shown to be especially attractive in this respect, as it prevents germination of clostridial spores. In vitro tests resulted in inhibition with killing kinetics similar to vancomycin (217).

Another application considered for lantibiotics is the prevention and treatment of bovine mastitis, which is usually treated with antibiotics. The emergence of antibiotic-resistant S. aureus, Streptococcus alagatiae, and Streptococcus uberis strains and the potential presence of antibiotic residues in milk makes alternative treatments desirable. A patent has been disclosed for the topical use of gallidermin and epidermin against mastitis (337), and nisin has been evaluated as a treatment for
mastitis since the 1940 (76). However problems due to teat irritancy were encountered, probably due to the presence of impurities. Nowadays, nisin is the active ingredient of two commercial products used in the prevention of mastitis, Consept®, which is applied as a teat dip, and WipeOut® (both from Applied Microbiology, Inc., New York, N.Y.), used as a teat wipe (76). Recently, a nisin-based formulation was shown to be very efficient in the treatment of mastitis in lactating dairy cattle (49). Also lacticin 3147 has been shown to have considerable potential when incorporated into a commercial teat seal, an oil-based formulation which forms a physical barrier against infection in the area of the teat canal and sinus (379).

Some research has focused on topical applications of lantibiotics against skin infections. As an example, gallidermin is considered as an excellent candidate for the topical treatment of juvenile acne, eczema, impetigo and cellulitis (487). Lacticin 3147 was also proposed for such applications (3).

Lantibiotics are also very resistant to acidic pH (86) and are thus good candidates as potential antibacterial agents against *H. pylori*, which has been strongly associated with peptic ulceration and gastric cancer (203). Moreover, nisin is also resistant to digestion by the gastric protease pepsin, but it is broken down in the intestine reducing its effect on the gut microflora (86). Indeed, nisin (Ambicin N) is currently being tested for this application (86).

Moreover, nisin has also been shown to have potential as a contraceptive, as intravaginal applications completely inhibited sperm motility (6). Duramycin is undergoing clinical evaluation for non-antimicrobial applications in cystic fibrosis and dry eye syndrome (300), and, according to Dawson (84), there is strong interest in the use of mutacin 1140 for prevention of dental caries and for prophylactic use against throat infections (167).

As all clinical trials performed to date have focused on topical applications against surface infections, rather than parenteral and oral applications against systemic infections, the toxicity issue remains that may limit systemic application (154). Nevertheless, lantibiotics are not considered to be membrane-active against eukaryotic cells, owing to the absence of anionic phospholipids and lipid II, to the presence of cholesterol in these membranes, and to the lack of a strong membrane potential gradient (37, 154, 156, 482). Even tough lantibiotics are among the more proteolysis-resistant antimicrobial peptides, due to their rigid structure, another disadvantage might be their potential lability to proteases, which could give rise to
unfavorable pharmacokinetics. In the case of nisin, as an example, its poor pharmacokinetic properties seem to be the key hindrance to its clinical development (37, 154).

However, in the last decades, great advances in the understanding of lantibiotic biosynthesis and properties have been achieved, which enable the implementation of rational mutagenesis strategies to alter individual residues for optimization of their antimicrobial activity by widening the active pH range, improving stability, and enhancing target affinity. Moreover, by taking advantage of the newly developed in vitro modification systems, tailor-made or hybrid peptides with novel activities might be generated to target particular pathogens.

7.2. Foodstuff applications

Bacteriocins of lactic acid bacteria have received particular attention in the last decades due to their potential application in the food industry as natural preservatives. Nisin is the only purified lantibiotic that has been licensed for utilization as a food preservative by the US FDA, and has been employed for over 50 years in more than 60 countries as a shelf-life extender in a broad range of products, ranging from processed and cottage cheese, liquid egg products, canned vegetables, diverse pasteurized dairy products, and salad dressings (458). It is also effective for inhibition of spoilage bacteria during beer and wine fermentations, and the exploitation of nisin-producing strains has been shown to improve certain vegetable fermentations (334).

According to Papagianni (334), biopreservatives can be delivered to a food in different ways; for example, lantibiotic-producing strains can be used as a starter or as an adjunct strain in a fermented food. In addition, a producer strain could be used as a protective culture on the surface of a suitable foodstuff, to protect against growth of undesirable bacteria. These delivery systems have the advantage of being extremely cost effective, as the producer can be used as starter- or protection-culture so that the lantibiotic is produced in situ (444). Alternatively, lantibiotics can be employed as food additive, and added as a purified or concentrated preparation, depending again on the specific food (444). An important example is the commercial preparation of nisin, Nisaplin (Aplin & Barrett Ltd., Applied Microbiology Inc.), which contains 2.5% nisin A and other ingredients consisting of salt and milk solids derived from the fermentation of a modified milk medium by nisin producing strains of L. lactis,
and is standardised to a concentration of 1 million international units per gram (86). Production of lacticin in a powdered form, incorporation into a food infant formula and successful protection against *Listeria monocytogenes* was also reported (303).

As nisin is produced by lactococcal cultures, its principal application has been in the manufacture of cheese and dairy products. In this type of product, the pathogen of primary concern is the Gram-positive bacterium *L. monocytogenes*, which is also capable of growing at refrigeration temperatures (107) and can survive the acidic conditions during cheese manufacturing. *L. monocytogenes* is an intracellular pathogen found in meat, poultry, seafood and cheese, which has been implicated in several outbreaks of food-borne disease. Listeriosis is found mainly among pregnant women and immunocompromised individuals, with symptoms of abortion, neonatal death, septicemia, and meningitis, and a mortality rate of about 24% (107). Nisin-producing lactococcal strains and Nisaplin have demonstrated the effectiveness of nisin as a bacteriostatic and bactericidal agent against *L. monocytogenes* in cheese, and the MICs ranged between 2.5 and 10 mg L\(^{-1}\) (109, 278). A system for controlling growth of *Listeria* in the production of surface ripened soft cheese was devised, which involves addition of nisin to the raw milk at levels ranging from 0.625 to 2.5 mg L\(^{-1}\), followed by a mild heat treatment, and then the addition of nisin producing starter cultures (86, 364). Besides, clostridial spores are often present in dairy and cheese ingredients, and are usually able to survive the heat treatment during processing. Nisin treatment has been shown to prevent spore outgrowth and to protect from cheese spoilage, as well as from toxin production by *C. botulinum* in cheese spreads (423).

Since direct addition of nisin to cheese-milk products is costly, the use of nisin-producing starters is the favourite strategy. However, no existing nisin-producing starters have the flavor-generating, acidifying activities required for the manufacture of most cheese types. Therefore, special nisin-producing starters have to be developed, i.e. the one used in the production of Gouda cheese (86). Since the ability to produce nisin is encoded on a transposon, this trait was transferred to industrial strains, which were then carefully selected for the above mentioned characteristics (86). Nisin has also been found to be effective in various food products including canned foods, whose storage at warm ambient temperatures without spoilage is facilitated by nisin addition, fish and meat, confectionary, brewing, wine, and pasteurized liquid egg.
The success of nisin has stimulated the search for novel potential biopreservatives. Enterocin has also been found to control the growth of *L. monocytogenes* in various meat products (121), and lacticin 3147 has been shown to be an effective biopreservative in many food applications (374). Moreover, as the genetic determinants of lacticin 3147 are encoded on a self-transmissible plasmid, the ability for production of lacticin 3147 has been transferred to different lactococcal hosts, many of which are derivatives of commercial starter strains (380, 444). These strains are suitable as alternative starters for cheese manufacture, as they can offer the product an ‘in-built’ protection against undesirable flora.

8. Production of lantibiotics

The largest issue of lantibiotic production is the high cost of manufacturing, which has limited both their testing for potential clinical applications and their development into commercial preparations.

Peptides tend to be very expensive drugs, costing between $100 and $600 per gram (an average daily dose for most systemic therapeutics) or more, if manufacturing is performed by solid-phase chemical synthesis (154). Moreover, chemical synthesis of lantibiotics is too complicated at present; therefore, less expensive lantibiotic-production platforms are still needed. Process development has followed various strategies: relying on wild-type strains, design of novel producers, or adaptation of process operations; however, none of these approaches have proven commercially feasible to date.

8.1. Fermentations

Extensive efforts have been dedicated to the optimization of the fermentative production of the lantibiotics nisin, epidermin, gallidermin, and Pep5 by their natural producing strains. According to Theobald (440), crucial points in achieving industrial standards are the optimization of the culture medium (135), the development of a suitable and reproducible process (110), the scale-up (oxygen transfer and mixing of the reactor) (363), and the implementation of a down-stream process (425).
8.1.1. Nisin

Nisin production has been studied in terms of fermentation parameters, such as media composition, optimal pH and host specificity. First reports on nisin batch fermentations date back to 1966, and titers achieved at that time were 50 mg L\(^{-1}\) of medium (187).

Nisin is regarded as a primary metabolite, as maximum nisin production is directly related to biomass formation and production is closely associated with growth (102, 223, 459) and affected by the type and level of carbon, nitrogen and phosphate sources and other nutritional factors (222, 459, 460). The final concentration of nisin produced is host-specific (223).

Sucrose metabolism and nisin production are genetically linked (199): the genetic determinants for nisin production and the ability to import sucrose via a phosphotransferase system have been shown to lie on the conjugative transposon Tn\(S276\) (359). Moreover, regulation of carbon metabolism has been reported to be a major control mechanism for nisin biosynthesis (459).

The production of Pep5, epidermin and gallidermin has been shown to be inhibited by low levels of added phosphate (180, 181). Phosphate control is a well-known regulatory mechanism of secondary metabolism (285), and optimal production of most antibiotics takes place at inorganic phosphate concentrations that are suboptimal for growth. However, nisin production levels were shown to be highly stimulated by added inorganic phosphate (15, 102).

Low specific growth rates are required for high specific nisin production rates. Indeed, when harder-to-digest sugars and nutrients were applied, to simulate nutrient limitation and therefore low specific growth rates, higher specific nisin production rates were obtained (222, 460). Moreover, cultivation under low nutrient conditions yielded higher specific nisin production rates than high nutrient cultivations (222). In addition, continuous culture experiments using lactose as carbon source have shown highest levels of nisin production (160 arbitrary units (AU) mL\(^{-1}\)) at low specific growth rates (\(\mu=0.25\) h\(^{-1}\)) (295).

The industrial production of nisin is carried out in batch fermentations based on milk as a substrate (360, 457), but the industrial production levels have never been reported to our knowledge. Using the natural producer \(L.\ lactis\ subsp.\ lactis\), nisin production levels obtained by fermentation generally reach 2'000-3'000 IU mL\(^{-1}\)
Moreover, comparing different natural \textit{L. lactis} subsp, \textit{lactis} strains, a nisin activity level of about 2'000-2'500 IU mL$^{-1}$ (50-62.5 mg L$^{-1}$) seems to be the maximum (459). According to de Vuyst and Vandamme (460), only a few investigators have reported higher nisin production levels, i.e. 3'640 IU mL$^{-1}$ (91 mg L$^{-1}$) with the strain \textit{L. lactis} subsp, \textit{lactis} MSU based on a complex medium containing molasses, yeast autolysate, KH$_2$PO$_4$ and K$_2$SO$_4$ (101), 4'000 IU mL$^{-1}$ (100 mg L$^{-1}$) with the strain \textit{L. lactis} subsp, \textit{lactis} 91 based on a medium containing whey enriched with potato starch syrup and a bacterial cell hydrolysate (434) and 6'750 IU mL$^{-1}$ (169 mg L$^{-1}$) with the strain \textit{L. lactis} subsp, \textit{lactis} 4, based on a milk medium enriched with CaCO$_3$ (209).

The positive correlation between nisin production levels and cell yield as demonstrated with varying concentrations of sucrose was also reported in batch fermentations with different organic nitrogen sources. Highest nisin production levels (2'500 IU mL$^{-1}$, 62.5 mg L$^{-1}$) were achieved using cotton-seed meal (460). Nitrogen sources such as cotton-seed meal, blood meal and fish meal are typical slowly metabolized and create a state of nitrogen limitation, resulting in low specific growth rates, and in the suppression of possible metabolic regulatory mechanisms such as the repression of catabolic enzymes and amino acid transport (1). Therefore, these nitrogen sources are often used in industrial antibiotic fermentations (48).

Other factors that were shown to affect nisin titers are the host-specific maximum concentration of nisin, caused by end-product inhibition of the producing strains (223, 457, 459), and the accumulation of lactic acid in the lactic acid fermentation process which leads to growth inhibition by a decrease of pH and toxic effect of the short chain organic acid. The nisin-toxicity issue was coped with by developing a two-phase batch strategy, in which some of the nisin produced was removed into the solvent phase, thereby reducing the concentration of nisin in the aqueous phase (24% increase in nisin produced per liter of fermentation volume) (221). More strategies have been developed to avoid problems related to lactic acid production and improve nisin production, which rely on lactic acid removal by coupling a microfiltration module to a continuous production bioreactor system (four-fold increase in nisin produced per liter of fermentation volume) (439), on lactate assimilation by the yeast \textit{Kluyveromyces marxianus} in a mixed-culture with \textit{L. lactis} (1.7-fold increase, maximal nisin titer 3'920 IU mL$^{-1}$, 98 mg L$^{-1}$) (411), on pH feedback controlled fed-batch fermentations (maximal nisin titer 2'660 IU mL$^{-1}$, 66.5 mg L$^{-1}$).
1) (275), and on *in situ* lactate removal from sucrose batch-fermentation using the anionic-exchange resin Amberlite IRA-67 (1.5-fold increase in nisin titer per liter of fermentation volume) (485).

Recently, an online removal of nisin by silicic acid coupled with a micro-filter module was developed to reduce detrimental effects caused by nisin-toxicity for the producer, proteolytic degradation of nisin, and product inhibition during fermentation. A significant increase in nisin was reported (7’445 IU mL⁻¹, 186 mg L⁻¹) when compared with batch fermentation without the online recovery (1’897 IU mL⁻¹, 47.5 mg L⁻¹) (351).

Immobilization of cells was reported to be advantageous over free-cell fermentations, as it allows greater volumetric productivity, long-term operating stability, improved process control, simplified cell recovery procedures, reduced contamination problems, decreased cell wash-out, and the ability to apply high dilution rates in continuous processes (186). Zezza and coworkers investigated nisin production in batch culture using *L. lactis* subsp. *lactis* NZ1 immobilized in coated alginate beads, but nisin titers were only 0.1% of free-cell fermentations (489). On the contrary, a study on the influence of different production media in continuous production of nisin by *L. lactis* cells immobilized on a fibrous matrix showed that higher concentrations of nisin were achieved with whey permeate supplemented with casein hydrolysate in continuous mode (30’000 AU mL⁻¹) if compared to batch fermentations with the same medium (13’000 AU mL⁻¹) (271).

In another study, the feasibility of producing lacticin 3147 and nisin continuously, using free and calcium alginate-immobilized *L. lactis* subsp. *lactis* strains, was investigated and compared with free-cell production (390). The authors reported earlier and higher bacteriocin production (5’120 AU mL⁻¹) in both nisin and lacticin 3147 free-cell bioreactors, but maximum productivity was maintained significantly longer (180 h) in bioreactors containing immobilized cells, possibly due to higher plasmid stability (390).

### 8.1.2. Epidermin

Production of epidermin has been optimized with the risk-class II, wild-type *S. epidermidis* Tü3298 and with a mutant, *Staphylococcus epidermidis* Tü3298/DSM 3095, that is resistant to concentrations of epidermin up to 1 g L⁻¹ and was isolated by repeated selection of resistant strains of wild-type *S. epidermidis* (181).
Epidermin was produced in submerged cultures in 20 or 200 L bioreactors with complex medium containing meat extract as a nitrogen source, and malt extract as a carbon source. Batch fermentations with wild-type *S. epidermidis*, which is sensitive against his own antibiotic (MIC: 10 µg mL\(^{-1}\)), led to maximum epidermin titers of 20 mg L\(^{-1}\) after 15 h incubation time. In contrast, batch fermentations with the resistant strain *S. epidermidis* Tü3298/DSM 3095 lead to titers of 80 mg L\(^{-1}\) after 46 h incubation (181).

Maximum antibiotic titers were achieved by the addition of 3% NaCl to the production medium, which lead to final concentrations of 225 mg L\(^{-1}\) (181). In batch-fermentations, epidermin production was coincident with the growth phase. The use of higher concentrations of both carbon and phosphate sources, which would have been necessary to extend the growth phase and to generate higher cell densities, was not taken into consideration for the batch approach, as the production of epidermin is known to underlie strong carbon-catabolite-repression and to be negatively regulated by the phosphate in the medium. Nevertheless, fed-batch fermentations were carried out with balanced glucose- and phosphate-feeding, but this did not lead to significant increase in epidermin yields (181).

A fed-batch fermentation with pH-dependent glucose and continuous phosphate and ammonia addition led to both significant higher biomass and higher yields of the antibiotic: The cell mass increased 3-fold and the maximum epidermin titer was 350 mg L\(^{-1}\) (181). Although the maximal cell number was reached after 28 h, the maximal antibiotic concentration was reached only after 72 h during the stationary phase. Nevertheless, epidermin production was mostly coupled to growth of the host, as 80% of the total epidermin were produced during the growth-phase (181). Scale-up of the combined-feeding fermentation to a 200 L pilot plant without further optimizations led to a reduction of the titer by 20% relative to the one achieved in the 20 L scale.

In order to prevent epidermin degradation by proteases and heat, a discontinuous adsorption strategy was developed as well: The whole fermentation broth was adsorbed to the resin Amberlite XAD-1180, and epidermin yield after elution amounted to 500 mg L\(^{-1}\), 50% more than in feeding fermentations without the intermittent adsorption steps (181). Besides, this first purification step resulted in a product with already a purity of 80% (181). However, discontinuous adsorption is not easily implemented a production plant. Therefore, continuous on-line adsorption of
epidermin during the fermentation process was performed using a cross flow filtration unit. The retentate was led back to the reactor while the filtrate was adsorbed on an Amberlite XAD-1180 column. Maximal epidermin yields eluting from the resin were reached after 80-90 h and amounted to 440 mg L\(^{-1}\) (181).

Production of epidermin was also possible with a “food-grade” recombinant Staphylocoocus carnosus strain with a two plasmids system (440). The strain S. carnosus TM300 [pTepiMA+, pRBgepSI] contained the genes for epidermin biosynthesis, modification, and immunity. However, only 70% of the wild-type production yields were observed and plasmid instability was observed (181).

8.1.3. Gallidermin

Gallidermin is produced naturally by the non-pathogenic, risk-class I strain S. gallinarum Tü3928. Optimization of gallidermin production in batch fermentations was reported, which led to a maximum gallidermin concentration of 250 mg L\(^{-1}\) (180). Similar to epidermin fermentations (181), strong acidification during the first hours of the fermentation process was observed due to the formation of acetate, which was metabolized further after the preferred carbon sources were exhausted. Even though malt extract could be replaced by a mixture of 0.2% glucose and 3% maltose, no other complex nitrogen source than meat extract could be used without loss of productivity (181).

In a further study, Ungermann reported the isolation of a resistant S. gallinarum strain that tolerated gallidermin concentrations up to 600 mg L\(^{-1}\). As tolerance was not lost during a monitoring period of three years, is very likely to be genetically encoded (448). Batch fermentations with this strain resulted in a final gallidermin titer of 170 mg L\(^{-1}\) (450). A continuous meat-extract feeding was developed, that increased the titer to 320 mg L\(^{-1}\). Further optimization involved the implementation of a product-removal strategy that, in contrast to the discontinuous adsorption developed for epidermin, was based on dialysis through a membrane with a cut-off of 10 kDa. Dialysis fermentations were performed in a membrane fermenter consisting of two chambers separated by the dialysis membrane. Chamber 1 contained 7.5 L production medium and was fed with meat-extract, whereas chamber 2 contained 1.2 L of 4.6 % NaCl. Chamber 2 was inoculated with S. gallinarum, and was fed with glucose (449). The batch dialysis process led to maximal gallidermin
yields of 330 mg L\(^{-1}\), corresponding to 2'142 mg L \((\text{bioactive volume})^{-1}\) gallidermin after 22 h, whereas with a 70 h-long dialysis fed-batch process (with glucose and meat extract feeding) the maximum production of gallidermin was 720 mg, corresponding to 5'100 mg L \((\text{bioactive volume})^{-1}\) (449). The dialysis reactor has the advantage of on-line product separation during the production phase but its major drawback is its unsuitability for scale-up that makes the development of an industrial-scale process impossible (440). Moreover, it enables an increase in volumetric productivity but not an increase in final titers, which is highly desirable in any biotechnological industrial-scale process.

According to Theobald (440), the difficulties for the development of a gallidermin production process are a significant fluctuation of gallidermin production whereas biomass formation is stable, which leads to low reproducibility of the production process, and the presence of meat extract in the production medium, which is the reason for high production downstream processing costs. Moreover, a possible risk of prion contamination (due to the meat extract) renders this component highly unsuitable from an industrial production perspective. In order to eliminate the problem of fluctuating productivity, a new medium was developed used for stock culture agar slants, which consisted of 0.5 % yeast extract, 0.1 % glucose and 0.5 % NaCl and stabilized production during a time period of about 200 days (441).

Another indispensable optimization step was the development of a novel production medium. The efforts focused on economic aspects of the medium under development and successfully reduced the cost of the medium by 93% compared to the meat extract-containing production medium. Although meat extract seemed to be critical for successful production, it was shown to be replaceable by a special yeast extract. In addition, three-fold lower concentrations of this yeast extract led to 20% higher product titers (300 mg L\(^{-1}\)) compared to the old medium composition (215). Also, addition of very high concentrations of CaCl\(_2\) to the production medium was shown to be beneficial for the final gallidermin titer. The final medium optimisation was carried out with a computer program using genetic algorithms for multiple parameter optimization (175), and yielded an optimized medium which lead to a cell dry weight of about 37 g L\(^{-1}\) and a gallidermin concentration of almost 300 mg L\(^{-1}\) (215).

Batch, fed-batch and continuous fermentations were tested with this novel production medium in stirred tank reactors. Best results were obtained either in batch
or in fed-batch cultivations with pulses of amino acids or maltose during the production phase (maximal gallidermin concentration approx. 400 mg L\(^{-1}\)) (214).

Moreover, a scale-up strategy was successfully tested up to a working volume of 200 L, which is based on a fed-batch process with the new production medium. Best results were achieved with a single maltose pulse during the late production phase leading to a final gallidermin concentration of 330 mg L\(^{-1}\), 20–30% more in comparison with the concentrations found in a non-pulsed pilot-scale fermentation (216). According to Theobald, the downstream processing was successfully optimized by adsorption of gallidermin to the resin Amberlite XAD-1180, a polymeric adsorbent resin that is used primarily in the purification and preparation of hydrophobic compounds (3).

### 8.1.4. Other lantibiotics

Pep5 production was optimized in batch fermentations with the wild-type strain \textit{S. epidermidis} 5 (180). Fermentation of this strain under the same conditions reported before for epidermin production, lead to maximal Pep 5 titer of 20 mg L\(^{-1}\). The culture medium was further optimized and the supplement of 10% meat extract together with 4% NaCl resulted in the production of 120 mg L\(^{-1}\) Pep 5 without prolonging the fermentation time more than 12 h (180).

Lactocin S production by \textit{Lactobacillus sake} L45 has been shown to depend on the growth stage of the bacteria, the pH of the medium, the presence of ethanol, and the aeration of the culture. Highest levels of bacteriocin production were observed in pH-controlled batch fermentations at pH 5, and reached 2'000-3'000 U mL\(^{-1}\), which represented an 8- to 10-fold increase in bacteriocin production compared with production during static batch culture fermentation. When 1% ethanol was included in the growth medium, a two- to fourfold increase in the bacteriocin yield was observed, whereas aeration during growth almost completely eliminated the production of lactocin S (304).

Finally, lacticin 3147 was produced on large-scale in batch fermentations with whey-based medium, followed by evaporation and spray-drying steps, resulting in a lacticin 3147-enriched whey powder (303).
8.2. Genetic strategies for lantibiotic overproduction

Molecular genetics has spawned a dramatic expansion of the biotechnology industry. Combination of traditional bioprocess engineering and DNA recombinant technology was successfully implemented for the improvement of antibiotic productivity and resulted in remarkable reduction of the production costs (87, 178, 420).

For overproduction of lantibiotics, various genetic engineering strategies have been developed in previous studies. These strategies will be discussed in detail in the following chapters and are usually based on: [i] The overproduction of inactive lantibiotic precursors (Chapter 2); [ii] the gene-dosage manipulation of lantibiotic biosynthesis, transport, immunity, and regulation genes (Chapter 3); and [iii] the heterologous production of lantibiotics (Chapter 4).

9. Aims of this study

Antibiotic resistance has spread dramatically among pathogens throughout the last two decades. Of particular concern is the increasing emergence of multi-resistant pathogens in healthcare settings. As the number of resistant pathogens continues to grow, the number of new antibiotics to fight them is steeply declining. Therefore, the development of new antimicrobial agents is essential.

So far, no lantibiotic has been developed into a clinical product, even though the clinical potential of many lantibiotics has been proven. The major obstacle to clinical development of lantibiotics is their generally very low production titer, which has triggered attempts to improve volumetric productivity, but generally failed to provide significant increase in titer.

Gallidermin is a tetracyclic, 21 amino acids class I lantibiotic peptide, which is produced by the non-pathogenic S. gallinarum Tü3928, and shows broad-spectrum activity against important pathogens like S. aureus, S. epidermidis, S. pyogenes, S. pneumoniae, S. faecalis, and P. acnes. In this study, various genetic engineering strategies have been followed with the aim of enhancing the production of gallidermin and to develop a production strain suitable for an industrial-scale production process:

[i] A novel two-step production strategy was developed which is based on the production of a non-toxic precursor of gallidermin. This is generated by a mutant strain impaired in the final proteolytic step. Fermentative production of this
precursor is followed by an enzymatic activation step after the fermentation that delivers the active lantibiotic. The overproduction capacity of the novel strain has been evaluated in non-optimized fermentations and shake-flasks experiments.

[ii] Genetic engineering strategies were followed with the aim to further increase the production of pregallidermin. A gene-dosage and a promoter-manipulation approach were evaluated. For the last approach, three Gram-positive promoters ($P_{mecA}$ and $P_{cap1A}$, $P_{aphIII}$) were characterized in both $S. gallinarum$ and $S. aureus$ RN4220.

[iii] Production of the gallidermin-precursor in various heterologous hosts, such as $B. subtilis$, $S. carnosus$, $S. aureus$ RN4220, $S. xylosus$, $S. arlettae$, and $E. coli$, was investigated.
Chapter 2

Circumventing the effect of product toxicity: Development of a novel two-stage production process for the lantibiotic gallidermin


1. Abstract

Lantibiotics such as gallidermin are lanthionine-containing polypeptide antibiotics produced by Gram-positive bacteria that might become relevant for the treatment of various infectious diseases. So far, self-toxicity has prevented the isolation of efficient overproducing strains, thus hampering their thorough investigation and preventing their exploitation in fields other than the food area.

We wanted to investigate the effect of lantibiotic precursor peptides on the producing strains in order to evaluate novel strategies for the overproduction of these promising peptides. In this study, gallidermin was chosen as a representative example of type A-lantibiotics. A Staphylococcus gallinarum Tüß928 mutant was constructed whose gene for the extracellular pregallidermin protease GdmP had been substituted by a kanamycin resistance gene. Mass spectrometry (MS) analysis indicated that this mutant produced fully post-translationally modified gallidermin precursors with truncated versions of the leader peptide, but not the entire leader as predicted from the gdmA sequence. In filter-on-plate assays, these truncated pregallidermins showed no toxicity against Staphylococcus gallinarum Tüß928 up to a concentration of 8 g L⁻¹ (corresponding to approximately 2.35 mM), while gallidermin produced clear inhibitory zones already at concentrations as low as 0.25 g L⁻¹ (0.12 mM). We could show that the lack in toxicity is entirely due to the presence of the truncated leader, since MS as well as bioassay analysis showed that the peptide resulting from tryptic cleavage of pregallidermins and gallidermin produced by S. gallinarum Tüß928 had identical mass and approximately the same specific activity. This demonstrates that even a shortened leader sequence is sufficient to prevent the toxicity of mature gallidermin. In non-optimized fermentations, the gdmP mutant produced pregallidermin to a 50% higher molar titer, suggesting that the absence of
self-toxicity has a beneficial effect on gallidermin production and giving a first confirmation of the suitability of the overproduction strategy.

2. Introduction

Antibiotic resistance has spread dramatically among pathogens throughout the last two decades. Of particular concern is the increasing emergence of multi-resistant pathogens in healthcare settings. Nowadays, about 70% of nosocomial infections in the US are resistant to at least one antibiotic (263), and in 2002 the first clinical isolate of Staphylococcus aureus with a high-level resistance to vancomycin, a drug of last resort, was isolated (53). As the number of resistant pathogens continues to grow, the number of new antibiotics to fight them is steeply declining (313). Therefore, the development of new antimicrobial agents should be encouraged.

Type-A lantibiotics are cationic, amphiphilic peptides that are produced by a wide range of Gram-positive bacteria and show bactericidal activity against other Gram-positive bacteria. They show a dual mode of action at nanomolar concentrations, which involves pore formation and inhibition of peptidoglycan biosynthesis by specific interaction with cell wall precursor lipid II (32, 163, 173). Lantibiotics are ribosomally synthesized and undergo posttranslational modifications resulting in the formation of unusual residues like the thioether amino acids lanthionine and methyllantionine, which are crucial for a proper functional structure (196). So far, no lantibiotic has been developed into a clinical product, even though nisin has proven its potential to treat peptic ulcers caused by Helicobacter pylori (127), gallidermin is effective against acne caused by Propionibacterium acnes and bovine mastitis caused by S. aureus (337, 487), and mersacidine can treat MRSA infections in mice (244). The major obstacle to clinical development of type-A lantibiotics is their generally very low production titer, which has triggered attempts to improve volumetric productivity, but generally failed to provide significant increase in titer.

Gallidermin is a tetracyclic, 21 amino acids type-A lantibiotic peptide, which is produced by the non-pathogenic S. gallinarum Tü3928 (212), and shows broad-spectrum activity against important Gram-positive pathogens like S. aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus faecalis, and P. acnes (180). Gallidermin (6L-epidermin) is closely related to epidermin, which is synthesized by the class II strain S. epidermidis
Tü3298 (2, 401). Biosynthesis, modification, secretion, and activation of epidermin have been broadly studied (43, 248, 338, 400-402, 454). Since gallidermin and epidermin have a high degree of identity on DNA and protein level, and several genes of the two gene clusters have been shown to be interchangeable (23, 327, 346), many assumptions on gallidermin biosynthesis and function have been made based on previous research on epidermin. The eleven genes responsible for epidermin synthesis are organized as a 13 kb gene cluster which is located on pTü32, a 54-kb plasmid of *S. epidermidis* Tü3298 (12, 400, 402). Epidermin formation starts with ribosomal synthesis of an extended prepropeptide encoded by the structural gene *epiA*, which is post-translationally modified by the gene products EpiB, EpiC, and EpiD (400). These are responsible for dehydration of Thr and Ser residues to dehydrobutyryl and dehydroalanine (DhB, DhA), for subsequent lanthionine and methyllanthionine formation, and for the formation of an S-aminovinyl–D-cysteine ring (22, 383). These post-translational modifications lead to the prepeptide. EpiT and EpiH, which show high homology to ATP binding cassette (ABC) transporters, should be responsible for active translocation of the prepeptide out of the cell (166, 346, 383).

However, as the DNA sequence of the *epiT* locus does not encode a functional subunit – having an internal deletion that causes a frameshift and a second deletion at the 3'-end – the translocation of epidermin through the cytoplasmic membrane is presumably accomplished by a host-encoded system (346). On the contrary, the homologous gene in the gallidermin synthesis cluster, *gdmT*, encodes for a fully functional protein (346). Removal of the leader peptide from the prepeptide leads to the mature lantibiotic. This reaction takes place extracellularly and is catalyzed by EpiP, a subtilisin-like serine protease (2, 383). The regulator EpiQ activates the expression of every gene encoded in the epidermin gene cluster but *epiP* and the promoterless *epiQ* (339).

The mechanism of action of lantibiotics results in toxicity against the producing strains (383). *S. epidermidis* Tü3298 has evolved a protection mechanism consisting of a specialized ABC-transporter EpiEFG that is thought to remove lantibiotic molecules from the cytoplasmic membrane (166, 329, 341, 388). However, this export system merely decreases the exposure to the lantibiotic and does not confer resistance to the producer (214). Due to this autotoxicity, epidermin as well as gallidermin titers in fermentations have generally remained low, in the order of 250 to 330 mg L$^{-1}$ (181, 440, 441, 449).
Operating under the hypothesis that gallidermin toxicity is the major obstacle to its overproduction, we developed a novel two-step production strategy which is based on 1) the overproduction of a potentially non-toxic precursor of gallidermin, such as pregallidermin, followed by 2) an enzymatic activation step leading to the active lantibiotic. Therefore, we investigated the production and the toxicity of pregallidermin, which was generated by a mutant strain impaired in the final proteolytic step of the synthesis pathway. As the biosynthesis of type-A lantibiotics is highly conserved, such a strategy might harbor great potential for a whole series of potentially clinically relevant molecules.

3. Material and methods

3.1. Bacterial strains and growth conditions.
Bacterial strains and plasmids are listed in Table 1. Escherichia coli strains were routinely grown in Luria-Bertani broth (387), whereas staphylococci were grown in B broth (219). All percentages refer to wt/vol unless otherwise mentioned. For production of gallidermin by S. gallinarum Tü3928 medium YE4 (215) or medium 21 (5% OHLY Cat yeast extract (Deutsche Hefewerke GmbH, Marl, Germany), 2% NaCl, 0.5% maltose, pH 7.2) were used. Media were supplemented when appropriate with ampicillin (100 µg mL\(^{-1}\)), chloramphenicol (10 µg mL\(^{-1}\)), kanamycin (50 µg mL\(^{-1}\)), or tetracycline (10 µg mL\(^{-1}\)), unless otherwise noted. All cultures were grown at 37°C and 225 rpm in a shaking incubator, unless otherwise noted.

3.2. Genetic procedures
Standard genetic procedures were performed as reported elsewhere (387). Staphylococcal chromosomal DNA was isolated using Qiagen Genomic Tips (Qiagen, Hilden, Germany) according to the protocol supplied by the manufacturer, except that prior to extraction cells were incubated with 20 µg mL\(^{-1}\) of lysostaphin at 37 °C for 1 h. Low-copy plasmid isolation from staphylococci was performed as described by Kies (218). In our hands, only staphylococcal plasmid DNA could be introduced into S. gallinarum, therefore plasmid DNA prepared from E. coli had to be first electroporated into the restriction negative S. aureus RN4220 as described elsewhere (393). Electrocompetent S. gallinarum cells were prepared and electroporation was carried out as described previously (165), except that the
Genepulser electroporator (BioRad, Hercules, CA, USA) was operated at 2.5 kV, 1000 Ω, and 1 µF.

Enzymes for molecular cloning were purchased from New England Biolabs (Ipswich, MA, USA). DNA purification steps were performed with JetQuick columns (Genomed GmbH, Löhne, Germany). PrimerSelect (Lasergene Package, DNASTar Inc.) software was utilized for primer design and oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Routine PCR amplifications were performed in 25 µL reactions containing 0.2 mM dNTPs, 0.2 µM of each primer, 1x PCR Buffer, 1.5 mM MgCl₂ and 0.070 U Taq DNA Polymerase (Fermentas, Vilnius, Lithuania). Standard cycling conditions were 94°C for 2 min followed by 30 cycles of: 94°C for 30 s, 52-55°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 10 min. Primers used in this study are listed in Table 2. Sequencing of cloned fragments was performed by GATC Biotech AG (Konstanz, Germany). Inverse PCR (iPCR) was performed as described elsewhere (408). PCR with primers designed on the epidermin cluster sequence were performed using the Expand Long Template PCR system (Roche, Basel, Switzerland). Southern blot analysis was performed as described elsewhere (387). DIG labeled probes were prepared with the PCR DIG probe synthesis kit (Roche, Basel, Switzerland) as described by the manufacturer. Detection was performed with anti-DIG-AP and the chemiluminiscent alkaline phosphatase CSDP substrate (Roche, Basel, Switzerland) following the manufacturer's instructions.

3.3. Sequencing of the complete gallidermin gene cluster

The sequencing strategy relied on the already available 4373 bp-long sequence (GenBank #U61158) that covers the central part of the gallidermin synthesis cluster (gdmF, gdmT, gdmH, gdmA, and a partial coding sequence of gdmE and gdmB) (346, 401). iPCR was performed using the primer pair GALL3, which was designed on the available sequence. Amplification of a Clal digest of S. gallinarum Tü3928 genomic DNA generated a 4 kb amplicon including gdmG and gdmB as verified by Southern-blotting with epiB- and epiG-derived amplicons as probes. The amplicon was cloned and sequenced by primer walking. A 4.4 kb-long fragment resulted from amplification with the primer pair epiB F/epiP R, which had been designed on the sequence of the epidermin gene cluster of S. epidermidis Tü3928 (GenBank #X62386, #X99127) (70, 400). Primers are detailed in Tab. 2. After sequencing, primer gdmseq F2 was
designed on the end of the partial \textit{gdmP} sequence and was used together with the primer episeq R2, which was designed on the sequence downstream of \textit{epiP}, in order to amplify a 1 kb fragment for sequencing. The sequences of the three novel fragments and the available part were assembled with SeqManII (Lasergene Package, DNASTar Inc.) and the final 12705 bp sequence was deposited in the GenBank database under the accession number DQ367437.

3.4. Development of an integration vector and construction and verification of a \textit{S. gallinarum}\textsuperscript{=}\textit{gdmP::aphIII} mutant strain

A fragment internal to \textit{gdmP} was amplified by PCR using the primer pair gdmP-PstI F and gdmP-\textit{Hind}III R. PCR amplification was performed with an annealing temperature of 58°C and generated a 651-bp long amplicon, which was digested with \textit{PstI} and \textit{Hind}III and cloned into pUC18\textit{NotI} to yield pUC18\textit{NotI}-P. An \textit{aphIII}-\textit{gdmQ} fusion was generated by overlap extension PCR (45, 172, 182, 183) by fusing the \textit{aphIII} gene without its terminator sequence to a fragment of \textit{gdmQ} (maintaining the \textit{gdmQ} Shine-Dalgarno sequence and start codon, Fig. 1). Amplification of the 364 bp-long sequence of \textit{gdmQ} was performed with the primer pair gdmQ-\textit{EcoRI}2 F and gdmQ R. The \textit{aphIII} gene was amplified from pDG782 by PCR using primers kan-\textit{ClaI} F and kan R. Overlap extension PCR was performed using a 1:1 molar mixture of the two amplicons as a template and the outer primers gdmQ-\textit{EcoRI}2 F and kan-\textit{ClaI} F. The resulting 1510 bp-long amplicon was digested with EcoRI and \textit{ClaI} and inserted into pUC18\textit{NotI}-P, generating plasmid pGV4. The \textit{E. coli}-\textit{Staphylococcus} shuttle vector pGV2 was constructed by excising a 1.4 kb Sacl-Nhel fragment containing a nuclease gene from pPSM1058 and replacing it by a Sacl-\textit{NotI}-SpeI linker fragment from the multiple cloning site of pGEM5zf(+). The knock-out vector pGV5 was assembled by cloning the approximately 2.2 kb \textit{NotI} fragment from pGV4 containing the \textit{gdmP} fragment and the \textit{aphIII}-\textit{gdmQ} fusion into pGV2. \textit{S. gallinarum} Tü3928 was transformed with pGV5 and transformants were grown at 30°C on selective B-agar plates containing 20 µg mL\textsuperscript{-1} chloramphenicol. Gene replacement was induced in \textit{S. gallinarum} Tü3928 [pGV5] as described previously (111), except that the first growth step at 30°C was repeated three times and cells were subsequently incubated at 42°C on B-agar plates containing 7.5 µg mL\textsuperscript{-1} kanamycin (determined in previous experiments to be the MIC value). Screening for strains with double homologous recombination was performed on B-agar plates containing either
20 µg mL⁻¹ chloramphenicol or 7.5 µg mL⁻¹ kanamycin. The site of integration was verified by PCR applying primers PROOF-K and PROOF-P, and primers PROOF-Q and PROOF-P to chromosomal DNA from *S. gallinarum* Tü3928 and *S. gallinarum* ΔgdmP::aphIII.

### 3.5. Gallidermin and pregallidermin analytics

Detection of gallidermin and pregallidermin was performed with a protocol adapted from Fiedler et al. (111). Culture supernatants were centrifuged at 16’000*g at 4°C for 10 min, filtered through a 0.2 µm cellulose-acetate membrane filter (Carl Roth GmbH, Karlsruhe, DE), and separated on a Prontosil Eurobond C18 5.0 µm column with guard column (Bischoff, Leonberg, Germany) operated in a La Chrom HPLC system (Merck, Germany) equipped with an L-7100 pump, L-7200 autosampler, L-7455 diode array detector (DAD) and L-7490 refractive index (RI) detector. Data collection was performed with a Merck-Hitachi model D-7000 chromatography station software. Injection volume was 10 µL and column temperature was maintained at 25°C. The mobile phase was composed of 0.1% (vol/vol) aqueous H₃PO₄ (solvent A) and acetonitrile (solvent B) and followed a linear gradient from 90% A/10% B to 45% A/55% B in 14 min at a flow rate of 1 mL min⁻¹. Regeneration of the column and equilibrium to start conditions was achieved in 5 additional minutes.

The identity of gallidermin produced in our laboratory by *S. gallinarum* Tü3928 was confirmed by comparing its LC/ESI-MS spectrum with that of commercially available gallidermin obtained from Alexis Biochemical Corp. (San Diego, USA). MS analysis was adapted from Kies et al. (219) and performed on a Finningan LCQ Deca LC/ESI-MS in cationic mode. A Bischoff Prontosil Eurobond C18 5.0 µm column was used as a stationary phase. The mobile phase consisted of 0.1% (vol/vol) aqueous trifluoroacetic acid (solvent A) and 100% acetonitrile (solvent B). Separation was performed with a gradient from 90% A/10% B to 45% A/55% B in 14 min at a flow rate of 1 mL min⁻¹. The relative abundance of ions in relation to the mass-to-charge (m/z) ratio of ions is indicated on the resulting spectra. The identity of the isolated pregallidermin and the composition of the tryptic digest of pregallidermin were verified by a different ESI-MS procedure. The samples were 20-fold diluted with 50% acetonitrile/0.2% aqueous formic acid and measured in the positive mode on a Q-Tof Ultima API mass spectrometer (Micromass, UK). The deconvoluted spectra were
obtained using the MaxEnt1 Software, and show the relative abundance of ions in relation to the mass of ions.

Bioassays were performed with the gallidermin sensitive strain *Kocuria rhizophila* as described for epidermin (391) or with *S. gallinarum* Tü3928. For the latter, a culture was incubated over night at 37°C, OD\(_{600}\) was adjusted to 0.1, and 100 µL were plated on LB-agar plates. 20 µL of enriched pregallidermin solutions (0.5 to 8 g L\(^{-1}\) in water) or commercial gallidermin solutions (0.13 to 8 g L\(^{-1}\)) were pipetted on sterile paper filters. As a control, water was used. Filters were air dried and then placed upside-down on *S. gallinarum* Tü3928 plates, which were incubated at 30°C for 24 hours. For the tryptic cleavage, a control bioassay with trypsin solutions (from 10 mg L\(^{-1}\) to 10 g L\(^{-1}\)) in sterile water, pH 6 was performed.

### 3.6. Tricine-SDS-PAGE analysis

Tricine-SDS-PAGE was performed as described previously (240, 391). *S. gallinarum\(\Delta\text{gdmP::aphIII}\)* and *S. gallinarum* Tü3928 cultures were grown in 20 mL of production medium 21 in 100 mL Erlenmeyer flasks. Samples were collected after 15 hours at a cell dry weight concentration of 10.9 g L\(^{-1}\) for *S. gallinarum\(\Delta\text{gdmP::aphIII}\)*, and 9.8 g L\(^{-1}\) for *S. gallinarum* Tü3928. 2 µl of the supernatant of the *S. gallinarum\(\Delta\text{gdmP::aphIII}\)* culture (containing pregallidermin) as well as 2 µl of the supernatant of the *S. gallinarum* Tü3928 culture (containing gallidermin) and 10 µl of solutions with different concentrations of commercial gallidermin (50 mg L\(^{-1}\) and 100 mg L\(^{-1}\)), and polypeptide SDS-PAGE molecular weight marker (BioRad, Hercules, CA, USA), were loaded onto acrylamide gels. This was composed of a 4% stacking gel and a 16.5% separating gel. As Coomassie staining is less sensitive than silver staining, and due to the small sizes of gallidermin and pregallidermin, the latter method was chosen. Blotting of the gels to 0.45 µm PROTRAN nitrocellulose membranes was performed in a Mini Trans-Blot cell (BioRad, Hercules, CA, USA) at 4°C, 0.8 mA/cm\(^2\) for 30 min. The transfer buffer was composed of 25.5 mM Tris, 192 mM glycine, 0.1% SDS in 20 % (vol/vol) methanol. Silver staining of the membranes was performed as described elsewhere (111).

### 3.7. Tryptic cleavage and activation of pregallidermin

Enriched pregallidermin was dissolved in water to a final concentration of 250 mg L\(^{-1}\), and pH was adjusted to 6.0. Trypsin (20 U mg\(^{-1}\), American Laboratories Inc., Omaha,
NE, USA) was added to a final concentration of 50 mg L\(^{-1}\), and proteolytic cleavage was carried out at 25\(^{\circ}\)C for 20 h.

3.8. Fermentations

S. gallinarum Tü3928 and S. gallinarum \(\Delta\)gdmP::aphIII from frozen stocks were reisolated on B-agar plates and incubated for 15 h at 37\(^{\circ}\)C. 3 mL precultures of medium 21 were inoculated from isolated colonies and grown for 12 h at 37 \(^{\circ}\)C and 190 rpm, from where they were inoculated into 100 mL of medium 21 in 500 mL Erlenmeyer flasks with one baffle under the same conditions. Finally, a 5 L stirred tank-bioreactor BioStat A (Braun Biotech Int., Melsungen, Germany) equipped with automatic recording of dissolved oxygen tension (DOT) (Pro 6000; Mettler-Toledo, Greifensee, Switzerland) and control of pH and temperature containing 3 L of medium 21 were inoculated with a 50 mL of a preculture. pH was controlled by the addition of 3 M NaOH or 3 M H\(_2\)SO\(_4\). Biomass formation was determined off-line by the measurement of optical density at 600 nm and cell dry weight determinations in triplicates. Foam was controlled by addition of silicon antifoam DF 204 (BASF, Ludwigshafen, Germany). The DOT was maintained above 50% first by adjusting stirrer speed, then by adjusting aeration. A feed of 50 % of maltose in water was initiated after the end of the batch phase after 6 h when the cells had reached a stationary phase.

3.9. Product isolation

Gallidermin and pregallidermin isolation was performed with a protocol adapted from Fiedler et al. (111). Gallidermin or pregallidermin were adsorbed from the cell-free fermentation broth onto XAD-7 (Fluka, Buchs, Switzerland; 4% vol/vol) over 90 min. The resin was filtered off, washed with water and eluted with methanol. The eluate was reduced \textit{in vacuo} until precipitation occurred. The precipitate was recovered by vacuum membrane filtration (pore size: 0.2 µm) and dissolved in sterile millipore water before further analysis. HPLC analysis at this point showed a purity of 85% (by peak area at 230 nm) for the isolated gallidermin and pregallidermin. The pregallidermin preparation was further purified by preparative HPLC on a Prontosil PREP 2025 C18 10.0 µm column with guard column (Bischoff, Leonberg, Germany). Injection volume was 0.8 mL and column temperature was maintained at 25\(^{\circ}\)C. The mobile phase was composed of 27.5\% of 0.1\% (vol/vol) aqueous trifluoroacetic acid and 72.5\% acetonitrile. The pregallidermin fraction was sampled and the eluate was
reduced in vacuo until precipitation occurred. The 96% pure (by peak area at 230 nm) pregallidermin precipitate was dried, weighed, and dissolved in sterile millipore water to a concentration of 10 g L\(^{-1}\). A dilution series of this solution was used to generate a standard curve for quantification of pregallidermin in supernatants by peak area at 230 nm.

Table 1: Strains and plasmids

<table>
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<th>Bacterial strains and plasmids</th>
<th>Genotype/relevant characteristic(s)</th>
<th>Reference/source</th>
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<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
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<td><em>E. coli</em> TOP10</td>
<td><em>E. coli</em> cloning host strain [F- mcrA (\Delta(mrr-hsdRMS-mcrBC)) (\Delta(lacZ\Delta M15) (\Delta(lacX74) (\Delta(recA1) araD139 (\Delta(araleu))) 7697 (gaU) (gaK) (fpsL) (Str) (endA1) (nupG)]</td>
<td>Invitrogen</td>
</tr>
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<td><em>S. aureus</em> RN4220</td>
<td>Intermediate strain for cloning</td>
<td>(243)</td>
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<td><em>S. gallinarum</em> Tü3928/DSMZ</td>
<td>Gallidermin producer, Tet(^{\prime})</td>
<td>(212); DSMZ, Braunschweig, Germany</td>
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<td>17239</td>
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<td></td>
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<tr>
<td><em>S. gallinarum</em> (\Delta gdmP::aphIII)</td>
<td>Pregallidermin producer, Tet(^{\prime}), Kan(^{\prime})</td>
<td>This study</td>
</tr>
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<td><em>S. epidermidis</em> Tü3298</td>
<td>Epidermin producer</td>
<td>(2); DSMZ</td>
</tr>
<tr>
<td><em>K. rhizophila</em></td>
<td>Gallidermin-sensitive indicator strain, previously known as <em>Micrococcus luteus</em></td>
<td>(238); DSMZ</td>
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<td><strong>Plasmids</strong></td>
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<td>pDG782</td>
<td><em>B. subtilis</em> kanamycin-resistance cassette vector, Amp(^{\prime}), Kan(^{\prime})</td>
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<td><em>E. coli</em> cloning vector, Amp(^{\prime})</td>
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<td>Stratagene</td>
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<tr>
<td>pPSM1058</td>
<td>Staphylococcal knock-out vector, pBT2 derivative, temperature sensitive ori(V), Cam(^{\prime}), Amp(^{\prime})</td>
<td>(276)</td>
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<td>pUC18NotI-P</td>
<td>pUC18NotI with a 651 bp (PstI-HindIII) fragment internal to (gdmP)</td>
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<td>pGV2</td>
<td>pPSM1053 in which a 0.9 kb (BamHI-SalI) fragment was replaced by a (NotI) oligo</td>
<td>This study</td>
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<tr>
<td>pGV4</td>
<td>pUC18NotI-P with a 1.5 kb (ClaI-EcoRI) fragment containing the Kan(^{\prime}) gene from</td>
<td>This study</td>
</tr>
</tbody>
</table>
Chapter 2 – Circumventing the effect of product toxicity

pDG782 fused to a part of gdmQ
pGV5
pGV2 with a 2.2 kb Ncol fragment from pGV4 containing the fragment internal to gdmP and the Kanr gene fused to a part of gdmQ
This study

Tab. 2: Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’→3’)</th>
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<td>GALL3 F</td>
<td>AAGGGATCTCCTCAGGCGAGAACACGAA</td>
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<tr>
<td>GALL3 R</td>
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<tr>
<td>epiB F</td>
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<tr>
<td>epiP R</td>
<td>TTGTTGTTGCAGCCTGGGAATGA</td>
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<td>Gdmseq F2</td>
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<td>Episeq R2</td>
<td>GCCGGGAATTCTGAGGTTGCTATATGAA</td>
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<tr>
<td>gdmP-PstI F</td>
<td>CATATCTGAGGTGTTGAGCGCATATAATC</td>
</tr>
<tr>
<td>gdmP-HindIII R</td>
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<tr>
<td>gdmQ-EcoRI F2</td>
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<td>kan R</td>
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<tr>
<td>PROOF-Q</td>
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</table>
4. Results

4.1. Sequencing of the gallidermin gene cluster
The central part of the sequence of the gallidermin synthesis gene cluster (gdmF, gdmT, gdmH, gdmA, and partial sequences of gdmE and gdmB) was already available (165, 338). We determined the DNA sequence of the remaining gaps in the gallidermin gene cluster by iPCR with primers designed on the available gdm sequence or by PCR with primers based on the equivalent epidermin cluster sequences. The determined DNA sequence was in perfect agreement with the sequence determined previously by Hille (165), which became available to us only after the sequencing of the gallidermin synthesis gene cluster had been completed. We could confirm that the epidermin and gallidermin cluster are highly homologous on both DNA and amino acid level and for further information we refer to the original work (165).

4.2. Construction and verification of a S. gallinarum ∆gdmP::aphIII mutant strain
The Shine-Dalgarno sequence of gdmQ overlaps with the gdmP stop codon (Fig. 1), thus gdmQ is translationally coupled to gdmP, and the gdmP promoter may serve as a promoter for transcription of both genes (165). To ensure expression of gdmQ in a gdmP deletion mutant, the Bacillus subtilis aminoglycoside phosphotransferase gene (aphIII) (147), conferring kanamycin resistance, including its constitutive promoter in the suitable orientation were introduced upstream of gdmQ into gdmP (Fig. 1). An internal gdmP fragment and a aphIII-gdmQ gene fusion were inserted into a derivative of the conditional shuttle vector pBT2, which is based on the temperature-sensitive replicon of pE194 (45). The resulting plasmid pGV5 was transferred into S. gallinarum Tü3928 and selection for double homologous recombination was performed by incubation at the permissive temperature and selection at the non-permissive temperature on kanamycin-containing plates. Resulting colonies were screened for double homologous recombination by replication onto chloramphenicol- and kanamycin-containing plates. Three colonies were found to be sensitive to chloramphenicol and resistant to kanamycin and assumed to be deletion mutants. These were further characterized by PCR analysis and all displayed the expected
amplicons. One of the three strains was named \textit{S. gallinarum} \textit{ΔgdmP::aphIII} and was further analysed and characterized in this study.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Construction of a \textit{S. gallinarum} Tü3928 mutant lacking \textit{gdmP}. Upper panel: \textit{gdmP} and \textit{gdmQ} and the sequence between them in detail, including the stop codon of \textit{gdmP}, the start codon of \textit{gdmQ}, and the Shine-Dalgarno sequence (SD). Lower panel: \textit{gdmP}, the kanamycine-resistance gene \textit{aphIII}, and \textit{gdmQ} in \textit{S. gallinarum} \textit{ΔgdmP::aphIII}. Arrows represent primers for verification of homologous recombination, and the sequences that were chosen for homologous recombination are shaded in light and dark grey. The promoters of \textit{gdmP} and \textit{aphIII} are represented by thick arrows. Expected lengths of amplicons are indicated (for details see text).}
\end{figure}

\section{Characterization of the \textit{S. gallinarum} \textit{ΔgdmP::aphIII} mutant strain}

Supernatants of \textit{S. gallinarum} \textit{ΔgdmP::aphIII} and \textit{S. gallinarum} Tü3928 cultures grown in medium YE4 for 18 hours were analyzed by HPLC for the presence of gallidermin or any precursor peptides. The supernatants from \textit{S. gallinarum} Tü3928 cultures contained only gallidermin, whereas no peak corresponding to gallidermin could be identified in supernatants of \textit{S. gallinarum} \textit{ΔgdmP::aphIII}. Instead, a novel peak was detected at a slightly smaller retention time. By analyzing the supernatants of \textit{S. gallinarum} \textit{ΔgdmP::aphIII} cultures with silver stained blots of tricine-SDS-polyacrylamide gels, we detected the presence of a roughly 4 kDa peptide (Fig. 2). This indicated that indeed the mutant did no longer produce gallidermin but produced a higher molecular weight precursor peptide. Interestingly, at comparable cell dry weight concentrations, the supernatant of \textit{S. gallinarum} Tü3928 contained remarkably more proteins than the supernatant of the mutant (Fig. 2), which might be
interpreted as a result from the interactions with gallidermin accumulating in the supernatant of the wild type culture.

Fig. 2: Tricine SDS-PAGE of 2 µl of the supernatant of a S. gallinarum ΔgdmP::aphIII culture Tü3928 (containing pregallidermin) (lane 2) and 2 µl of the supernatant of a S. gallinarum culture (containing gallidermin) (lane 3). 1 µg (lane 4) and 0.5 µg (lane 5) of commercial gallidermin as well as the polypeptide SDS-PAGE molecular weight marker (lane 1) were loaded. After electrophoresis, the separated proteins were blotted onto a nitrocellulose membrane and silver-stained.

In order to produce larger amounts of gallidermin and the potential precursor peptide 3 L fermentations were carried out with the wild type and the mutant strain. These were subsequently enriched and their identity was confirmed by mass spectrometry. Gallidermin that had accumulated in the supernatant of S. gallinarum Tü3928 resulted in a mass ion peak (M+H)$^+$ at 2167.2 ±2 Da, which was identical to the one obtained with commercially available gallidermin and is in excellent agreement with the signal expected from the predicted 21 amino acid peptide (2165 Da) (Fig. 3 A, B). The MS spectrum of the compound isolated from the supernatant of S. gallinarum ΔgdmP::aphIII revealed the presence of two peptides of 3407.4 Da and 3620.5 Da (Fig. 3 C), corresponding well to a 33 and a 36 amino acids-long pregallidermin fragment (Fig. 3 C). The expected form, consisting of the entire GdmA peptide, would have resulted in a mass peak of 5608 kDa but was surprisingly not detected. Moreover, remarkably different proportions of the two peptides were
present in the supernatant, with the larger one reaching only 10% of the MS spectrum intensity (Fig. 3 C). In the following, we will apply the name “pregallidermin” to the mixture of the two obtained precursor peptides.

Fig. 3: Identity of the peptides produced by *S. gallinarum* Tü3928 and *S. gallinarum*Δ*gdmp*::*aphIII*. A) LC/ESI-MS spectrum of commercial gallidermin and B) LC/ESI-MS spectrum of gallidermin produced in this study. The spectra show the relative abundance of ions in relation to the mass-to-charge (m/z) ratio of ions. The detection range was limited to below 2000, thus the double charged molecular ion is detected. C) Upper panel: nanoESI-MS of pregallidermin variants produced by *S. gallinarum*Δ*gdmp*::*aphIII*. The two peaks correspond to 33 amino acid and 36 amino acid pregallidermin fragments. This and the spectrum in D show the relative abundance of ions in relation to the mass of ions. Lower panel: amino acid sequences of the 30 amino acids-long pregallidermin leader peptide as expected from *gdmA* sequence (C1), and the 14 and 12 amino acids-long truncated peptides of pregallidermin variants from culture supernatant of *S. gallinarum*Δ*gdmp*::*aphIII* (C2, C3). D) NanoESI-MS of gallidermin generated by tryptic cleavage of pregallidermin. The peak at 2165 corresponds to that of commercial gallidermin. The spectrum shows the relative abundance of ions in relation to the mass of ions.
Chapter 2 – Circumventing the effect of product toxicity

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4.4. Characterization of activity of pregallidermin

In order to evaluate the antimicrobial activity of the enriched compounds, we investigated their effect on the indicator strain *Kocuria rhizophila* ATCC 9341 and on *S. gallinarum* Tü3928. While supernatants of *S. gallinarum* Tü3928 cultures displayed the expected activity on *K. rhizophila*, supernatants from cultures of *S. gallinarum ΔgdmP::aphIII* showed no antimicrobial activity on such plates (results not shown). By systematically increasing the concentration of the purified peptides it was shown that solutions with as much as 8 g L⁻¹ pregallidermin did not lead to the formation of inhibitory zones (Fig. 4 A). In contrast, inhibitory zones were observed when spotting solutions with 0.25 g L⁻¹ of gallidermin (Fig. 4 B).

4.5. Proteolytic cleavage with trypsin

In order to confirm that pregallidermin enriched from *S. gallinarum ΔgdmP::aphIII* supernatants could be transformed into active gallidermin and that the absence of a self-toxicity was only due to the presence of the truncated leader peptide, we investigated proteolytic cleavage of pregallidermin. Analysis of the cleavage sites of known proteases and of the pregallidermin sequence indicated that trypsin should be a suitable candidate for the biotransformation. Reactions were performed where 75 nmol of pregallidermin were treated with 1 U of trypsin. After 20 h total conversion of pregallidermin had taken place, since the pregallidermin peak could no longer be detected by HPLC analysis. Instead, a novel peak was detected at a retention time identical to that of gallidermin. The reaction was analyzed by ESI-MS, and a molecular ion peak corresponding exactly to the expected mass of gallidermin of 2165 Da was obtained (Fig. 3 D). To further confirm these results, we used equivalent amounts of gallidermin treated with trypsin and of commercially available gallidermin in bioassays with *K. rhizophila*. Indeed, the gallidermin generated by trypsin treatment showed similar specific antimicrobial activity as the commercially available gallidermin, while the control solution containing trypsin but no pregallidermin did not lead to the formation of an inhibition zone (Fig. 4 C). Furthermore, trypsin solutions from 10 mg L⁻¹ to 10 g L⁻¹ were used in a control bioassay, to exclude bioactivity of trypsin (Fig. 4 D).
4.6. Effect on production titer

The results discussed above indicated that *S. gallinarum ΔgdmP::aphIII* indeed produces pregallidermin that – within the range of our analysis – is not toxic to *S. gallinarum* Tü3928 strains. A truncated form of the leader sequence confers inactivity to the peptides, even though their gallidermin moiety appears to be fully mature. In view of our goal of overproducing gallidermin, we investigated whether the elimination of *gdmP* and the resulting switch from the production of a toxic peptide to a non-toxic precursor would be sufficient to increase concentration of the product. In order to determine final cultivation titers we carried out 3 L fed-batch fermentations with the wild-type and the mutant *S. gallinarum* strains. Gallidermin accumulated to a maximum of 280 mg L\(^{-1}\) in *S. gallinarum* Tü3928 fermentations (Fig. 5 A), whereas pregallidermin accumulated to 630 mg L\(^{-1}\) in *S. gallinarum ΔgdmP::aphIII* supernatants (Fig. 5 B), corresponding to a gallidermin concentration of 420 mg L\(^{-1}\). Therefore, an increase of 50% has already been achieved in these initial, non-optimized fermentations.
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Fig. 4: Bioassays to establish the toxicities and specific activities of different peptide preparations. 20 µL of solutions with different concentrations of pregallidermin/gallidermin were spotted on filters and placed on agar plates. Upper panel: Toxicity test of A) enriched pregallidermin and B) commercial gallidermin against S. gallinarum Tü3928. K: control (sterile water). Lower panel: C) Bioactivity of 1) commercial gallidermin and 2) gallidermin produced by proteolytic cleavage of pregallidermin with trypsin against the indicator strain K. rhizophila. K: control (sterile water for gallidermin and the trypsin solution for pregallidermin). D) Bioactivity of trypsin at different concentrations against the indicator strain K. rhizophila.
Fig. 5: Results from 3 L fed-batch fermentations performed to overproduce gallidermin and pregallidermin. A) Wild-type *S. gallinarum* Tü3928 fermentations lead to a maximal yield of gallidermin of 280 mg L⁻¹ and B) 3 L fed-batch fermentations with the mutant *S. gallinarum ΔgdmP::aphIII* strain lead to a maximal yield of pregallidermin of 630 mg L⁻¹, which corresponds to a gallidermin concentration of 420 mg L⁻¹. After 17 hours, 15 g (NH₄)₂SO₄ were added (X).
5. Discussion

Overproduction of lantibiotics has so far met with only very limited success, which might be attributed to the toxicity of the molecules for its own producer. Fundamentally, there are three possible strategies to overcome this bottleneck and increase the amount of active molecule produced per reactor volume: [i] Rapid removal of the active molecule from the cells (by selective in situ product removal (180, 448-450) or removal of the supernatant from the reactor (21, 439), [ii] the isolation of producer strains that are insensitive to (elevated concentrations of) the active molecule (448), and [iii] the production of a non-toxic variant of the molecule that can be easily transformed into the active form after its production. Although [i] has led in some cases to increases in volumetric productivities (21, 449), it did not lead to increases in product concentration, which would be advantageous from the perspective of product purification. Moreover, no indications of success of [ii] are available from the literature. This might be traced back to the multiple modes of action of type A-lantibiotics on their Gram-positive producers. Consequently, we investigated the feasibility of production of a non-toxic variant.

An easy strategy towards this aim might consist in the elimination of the final post-translational modification step, i.e. extracellular proteolytic cleavage of the leader sequence from Type-A lantibiotic precursor peptide. This approach is supported by previous studies on nisin showing that N-terminal extensions are detrimental to the activity of the lantibiotic. The modified but uncleaved nisin Z precursor peptide has been found to be completely inactive against M. flavus (93), and a precursor in which the leader peptide of nisin was exchanged for the subtilin leader segment did not show any activity against lipid II-containing liposomes (473).

Consequently, we investigated this rationale for gallidermin, which has been shown to exhibit a particularly promising antimicrobial profile (82, 337, 487). Specifically, S. gallinarum ΔgdmP::aphIII was generated by insertional disruption of gdmP, which encodes a subtilisin-like serine protease responsible for gallidermin activation. Gallidermin could not be detected by HPLC analysis of culture supernatants of the engineered strain, while a new peak was observed implying the production of pregallidermin. Two pregallidermin variants were identified, which had a higher molecular mass than gallidermin (Fig. 2) and were shown to be truncated versions of the mature pregallidermin (Fig. 3). Their molecular weights corresponded
to prepeptides having respectively a 12 and a 14 amino acid-long leader peptide. As no alternative start codons are present in the \textit{gdmA} sequence, these truncated prepeptides are likely to be generated by the action of other proteases. The two variants were present in different proportions in supernatants, with the larger one reaching only 10\% of the spectrum intensity. These results were in agreement with a previous study reporting that two preepidermin fragments were detected in supernatants of a \textit{S. epidermidis} $\Delta$\textit{epiP} mutant strain (219). Furthermore, it resulted that the truncated pregallidermins did not show self-toxicity even at higher concentrations than the ones expected in overexpression experiments (Fig. 4).

Still, the possibility remained that the produced prepeptides did not carry a mature gallidermin moiety, but a non-functional variant of identical mass. However, hydrolysis of pregallidermin by tryptic cleavage resulted in fully active gallidermin of exactly the expected mass and, within the accuracy of a bioassay, identical specific activity to commercially available gallidermin (Fig. 3 and 4). This indicates that the truncated leader sequence is sufficient to prevent gallidermin toxicity. Furthermore, this suggests a simple processing strategy to obtain efficiently gallidermin from pregallidermin by biotransformation.

Finally, we looked for a first indication of the beneficial effects that elimination of product toxicity might have on gallidermin production. For this, we applied the same protocol as for gallidermin production from \textit{S. gallinarum} Tü3928 to the fermentation of the mutant strain. Even in these non-optimized settings, the mutant strain produced gallidermin to a 50\% higher concentration (in mol L$^{-1}$) than the wild-type strain. In the case of the wild-type strain gallidermin concentrations increased over the first 10 h of the fermentation and leveled then off. Biomass concentration increased afterwards only slightly and at significantly reduced specific growth rates, suggesting adverse effects of the accumulating gallidermin. On the contrary, production of pregallidermin with the mutant continued for another 10 h and leveled only out as the biomass stopped to increase, indicating a medium limitation rather than self-toxicity as the pregallidermin has been shown to be not toxic at these concentrations. This resulted in a higher final pregallidermin concentration, which was also – in molar terms – higher than the gallidermin concentration achieved in the first fermentation.

In summary, the fundamentals of a novel overproduction strategy for type-A lantibiotics based on the elimination of their self-toxicity were established in this study.
Exemplified by the overproduction of pregallidermin as a non-toxic gallidermin variant, this strategy might be of much broader usefulness, as type-A lantibiotics are synthesized through an almost identical production pathway. In fact, they are all ribosomally synthesized as inactive prepeptides that are post-translationally modified, then exported and proteolytically activated extracellularly. Current efforts in our laboratory are directed to the optimization of the fermentation process and of the production strain to further enhance the production titer. We reason the production strategy illustrated in this study can be the foundation of a process suitable for an industrial scale production of pharmaceutical grade type A-lantibiotics, including the widely used nisin, which could play a significant role in the battle against antibiotic resistant pathogens, such as methicillin- and vancomycin-resistant S. aureus.

6. Acknowledgements

G. Valsesia carried out the sequencing, the generation of the mutant strain and other molecular biology parts of this work and and wrote the article. G. Medaglia developed the HPLC method, the tryptic cleavage protocol and performed the fermentations.

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We are indebted to A. Dumoulin, M. Bischoff, B. Berger-Bächi, D. Hilvert. and P. Hunziker for their invaluable help with Staphylococcus molecular biology and MS analyses.
Chapter 3
Towards overproduction of pregallidermin by means of increasing mRNA template concentration: Gene dosage increase and promoter engineering of the pregallidermin cluster genes

1. Abstract

Genetic engineering strategies that have proved successful for overproduction of lantibiotics are usually based on the provision of additional copies of the lantibiotic biosynthesis, transport, immunity, and regulation genes. However, overproduction has always been limited to titers in the low mg L\(^{-1}\) range. In this study, we investigated enhancing gallidermin or pregallidermin production by \textit{Staphylococcus gallinarum} Tü3928 or the GdmP-protease deficient \textit{S. gallinarum} \textit{ΔgdmP::aphIII} by following two different genetic strategies: [i] increasing the gene dosage of the pregallidermin production cluster; and [ii] modification of the promoters involved in expression of the pregallidermin production genes. However, none of these strategies led to increased (pre)gallidermin titers. On the contrary, (pre)gallidermin concentrations were in most cases significantly reduced if compared to the titers achieved with \textit{S. gallinarum} Tü3928 and \textit{S. gallinarum} \textit{ΔgdmP::aphIII}, a mutant that had the GdmP protease deleted and produced a gallidermin precursor. Moreover, these attempts were significantly frustrated by instability and recombination of plasmid vectors in either of the two strains. In order to reduce the chances of homologous recombination, we constructed a deletion mutant of \textit{S. gallinarum} that lacked the entire pregallidermin gene cluster. However, the production plasmids tested were unstable even in the cluster-free mutant, and no positive effects on pregallidermin production were observed. We conclude that \textit{S. gallinarum} in its current state does not seem to be a suitable host for the straightforward application of standard genetic engineering manipulations based on the employed plasmid-tools.
2. Introduction

*Staphylococcus gallinarum* Tü3928 (95) is the natural producer of the lantibiotic gallidermin. Gallidermin biosynthesis starts with the production of an extended prepropeptide encoded by the structural gene *gdmA*. This prepropeptide is then post-translationally modified by the enzymes GdmB, GdmC, and GdmD, and exported as pregallidermin by the action of the transporters GdmT and GdmH. In the extracellular space, pregallidermin is activated to gallidermin by the action of the serine protease GdmP (165, 339). Transcription of the genes of the gallidermin cluster is very likely to be activated by the regulator GdmQ (165).

Previously, we developed a two-step strategy for increased production of gallidermin, which is based on the overproduction of the inactive, fully post-translationally modified pregallidermin by the mutant strain *S. gallinarum* ∆*gdmP::aphIII* (Chapter 2). Effectively, this strategy enables contemplation of the problem of gallidermin overproduction as a problem of plain peptide overexpression, as the antibiotic activity of gallidermin does no longer interfere with its production. This should allow the application of the well-established biotechnological toolbox of gene overexpression to the overproduction problem. In addition, some genetic strategies have offered a valid approach for lantibiotic overproduction in the past (74, 160, 369).

As the genes of lantibiotic clusters are subject to transcriptional activation (146, 293) one possible strategy might rely on overproduction of the transcriptional activator. In a previous study this strategy was followed: A plasmid carrying the gene of transcriptional activator of the epidermin gene cluster EpiQ, which shows 78% identity on amino acid level to the activator of the gallidermin cluster, GdmQ (165), was introduced into *S. gallinarum* Tü3928 under the control of the strong constitutive promoter of the arsenic-resistance operon (372). The specific gallidermin production of the recombinant strain was indeed increased from 30 mg g⁻¹ of cell dry weight (CDW) to 50 mg g⁻¹, but no change in final titers was reported. Moreover, Peschel and coworkers reported a two-fold increase in specific epidermin production (from 4.6 to 10.1 mg g⁻¹ CDW) due to the presence of a plasmid carrying *epiQ* in *S. epidermidis* but, once more, no change in final titers was reported (339). As the regulation of the GdmQ synthesis has already been changed in our *S. gallinarum* ∆*gdmP::aphIII* production strain and brought under the control of the *aphIII* promoter, we did not
take this strategy further into consideration for pregallidermin overproduction in this study.

Another approach relies on a gene dosage effect for lantibiotic overproduction, and has been successfully implemented for nisin, subtilin, and lactacin 3147 (57, 74, 160). These studies demonstrated that the provision of additional copies of the regulatory or immunity genes can result in increased concentrations of the peptide. For example, increasing the gene dosage lead to 1.5- to 1.7-fold-higher titers of nisin and subtilin (25000 AU mL⁻¹ and 9 mg L⁻¹, respectively) (57, 160). Moreover, provision of additional copies of the biosynthetic/transport and of the regulator genes was required for 2.7-fold overproduction of LtnA1 and 6.6-fold overproduction of LtnA2, the two prepeptides of the two-component lantibiotic lacticin 3147, whereas the presence of additional copies of the two structural genes was not required (74). However, final titers were not reported.

In this study, we investigated the gene dosage strategy discussed above for the production of pregallidermin, and we provided an alternative approach to gene overexpression by applying strong promoters for selected genes in the pregallidermin production cluster.

3. Materials and methods

3.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Tab. 1. *Escherichia coli* strains were routinely grown in Luria-Bertani broth (387), whereas staphylococci were grown in B broth (219) or in Brain Hearth Infusion (BHI) broth (Difco Laboratories, Detroit, MI). All percentages refer to wt/vol unless otherwise mentioned. For production of gallidermin by *S. gallinarum* strains medium #21 (Chapter 2) was used. Media were supplemented when appropriate with ampicillin (100 µg mL⁻¹), chloramphenicol (10 µg mL⁻¹), kanamycin (50 µg mL⁻¹), tetracycline (10 µg mL⁻¹), or erythromycin (5 µg mL⁻¹), unless otherwise noted. Shake flasks experiments were performed with 20 mL cultures in 100 mL Erlenmeyer flasks with 4 baffles, which were incubated at 37°C and 190 rpm in a shaking incubator (amplitude: 25 mm), unless otherwise noted.
3.2. Genetic procedures

Standard genetic procedures were performed as reported elsewhere (Chapter 2). Staphylococcal chromosomal DNA was isolated according to previously described protocols (10). Plasmid minipreps from staphylococci were performed as described elsewhere (455), with a modification. Briefly, 15 mL overnight cultures were centrifuged at 4000*g, 4°C for 10 min. Pellets were washed with 1 mL of ddH2O and then resuspended in 100 µL of a solution of pH 8 containing 50 mM Tris-HCl and 10 mM EDTA (TE8 solution). An aliquot of 50 µL of a lytic enzyme mixture (50 g L⁻¹ lysozyme (Sigma-Aldrich, Buchs, Switzerland), 200 U mL⁻¹ mutanolysin (Sigma-Aldrich, Buchs, Switzerland), 0.3 g L⁻¹ lysostaphin in water) were added, and then solutions were incubated for 2 h at 37°C in a shaking incubator (speed: 150 rpm, amplitude: 25 mm). An aliquot of 200 µL of TE8 solution was added and cells were lysed by addition of 300 µL of 0.2 N NaOH/1% SDS in water for 10 min at room temperature (RT). Then, 300 µL of 3 M potassium acetate were added, the solutions were incubated for 15 min on ice, and centrifuged at 14000*g, 4°C for 15 min. The supernatants were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) (Fluka, Buchs, Switzerland) and plasmid DNA was purified through a JetQuick column (Genomed GmbH, Löhne, Germany). Low-copy plasmid isolation from staphylococci was performed as described by Kies (218). In our hands, only staphylococcal plasmid DNA could be introduced into S. gallinarum strains, therefore plasmid DNA prepared from E. coli had to be first electroporated into the restriction negative S. aureus RN4220 as described elsewhere (393). Electrocompetent S. gallinarum and S. gallinarum ΔgdmP::aphIII cells were prepared and electroporation was carried out as described previously (Chapter 2).

High fidelity PCR amplifications were performed using the Expand High Fidelity PCR system (Roche, Rotkreuz, Switzerland). PCR was performed in 50 µL reactions, which contained 100 ng of template DNA, 0.3 µM of each oligonucleotide, 0.2 µM of each dNTP, 2.6 U of Expand High Fidelity Enzyme mix, and 1x Expand High Fidelity buffer (containing 1.5 mM MgCl₂). Cycling conditions were 94°C for 2 min followed by 30 cycles of: 94°C for 10 sec, 50-59°C for 30 sec, 68°C for 8 min; and a final extension step at 68°C for 7 min. Primers used in this study are listed in Tab. 2.
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3.3. Construction of the production plasmids, of the promoter-test plasmids, and of an integration vector

Isolation of the pregallidermin gene cluster and construction of the production plasmid pCLUST2S: S. gallinarumΔgdmP::aphIII genomic DNA was isolated and digested with HpaI and SbfI, which was predicted to produce one 10.5 kb fragment that contained the pregallidermin gene cluster including the kanamycin resistance gene aphIII. The population of fragments was cloned into pUC18-NotI (274) previously digested with HincII and PstI. The plasmid carrying the pregallidermin gene cluster (pCLUST1) was selected for on kanamycin-containing agar plates. Next, the E. coli-Staphylococcus shuttle vector pALCNotI was constructed by excising the multiple cloning site from pALC2084 AatII to SbfI (16) and replacing it by an AatII-EcoRI-KpnI-EcoRV-NotI-BamHI-SbfI linker generated by annealing of 5’-phosphorylated oligonucleotides oligoNotI 1 and 2. Then, the production vector pCLUST2S was assembled by cloning the approximately 10.5 kb NotI fragment containing the pregallidermin gene cluster from pCLUST1 into pALCNotI. Correctness of the plasmid was verified by restriction analysis with HindIII, SpeI, Clal, SacI, PvuII, and HincII.

Introduction of the production plasmid pCLUST2S into S. gallinarum and S. gallinarumΔgdmP::aphIII and verification of the transformants: S. gallinarumΔgdmP::aphIII and S. gallinarum were transformed with pCLUST2S and transformants were grown at 30°C on selective B-agar plates containing 10 µg mL⁻¹ chloramphenicol. Plasmid DNA was isolated from S. gallinarum[pCLUST2S] and S. gallinarumΔgdmP::aphIII[pCLUST2S] from 15 mL B-medium cultures following the modified method described above.

Construction of the promoter-test plasmids: Three promoter sequences, P_aphIII, P_meca, and P_cap1A, were amplified by PCR with the primers listed in Tab. 2 using S. gallinarumΔgdmP DNA (P_aphIII) carrying the aphIII gene, pALCP_mecaAgdmTH (P_meca) or pALCP_cap1A_gdmTH (P_cap1A) as templates. High fidelity PCR amplifications were performed with the primer pairs P_aphIII F/R, P_cap1A F/R, and P_meca F/R, was performed with temperatures of annealing of 50-53°C. The resulting amplicons were digested with KpnI and Ncol and cloned into equally digested pSPluc+NF (Promega, Madison, WI) to yield the plasmids pP_aphIII-luc, pP_meca-luc and pP_cap1A-luc. The promoter-luciferase gene cassettes were then re-excised with KpnI and EcoRI and inserted into
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equally digested pALCNotI, to generate the staph-compatible promoter-probe plasmids pALCP<sub>aphIII</sub>, pALCP<sub>mecA</sub>, and pALCP<sub>cap1A</sub>. The accuracy of the final plasmids was confirmed by sequencing (Fasteris SA, Plan-les-Ouates, Switzerland). The plasmids were introduced by electroporation in <i>S. aureus</i> and <i>S. gallinarum</i> as described previously (Chapter 2).

**Construction of the promoter-engineered production plasmids:** The transporter gene <i>gdmH</i> was first amplified with the primer pair NotI-gdmH F and Spht-gdmH R. High fidelity PCR amplification was performed with a temperature of annealing of 55°C, and generated a 990 bp-long amplicon, which was digested with NotI and Spht and cloned into equally digested pALCNotI to yield pALC-H. The <i>gdmT</i> sequence was amplified with the primer pairs KpnI-P<sub>mecA</sub>gdmT F/NotI-gdmT R and KpnI-P<sub>cap1A</sub>gdmT F/NotI-gdmT R at an annealing temperature of 58.5°C, and generated two 1.3 kb-long amplicons. These were digested with NotI and KpnI and inserted into likewise digested pALC-H to generate the plasmids pmTH and pcTH. Amplification of the biosynthesis genes sequence (from <i>gdmA</i> to <i>gdmD</i>) and introduction of P<sub>mecA</sub> and P<sub>cap1A</sub> promoters was carried out with the two primer pairs AatII-P<sub>mecA</sub>gdmA F/KpnI-gdmD R and AatII-P<sub>cap1A</sub>gdmA F/KpnI-gdmD R with an annealing temperature of 58.5°C. The resulting 4.9 kb-long amplicons (mAD, cAD) were digested with AatII and KpnI and cloned into equally digested pALCNotI to yield pcAD and pmAD. After digestion with AatII and KpnI the cAD amplicon was also inserted into similarly digested pmTH to generate the plasmid pmTHcAD. The sequence of the structural gene <i>gdmA</i> was amplified using the primer pairs AatII-P<sub>cap1A</sub>gdmA F/KpnI-gdmA R and AatII-P<sub>mecA</sub>gdmA F/KpnI-gdmA R. The 259 bp-long PCR products were digested with AatII and KpnI, and cloned into equally digested pALCNotI, yielding the plasmids pcA and pmA. The plasmids were verified by restriction analysis with Scal and HindIII. Correctness of the promoters was verified by sequencing, which was outsourced to Fasteris SA (Plan-les-Ouates, Switzerland).

**Development of the integration vector pGV6:** The gdmG-gdmE sequence was amplified from genomic DNA of <i>S. gallinarum</i> by high fidelity PCR using the primers Scal-outG F and KpnI-E R. PCR amplification was performed at a temperature of annealing of 53°C, and generated to a 1500 bp-long amplicon that was digested with KpnI and Scal and cloned into equally digested pUC18NotI-P (Chapter 2), to yield the plasmid pUC18NotI-P-EG. The erythromycin resistance gene <i>ermC</i> was amplified by
high fidelity PCR using pMAD (7) as a template with the primer pair \textit{SalI}-ermC F and \textit{XbaI}-ermC R. PCR amplification was performed with a temperature of annealing of 55°C and generated a 1400 bp-long amplicon, which was digested with \textit{XbaI} and \textit{SalI} and then cloned into equally digested pUC18\textit{NotI}P-EG, to generate the plasmid pUC-PEGermC. The 3550 bp-long \textit{NotI} fragment was then inserted into \textit{NotI}-digested pGV2 (Chapter 2), generating the homologous recombination vector pGV6.

3.4. Characterization of the activity of P\textit{mecA}, P\textit{cap1A}, and P\textit{aphIII} in \textit{S. aureus} and \textit{S. gallinarum} by means of the luciferase assay

To verify the operation and relative strengths the P\textit{aphIII}, P\textit{cap1A}, and P\textit{mecA} promoters, production of the luciferase reporter Luc was determined by measuring its capacity to emit light in the early-log, mid-exponential, late-exponential, and stationary phase. Bacterial cells from overnight cultures were diluted in BHI to an an optical density at 600 nm (OD\textsubscript{600}) of 0.05. The cells were harvested at different optical densities (OD\textsubscript{600} of 3, 8, 12, 14) at 13,000 x g for 1 min at room temperature, and the cell pellets were conserved at -80°C. Pellets were then thawed and resuspended in 0.1 M sodium phosphate buffer (pH 7.0) to an OD\textsubscript{600} of 10. Luciferase activity was then determined by rapidly mixing the resuspended cells (10 µL) with an equal volume of luciferase assay reagent (containing the substrate beetle luciferin and the cosubstrates ATP, Mg\textsuperscript{2+}, and coenzyme A) (Promega, Madison, WI). Luminescence was measured on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) for 10 s with a delay of 2 s. Bacteria carrying pALC\textit{NotI} were used as negative controls, and background was measured by light emission of sterile medium. Samples were analyzed in triplicate.

3.5. Construction and verification of a \textit{S. gallinarum\Delta cluster} mutant strain

\textit{S. gallinarum\Delta gdmP::aphIII} was transformed with pGV6 and transformants were grown at 30°C on selective B-agar plates containing 5 µg mL\textsuperscript{-1} erythromycin. Gene replacement was induced in \textit{S. gallinarum \Delta gdmP::aphIII}[pGV6] as described previously (111), except that the first growth step at 30°C was repeated three times and cells were subsequently incubated at 42°C on B-agar plates containing 1 µg mL\textsuperscript{-1} erythromycin (MIC value had been determined in previous experiments to be 0.1 µg mL\textsuperscript{-1}). Screening for strains with double homologous recombination was
performed on B-agar plates containing 20 µg mL\(^{-1}\) chloramphenicol, 30 µg mL\(^{-1}\) kanamycin or 1 µg mL\(^{-1}\) erythromycin. A total of 55 colonies were found to be sensitive to chloramphenicol and kanamycin while resistant to erythromycin and assumed to be deletion mutants. Staphylococcal DNA for colony PCR was extracted using the InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The potential mutants were further analysed by colony PCR with the primer pairs SacI-outG F/KpnI-E R, gdmB F/R, gdmQ F/R, gdmF F/R, gdmH F/R. A total of 26 mutants displayed the expected amplicon pattern (absence of the gdmB, gdmQ, gdmF, and gdmH amplicons and presence of the gdmEG PCR product) (data not shown). Five of them, as well as *S. gallinarum*ΔgdmP::aphIII (as a control), were further characterized by PCR analysis with the primer pairs outG F proof/ermCinv R proof, XbaI-ermC R/gdmP R proof, outG F proof/gdmF R, and aphIII R proof/gdmP R proof for verification of the complete deletion of the cluster. Three of them displayed the expected amplicons and one was named *S. gallinarum*Δcluster. *S. gallinarum*Δcluster was electroporated with pCLUST2S and transformants were selected for on B-agar plates containing 10 µg mL\(^{-1}\) chloramphenicol incubated at 30°C.

Partial shotgun sequencing of a *S. gallinarum* genomic library resulted in the enriched presence of phage-specific sequences in the library. BlastX analysis of these sequences showed 41% sequence identity of one sequence to the endonucleases of *S. aureus* phages Φ13 and PVL. The primer pair phage1/2 was designed on this sequence, and PCR was performed using genomic DNA of the bacterial strains used in this study (*S. gallinarum*, *S. aureus*, *S. carnosus*, *S. xylosus*, *S. arlettae*, *S. epidermidis*, *B. subtilis*, *E. coli*). Amplification of *S. gallinarum* DNA resulted in a 980 bp product, whereas PCR with DNA from other bacterial strains did not produce any amplicons. Therefore, amplification with the primer pair phage1/2 was deemed specific for *S. gallinarum* strains within this set of strains, and could be applied to verify the identity of *S. gallinarum*Δcluster[pCLUST2S]. Colony PCR on transformants with primer pair phage1/2 was complemented by gdmB F/gdmB R.
3.6. Plasmid curing from *S. gallinarum* and *S. gallinarumΔgdmP::aphIII*

In order to easily verify the presence of transformed plasmids, the generation of a plasmid-cured strain would be very advantageous. From previous works (165) it is known that the genetic determinant of tetracycline resistance is located on a plasmid in *S. gallinarum*. Hence, we tried to take advantage of this information by using sensitivity to tetracycline as a marker for plasmid curing. Plasmid curing was attempted by EtBr treatment as described elsewhere (114). Briefly, 0.1 mL of overnight cultures of *S. gallinarum* and *S. gallinarumΔgdmP::aphIII* were added to vessels with trypticase soy broth (Becton Dickinson GmbH, Heidelberg, Germany) containing EtBr in concentrations increasing by a factor 2 per step from 0.2 to 25.6 µg mL\(^{-1}\). After a 24 h incubation at 37°C the cultures that contained a concentration of EtBr subinhibitory to cell growth were plated onto trypticase soy agar (TSA) plates. After 18 h of incubation, colonies were replica-plated onto tetracycline-containing and non-selective TSA plates to detect antibiotic-sensitive segregants. Curing was also attempted during protoplast formation (143). *S. gallinarumΔgdmP::aphIII* and *S. gallinarum* cultures were grown to an optical density at 600 nm of 0.5 in 10 mL tetracycline-containing B-broth. Cultures were centrifuged, resuspended in 2 mL SMM solution (0.5 M sucrose, 0.02 M sodium maleate, 0.02 M MgCl\(_2\)), and lysostaphin was added to a concentration of 50 µg mL\(^{-1}\). After incubation at 37°C for 1 h, protoplasts were diluted with 5 mL SMM, centrifuged, resuspended in 1 mL SMM, and then plated on DM3 regeneration plates (132), which were incubated at 37°C for 24 h. Regenerated colonies were scored for plasmid curing by differential plating and in part by plasmid isolation for verification.
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<table>
<thead>
<tr>
<th>Bacterial strains and plasmids</th>
<th>Genotype/ relevant characteristic(s)</th>
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<td><em>E. coli</em> cloning host $[$F- mcrA $\Delta$ <em>mrr-hsdRMS-mcrBC</em>) $\varphi$ 80lacZ $\Delta$ M15 $\Delta$ lacX74 recA1 araD139 $\Delta$ (araleu) 7697 galU galK rpsL (Str$^r$) endA1 napG$]$</td>
<td>Invitrogen</td>
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<td><em>S. aureus</em> RN4220</td>
<td>Restriction-deficient, modification positive <em>S. aureus</em></td>
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<td>Gallidermin producer; Tet$^r$</td>
<td>(95); DSMZ, Braunschweig, Germany</td>
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<td><em>S. gallinarum</em> $\Delta$gdmP::aphIII</td>
<td>Pregallidermin producer; Tet$^r$ Kan$^r$</td>
<td>Chapter 2</td>
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<td><em>S. gallinarum</em> $\Delta$cluster</td>
<td>Cluster-deleted mutant originating from <em>S. gallinarum</em> $\Delta$gdmP::aphIII; Tet$^r$, Erm$^r$</td>
<td>This study</td>
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<td><em>K. rhizophila</em></td>
<td>Gallidermin-sensitive indicator strain, previously known as <em>Micrococcus luteus</em></td>
<td>(238); DSMZ</td>
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<td>pUC18NotI</td>
<td>Cloning vector; Amp$^r$</td>
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<td>pUC18NotI with a part of gdmP; Amp$^r$</td>
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<td>pALC2084</td>
<td><em>Staphylococci</em> expression vector containing the tetR repressor gene and the tetO/xyl promoter. Cm$^r$, Amp$^r$</td>
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<td>pALC-H</td>
<td>pALCNotI with a fragment containing gdmH; Cm$^r$, Amp$^r$</td>
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<td>pmTH</td>
<td>pALCNotI with P<em>mech</em>-gdmT::gdmH; Cm$^r$, Amp$^r$</td>
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<td>pmAD</td>
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**pcAD**
- pALCNotl with $P_{\text{cap1A}}$::$\text{gdmABCD}$; Cm$^{\prime}$, Amp$^{\prime}$
  - This study

**pmA**
- pALCNotl with $P_{\text{mecA}}$::$\text{gdmA}$; Cm$^{\prime}$, Amp$^{\prime}$
  - This study

**pcA**
- pALCNotl with $P_{\text{cap1A}}$::$\text{gdmA}$; Cm$^{\prime}$, Amp$^{\prime}$
  - This study

**pmTHcAD**
- pALCNotl with $P_{\text{cap1A}}$::$\text{gdmABCD}$; Cm$^{\prime}$, Amp$^{\prime}$
  - This study

**pCLUST1**
- pUC18Notl with a 10.5 kb NotI fragment containing $\text{gdmABCD}$, $\text{gdmTH}$, $\text{gdmQ}$ and $\text{aphIII}$; Kan$^{\prime}$, Amp$^{\prime}$
  - This study

**pCLUST2S**
- pALCNotl containing the 10.5 kb NotI fragment from pCLUST1; Cm$^{\prime}$, Kan$^{\prime}$, Amp$^{\prime}$
  - This study

**pUC18Notl-P-EG**
- pUC18Notl-P with $\text{gdmG}$ and a part of $\text{gdmE}$; Amp$^{\prime}$
  - This study

**pUC18Notl-PEGermC**
- pUC18Notl-P-EG containing the erythromycin resistance gene $\text{ermC}$; Amp$^{\prime}$, Erm$^{\prime}$
  - This study

**pGV6**
- pGV2 with a 3550 bp NotI fragment containing the fragment internal to $\text{gdmP}$, the Erm$^{\prime}$ gene, and the sequence including $\text{gdmG}$ and a part of $\text{gdmE}$; Cm$^{\prime}$, Amp$^{\prime}$, Erm$^{\prime}$
  - This study

**pSP\textit{luc}+\textit{NF**}
- Promoter-probe vector containing the firefly luciferase gene, Amp$^{\prime}$
  - Promega, Madison, WI.

**pP\textit{aphIII-luc**}
- pSP\textit{luc}+\textit{NF} with the $P_{\text{aphIII}}$ promoter upstream of $\text{luc}$; Amp$^{\prime}$
  - This study

**pP\textit{mecA-luc**}
- pSP\textit{luc}+\textit{NF} with the $P_{\text{mecA}}$ promoter upstream of $\text{luc}$; Amp$^{\prime}$
  - This study

**pP\textit{cap1A-luc**}
- pSP\textit{luc}+\textit{NF} with the $P_{\text{cap1A}}$ promoter upstream of $\text{luc}$; Amp$^{\prime}$
  - This study

**pALC\textit{aphIII**}
- pALCNotl containing the the $P_{\text{aphIII}}$::$\text{luc}$ cassette from pP\textit{aphIII-luc}; Cm$^{\prime}$, Amp$^{\prime}$
  - This study

**pALC\textit{mecA**}
- pALCNotl containing the the $P_{\text{aphIII}}$::$\text{luc}$ cassette from pP\textit{mecA-luc}; Cm$^{\prime}$, Amp$^{\prime}$
  - This study

**pALC\textit{cap1A**}
- pALCNotl containing the the $P_{\text{aphIII}}$::$\text{luc}$ cassette from pP\textit{cap1A-luc}; Cm$^{\prime}$, Amp$^{\prime}$
  - This study
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<td>gdmB R</td>
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<td>gyrB R</td>
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4. Results

4.1. Investigations on a gene dosage effect

In an attempt to increase the pregallidermin gene dosage, a plasmid containing the whole pregallidermin gene cluster was generated and used for transformation of pregallidermin and gallidermin producing strains. In order to isolate the pregallidermin gene cluster we took advantage of the aminophosphotransferase gene (aphIII) that had already been inserted in the cluster to delete the gene encoding for the serine protease GdmP (Chapter 2). An *E. coli* transformant carrying the pregallidermin gene cluster, containing the biosynthesis genes *gdmABCD*, the transport genes *gdmTH*, and the regulatory gene *gdmQ* was selected from a library of *S. gallinarum*Δ*gdmP::aphIII* genomic DNA by resistance against kanamycin. The cluster was transferred to pUC18**Not**I and named pCLUST1. It was then transferred to an *E. coli-staphylococci* shuttle to yield the production plasmid pCLUST2S (Fig. 1), which then was electroporated into *S. gallinarum*Δ*gdmP::aphIII* and *S. gallinarum*. Transformants were selected for resistance against chloramphenicol and tetracycline, the former to select for the presence of the plasmid and the latter to exclude contaminations, as *S. gallinarum* strains are resistant to tetracycline. The growth behaviour of *S. gallinarum*Δ*gdmP::aphIII* [pCLUST2S] and *S. gallinarum* [pCLUST2S] did not differ significantly from the original strains (data not shown). Nevertheless, in shake flasks experiments with production medium #21, gallidermin production by *S. gallinarum* [pCLUST2S] was only 25 % of that of the wild-type strain (18 mg L⁻¹ ± 4.1 vs. 72 mg L⁻¹ ± 6.3), and pregallidermin production was only approximately 60 % (28 mg L⁻¹ ± 5.5 vs. 49 mg L⁻¹ ± 2.2). Moreover, pregallidermin production by *S. gallinarum*Δ*gdmP::aphIII* [pCLUST2S] was impaired: HPLC analysis of the culture supernatants showed a decrease of pregallidermin concentration from 95 mg L⁻¹ ± 7.4 for *S. gallinarum*Δ*gdmP::aphIII* to 15 mg L⁻¹ ± 5.2 for *S. gallinarum*Δ*gdmP::aphIII* [pCLUST2S]. Pregallidermin was not detected in silver stained blots of tricine-SDS-polyacrylamide gels of supernatants of *S. gallinarum*Δ*gdmP::aphIII* [pCLUST2S] cultures (Fig. 2), and no antibiotic activity could be detected in bioassays against the sensitive strain *K. rhizophila* after trypsinisation (data not shown). Re-testing of the resistance profiles of *S. gallinarum*Δ*gdmP::aphIII* [pCLUST2S] and *S. gallinarum* [pCLUST2S] transformants against tetracycline, chloramphenicol, and kanamycin indicated that
S. gallinarumΔgdmP::aphIII[pCLUST2S] transformants were tetracycline and kanamycin resistant, but had lost their resistance to chloramphenicol, which would have been expected due to the presence of a cat gene on pCLUST2S. S. gallinarum[pCLUST2S] transformants, in contrast, were chloramphenicol and tetracycline resistant but unexpectedly kanamycin sensitive, which was at odds with the expected presence of the aphIII gene on pCLUST2S. Plasmid pCLUST2S could not be recovered from either of the two strains S. gallinarumΔgdmP::aphIII[pCLUST2S] and S. gallinarum[pCLUST2S] (data not shown). However, plasmid patterns from transformed S. gallinarum strains are difficult to compare due to the presence of various natural plasmids in the original strain. Despite repeated efforts, we did not succeed in producing a plasmid-free variant of S. gallinarum or S. gallinarumΔgdmP::aphIII (see Materials and Methods for details).
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Fig. 1: Scheme of isolation of the pregallidermin gene cluster and of subsequent construction of pCLUST2S (approximately 15 copies/cell).
4.2. Characterization of P_{aphIII}, P_{cap1A}, and P_{meca} in S. gallinarum and S. aureus.

In order to rationally increase the mRNA production from gallidermin synthesis cluster genes, we investigated the relative strengths of additional constitutive promoters. Specifically, the activities of two strong staphylococcal promoters (P_{cap1A} and P_{meca}) and of a Streptococcus faecalis promoter (P_{aphIII}) were characterized in this study. The promoter P_{aphIII} drives the recombinant kanamycin resistance gene used for the elimination of \( gdmP \) and the activation of \( gdmQ \).

Strong promoters have been frequently associated with transcription of genes encoding virulence factors or antibiotic resistance determinants in pathogenic bacterial strains (287): The promoter P_{cap1A} (Fig. 3) naturally drives transcription of the type 1 capsule polysaccharide gene \( cap1A \) in S. aureus (310) and is fundamental for the biosynthesis of serotype 1 capsule, an important virulence factor in staphylococcal infections. This promoter was previously described as a strong promoter, as it was reported to display 45- to 198-fold higher activities than other promoters of the capsule gene cluster (330). The second promoter, P_{meca} (Fig. 3), is responsible for expression of the \( mecA \) gene in S. aureus, which encodes for the
methicillin resistance that relies on the production of a low-affinity penicillin-binding protein (PBP2) (466).

The last promoter that was characterized in this study, P_{aphIII}, is involved in the conferral of aminoglycoside resistance to *Streptococcus faecalis*, other Gram-positive bacteria and *E. coli*, as it drives transcription of a 3"5" aminoglycoside phosphotransferase gene of type 3 (147, 442, 443).

In order to verify the activity of these promoters, P_{cap1A}, P_{mecA}, and P_{aphIII} were fused to the sequence of the firefly luciferase gene and placed on the *E. coli*-staphylococci shuttle plasmid pALCNotl to generate the plasmids pP_{aphIII}, pP_{cap1A}, and pP_{mecA}. Both *S. aureus* RN4220 and *S. gallinarum* were transformed with the promoter-plasmids and with pALCNotl as a control.

Fig. 3: Promoter regions of A) the *S. aureus* capsular polysaccharide gene *cap1A* (330), B) the *S. aureus* mecA gene, conferring oxacillin resistance (466), and C) the *S. faecalis* aphIII gene (443). Underlined sections represent the putative promoter (-35, -10) and Shine–Delgarno (SD) sequences. Where known, transcription start point was indicated by +1.

The expression of the luciferase gene from the promoters during growth was monitored in both *S. aureus* and *S. gallinarum* strains by measuring luciferase activity (Fig. 4). Luciferase activity values were similar in both strains; they reached maximum levels during early- or middle-exponential phase (i.e. at 5-7 h) and kept declining thereafter, as the cultures reached stationary phase (i.e. at 12 h). P_{mecA} was
markedly the strongest promoter and had very high levels of activity compared to $P_{cap1A}$ (56-fold higher increase in luciferase activity in both strains) and $P_{aphIII}$ (1840- and 1390-fold in *S. aureus* and in *S. gallinarum*, respectively) (Fig. 4). The same trend was confirmed by real-time RT-PCR analysis (data not shown).

![Fig. 4: Activity of cap1A, mecA, and aphIII promoters was determined by measuring the luciferase activity of the promoter-luc fusions. Results are expressed relatively to the calibrator sample (*S. aureus*[pP$_{aphIII}$] at an OD$_{600}$ of 14).](image)

### 4.3. Promoter engineering in *S. gallinarum* and *S. gallinarumΔgdmP::aphIII* with the $P_{cap1A}$ and $P_{mecA}$ promoters

The natural promoters of the pregallidermin genes *gdmA* and *gdmT* were exchanged for the two strong promoters $P_{cap1A}$ and $P_{mecA}$, in order to provide increased mRNA levels of the corresponding genes. At the same time, this eliminated regulation of the corresponding genes by the transcriptional activator GdmQ. The promoters were inserted before the sequence of *gdmA* and placed on the *E. coli*-staphylococci shuttle plasmid pALCNotl to generate the plasmids pcA and pmA. Similarly, the biosynthesis genes *gdmA-gdmD* were placed as an operon under the control of these promoters on pALCNotl (pcAD and pmAD). The same was done with the transporter genes *gdmH* and *gdmT*, whose promoterless sequences were cloned so that both genes
had the same direction and were under the control of the new promoters, generating pcTH and pmTH. Additionally, the biosynthesis genes under control of P\textsubscript{cap1A} were introduced into pmTH. This led to production plasmid pmTHcAD, which contained all genes required for biosynthesis and export of pregallidermin.

\textit{S. gallinarum}\textsuperscript{\text{\textgdmP::aphIII}} and \textit{S. gallinarum} were transformed by electroporation with the P\textsubscript{cap1A} promoter-engineered plasmids pcA, pcAD, and pcTH. Colonies appeared after 24-72 h and were verified on tetracycline-containing agar plates. Overproduction of pregallidermin or gallidermin by the transformants was tested in shake flasks experiments. Growth of \textit{S. gallinarum} strains carrying the P\textsubscript{cap1A} promoter plasmids (pcA, pcAD, pcTH), as well as of \textit{S. gallinarum}\textsuperscript{\text{\textgdmP::aphIII}[pcAD]}, was compromised. In contrast, \textit{S. gallinarum}\textsuperscript{\text{\textgdmP::aphIII}[pcA]} and \textit{S. gallinarum}\textsuperscript{\text{\textgdmP::aphIII}[pcTH]} had a longer lag-phase than \textit{S. gallinarum}\textsuperscript{\text{\textgdmP::aphIII}[pCLUST2S]} or \textit{S. gallinarum}\textsuperscript{\text{\textgdmP::aphIII}} but grew to only slightly lower or comparable optical densities. Cultures of those strains that could grow under these experimental conditions were tested for overproduction of gallidermin or pregallidermin in shake flasks. \textit{S. gallinarum}\textsuperscript{\text{\textgdmP::aphIII}[pcA]} grew to slightly lower optical densities than \textit{S. gallinarum}\textsuperscript{\text{\textgdmP::aphIII}}, and did not seem to produce pregallidermin (Fig. 5). Even \textit{S. gallinarum}\textsuperscript{\text{\textgdmP::aphIII}[pcTH]}, which grew to similar optical densities as the unmodified strain, produced significantly less pregallidermin (20 mg L\textsuperscript{-1} instead of 100 mg L\textsuperscript{-1}) (Fig. 5).
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![Graph showing growth (OD) and pregallidermin (pregall) production profiles of S. gallinarum Δgdmp::aphIII, S. gallinarum Δgdmp::aphIII[pCLUST2S], S. gallinarum Δgdmp::aphIII[pcA], and S. gallinarum Δgdmp::aphIII[pcTH] in production medium #21 after 11, 15, 18, and 36 hours. Detection limit: 10 mg L⁻¹.](image)

Fig. 5: Growth (OD) and pregallidermin (pregall) production profiles of S. gallinarum Δgdmp::aphIII, S. gallinarum Δgdmp::aphIII[pCLUST2S], S. gallinarum Δgdmp::aphIII[pcA], and S. gallinarum Δgdmp::aphIII[pcTH] in production medium #21 after 11, 15, 18, and 36 hours. Detection limit: 10 mg L⁻¹.

Plasmid isolation from the transformants was performed to check for recombination. Although plasmid patterns are difficult to compare, we conclude that all plasmids underwent recombination but pcTH in S. gallinarum and pcA in both strains, as only the bands corresponding to these plasmids were still present once the plasmid preparations were analyzed on agarose gels (data not shown). Surprisingly, even though the bands of the introduced plasmids are missing in plasmid isolations from S. gallinarum Δgdmp::aphIII[pCLUST2S] and S. gallinarum Δgdmp::aphIII[pcAD], the plasmid patterns were not identical to the one of S. gallinarum Δgdmp::aphIII. As the gallidermin genes were reported to be chromosomally encoded (165, 383), we expected recombination as a result of homologous recombination to occur between genes encoded on the recombinant plasmids and the chromosome, not between the plasmids.
4.4. Development of the integration vector pGV6, construction and verification of a *S. gallinarum*Δ*cluster* mutant strain

Motivated by the frequent problems that we attributed in part to homologous recombination, we proceeded to construct a strain that lacked substantial parts of the pregallidermin production cluster and therefore should also be limited in its capacity to exploit recombinant plasmid-based sequences for homologous recombination. We selected a 700 bp-long internal gdmP sequence and a 1.5 kb fragment including the entire gdmG and a part of the gdmE gene as homologous sequences for deletion of the pregallidermin gene cluster from *S. gallinarum*ΔgdmP::aphIII. For selection of the recombinants, the staphylococcal methylase gene ermC from pMAD (7), conferring erythromycin resistance, was cloned between the two homologous sequences. The recombination cassette was inserted into pGV2, a derivative of the conditional shuttle vector pBT2, which is based on the temperature-sensitive replicon of pE194 (179) (Fig. 6 A). The resulting plasmid pGV6 was transferred into *S. gallinarum*ΔgdmP::aphIII and selection for double homologous recombination was performed. This resulted in the mutant strain *S. gallinarum*Δ*cluster*. After verification of the absence of amplification signals for the genes gdmB, gdmQ, gdmH, and gdmF by PCR analysis (data not shown), we confirmed the expected pattern from control PCR-reactions (Fig. 6 B, C), and we could also confirm that the strain did not produce (pre)gallidermin in shake flask experiments with production medium #21. Indeed, no (pre)gallidermin bands could be detected in HPLC analysis and no antibiotic activity against the sensitive strain *K. rhizophila* could be recovered from the supernatant, irrespective of trypsin treatment.
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Fig. 6: A) Schematic diagram of homologous recombination strategy for deletion of the pregallidermin gene cluster using vector pGV6. B) PCR amplicons expected for the mutant S. gallinarumΔcluster in comparison to the original strain S. gallinarumΔgdmP::aphIII. C) PCR verification of S. gallinarumΔcluster strain was performed with primer pairs outG F proof/gdmF R, aphIII R proof/gdmP R, XbaI-ermC R/gdmP R proof, and ermCinv R proof/outG F proof on DNA from (lane 1) S. gallinarumΔgdmP::aphIII, (lane 2) pGV6, (lane 3) S. gallinarumΔcluster. Water was used in no template control reactions (lane 4), and 2-Log DNA Ladder was loaded (lanes a).
4.5. Generation and characterization of the overproducers

*S. gallinarumΔcluster[pCLUST2S]* and *S. gallinarumΔcluster [pmTHcAD]*

In order to investigate whether the elimination of most parts of the pregallidermin production cluster had indeed any effect on the success of plasmid-based genetic engineering strategies in *S. gallinarum*, the cluster-free mutant *S. gallinarumΔcluster* was transformed by electroporation with the overproduction plasmids pCLUST2S and pmTHcAD. Surprisingly, *S. gallinarumΔcluster[pCLUST2S]* transformants were resistant to chloramphenicol but sensitive to kanamycin. Therefore, to verify the presence of the pregallidermin gene cluster, colony PCR was performed on the biosynthesis gene *gdmB*, while amplification of a phage sequence specific for *S. gallinarum* strains was performed in order to verify their identity. Almost all tested transformants as well as the positive control *S. gallinarumΔgdmP::aphIII* possessed *gdmB* and the phage-specific gene, whereas *gdmB* was not detected in the control *S. gallinarumΔcluster* (Fig. 7). Nevertheless, PCR analysis showed that the *aphIII* gene conferring kanamycin resistance was no longer present in the recombinant strain (data not shown) and no plasmid of the expected size could be isolated from *S. gallinarumΔcluster[pCLUST2S]* (Fig. 8A). The strain showed a prolonged lag phase (15 h) in shake flask experiments with production medium #21, and did not reach the same optical densities as the controls *S. gallinarumΔcluster* or *S. gallinarumΔcluster[pALCNotl]* (OD$_{600}$ 25 vs. 30). HPLC analysis of the supernatant from a 18 h culture of *S. gallinarumΔcluster[pCLUST2S]* confirmed that pregallidermin production was restored, even though at a very low level (data not shown). In contrast, on tricine-SDS-gels, no pregallidermin was detected in the supernatants of *S. gallinarumΔcluster[pCLUST2S]*, *S. gallinarumΔcluster* or *S. gallinarumΔcluster[pALCNotl]* (Fig. 9). Still, *S. gallinarumΔcluster[pCLUST2S]* supernatants were slightly active in bioassays, although no definite and clear inhibition zones were present, even upon trypsin treatment of the supernatant (data not shown).

*S. gallinarumΔcluster* cells were also transformed with pmTHcAD, which carries the whole set of genes for biosynthesis, modification and transport of pregallidermin under control of the *S. aureus* promoters P$_{cap1A}$ and P$_{mecA}$. Transformants were selected for on chloramphenicol-containing B-agar plates, and then plasmid isolation was performed to verify the presence of an intact pmTHcAD.
Once more, no plasmid of expected size could be recovered from the transformants, whereas pALCNotI isolation from *S. gallinarum*Δ*cluster*[pALCNotI] showed the expected pattern on a 0.8% agarose gel (Fig. 8 B). These results suggest that pALCNotI without additional DNA is neither unstable nor toxic, and that recombination is likely to have taken place. Growth behaviour of *S. gallinarum*Δ*cluster*[pmTHcAD] in shake flasks experiments with production medium #21 was similar to the one of *S. gallinarum*Δ*cluster*[pCLUST2S], which was discussed above. Pregallidermin production by *S. gallinarum*Δ*cluster*[pmADcTH] was also investigated by HPLC and tricine-SDS-PAGE analysis and on bioassays against the sensitive strain *K. rhizophila*. Interestingly, a peptide of the same size as gallidermin was detected in silver-stained blots of tricine-SDS-PAGE (Fig. 9), which showed antibiotic activity already prior to trypsinisation in bioassays (Fig. 9). Both HPLC analysis and bioassays of supernatants of cultures of *S. gallinarum*Δ*cluster*[pmADcTH] showed a restored gallidermin production at levels comparable to the wild-type *S. gallinarum* strain (HPLC data not shown, Fig. 10).

![Fig. 7: Colony PCR of four colonies of candidate transformants (1, 2, 3, 4), which were analysed for the presence of the *gdmB* and a phage-specific sequence. Expected amplicons were 0.9 kb for *gdmB* and 0.98 kb for the phage fragment. As controls *S. gallinarum*Δ*gdmP:*aphIII (K1), *S. gallinarum*Δ*cluster* (K2) and water (K3) were used as templates. 2-log DNA ladder was also loaded (M).](image)
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Fig. 8: 0.8% agarose gel of A) (1) plasmids isolated from S. gallinarum ∆cluster[pCLUST2S] (a. digested with BamHI, b. undigested), and (2) plasmids isolated from S. aureus[pCLUST2S] (a. digested with BamHI, b. undigested). 2-log DNA ladder was also loaded (lane 3). B) Plasmids isolated from S. gallinarum ∆cluster (lane 2), S. gallinarum ∆cluster[pmTHcAD] (lane 3), S. aureus[pmTHcAD] (lane 4), S. gallinarum ∆cluster[pALCNotI] (lane 5), and S. aureus[pALCNotI] (lanes 6, 7). 2-log DNA was loaded (lanes 1, 8).

Fig. 9: Tricine-SDS-PAGE of supernatants of 16 h cultures of S. gallinarum (lane 2), S. gallinarum[pCLUST2S] (lane 3), S. gallinarum::gdma::aphIII (lane 4), and S. gallinarum::gdma::aphIII[pCLUST2S] (lane 5), S. gallinarumΔcluster (lane 6), S. gallinarumΔcluster[pALCNotI] (lane 7), S. gallinarumΔcluster[pCLUST2S] (lane 8), and S. gallinarumΔcluster[pmTHCpAD] (lane 9). Polypeptide SDS-PAGE molecular weight marker was also loaded (lanes 1, 10).
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Fig. 10: Bioassay of *S. gallinarum* ∆cluster [pmTHcAD] supernatants from 1) 8 h, 2) 11 h, 3) 14 h, 4) 19 h), 5) 22 h, 6) 34 h cultures. Trypsin-treated (left), and untreated (right) supernatants were spotted. As negative controls the supernatants of 19 h cultures of *S. gallinarum* ∆cluster (K1 a: trypsin-treated, b: untreated), *S. gallinarum* ∆cluster [pALCNotI] (K2 a: trypsin-treated, b: untreated), and *S. gallinarum* were also spotted (K3).

5. Discussion

Based on the production of a gallidermin precursor peptide, pregallidermin, a successful gallidermin overproduction strategy has been developed previously, which takes advantage of the absence of toxic effects of pregallidermin in order to increase production titers (Chapter 2). Therefore, we reasoned that further optimization of pregallidermin overproduction can be approached along the lines typical for peptide overexpression, for which genetic engineering technology has offered excellent solutions in the past (96, 192, 350, 467). Essentially, we wanted to work on two strategies to further increase pregallidermin production titers, aimed at increasing the production of the corresponding mRNAs for pregallidermin production: [i] gene dosage increase of the biosynthesis and transporter genes, and [ii] manipulation of the promoters of the biosynthetic and transporter genes.

In previous studies, an enhancement of the gene dosage of lantibiotic genes had been successfully implemented and led to increased lantibiotic production (57, 74, 160). Therefore our first approach relied on the introduction of the plasmid pCLUST2, containing part of the pregallidermin cluster *gdmH* to *aphIII* and typically showing a plasmid copy number of 15 (4), into *S. gallinarum* and
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S. gallinarum\textsuperscript{Δ}gdmP::aph\textsuperscript{III}. Unfortunately, the resulting strains were negatively impacted with respect to pregallidermin or gallidermin production and the plasmid could not be reisolated from either of the two strains.

With respect to the second strategy, first we characterized the activity of three promoters derived from Gram-positive bacteria, $P_{cap1A}$, $P_{mecA}$, and $P_{aph\textsuperscript{III}}$. The promoter activities remained generally the same, with $P_{mecA}$ showing highest activity, followed by $P_{cap1A}$ and by $P_{aph\textsuperscript{III}}$, whether they were investigated for the genetically easily accessible \textit{S. aureus} RN4220 or for the biotechnologically more relevant but genetically less accessible \textit{S. gallinarum} Tü3928. The two stronger promoters, $P_{cap1A}$ and $P_{mecA}$, were chosen as substitutes of the natural gallidermin promoters, and plasmids were constructed with different combinations of the biosynthesis and transporter genes under control of these promoters. However, recombinant production strains harboring the promoter-engineered production plasmids showed impaired growth. Thus, production tests could be carried out only for strains resulting from the insertion of pcA or pcTH into \textit{S. gallinarum}\textsuperscript{Δ}gdmP::aph\textsuperscript{III}. Again, pregallidermin production was lower and plasmids could not be recovered. Even though promoter activity is one of the important parameters that have an impact on protein production (433), intense overproduction of the protein of interest might be toxic to the bacterial cell (210). Moreover, strong promoters seem to increase plasmid instability (54).

Apparently, \textit{S. gallinarum} can very efficiently modify recombinant plasmids, and the fact that the sequences on the plasmids had in these experiments identical equivalents on the chromosome pointed our suspicion towards homologous recombination. Indeed, homologous recombination in \textit{Staphylococcus} strains is a phenomenon that has been shown to take place easily even between homologous sequences as short as 350 bp (28, 451). Moreover, recombination in Gram-positive bacteria has been shown to be stimulated by the use of rolling-circle (RC) plasmids as vectors for homologous recombination (28). Here, it is interesting to note that the majority of small plasmids isolated from \textit{S. aureus}, e.g. pC194, pE194, and pT181, replicate via an asymmetric rolling circle mechanism, and so does the pC194-derivative pALC\textsuperscript{Not}\textsuperscript{I} (this study). Gruss and coworkers observed that the RC replication mechanism is error-prone when foreign DNA is present on ssDNA plasmids (142), as after the insertion of even relatively small foreign DNA fragments they often exhibit structural or segregational instability (39). The G+C content of
foreign DNA, its transcriptional activity, and the presence of secondary structures also influence stability of those plasmids (141) and results in high frequencies of homologous (28, 317), site-specific (123), and illegitimate recombination (136, 200, 298, 470). These recombinatory events result in: [i] Linear multimeric species of plasmids (high molecular-weight plasmid multimers, HMW) (142); [ii] deletions arising from aberrant replicative initiation or premature termination (137, 138, 298); or [iii] deletions arising from homologous recombination between direct repeats in the foreign DNA during elongation (296). In addition, released circular ssDNA (with or without foreign DNA) is a reactive recombination intermediate, and seems to stimulate intermolecular homologous recombination (141) leading for example to the presence of linearised pC194 in the chromosome of *Streptococcus pneumoniae* (470). Recombination has also been suggested to have taken place between inverted repeats on the plasmid and on the chromosome (470). Moreover, pE194 was shown to be capable of integration into the genome of *B. subtilis* in the absence of the major homology-dependent RecE recombination system, and integration into the chromosome was explained to occur by recombination at one of several short (5 to 15 bp) nucleotide sequence identities by a single recombination event (89). Since all the plasmids used in this study are derivatives of pC194, instability of the promoter-engineered plasmids as well as the production plasmid pCLUST2S in *S. gallinarum* and *S. gallinarum*\(_\Delta gdmP::aphIII\) might be due to one or a combination of the events listed above.

In order to reduce the chance of RecA-mediated homologous recombination, a novel mutant – *S. gallinarum*\(_\Delta\)cluster – was generated that lacked the entire gallidermin gene cluster. Gene dosage experiments were repeated with this knock-out strain with the production plasmid pCLUST2S and the promoter-engineered plasmid pmTHcAD, which carry the minimal set of genes necessary for pregallidermin production. Once more, plasmid isolation from the recombinant strains *S. gallinarum*\(_\Delta\)cluster[pCLUST2S] and *S. gallinarum*\(_\Delta\)cluster[pmADcTH] evidenced the absence of the expected plasmid patterns.

Under the assumption that we can exclude RecA-mediated homologous recombination events, this observation leads us to consider the formation of high molecular-weight plasmid multimers (HMW): Plasmid patterns of the recombinants were either identical (*S. gallinarum*\(_\Delta\)cluster[pCLUST2S]) or very similar
\( S. gallinarum\_\text{\textregistered} \text{cluster}[\text{pmTHcAD}] \) to the original strain \( S. gallinarum\_\text{\textregistered} \text{cluster} \), suggesting that the original production plasmids are not present in these strains. The formation of high molecular-weight plasmid multimers would have escaped our attention, as they are usually only detected by Southern hybridization as they are predominantly linear, migrate together with the chromosomal DNA on agarose gels (142), and fail to be captured in standard plasmid isolation. This could explain the absence of the expected plasmid patterns on agarose gels. Moreover, the presence of HMW was shown to drastically slow down cell growth (141), and would explain the prolonged lag-phase of \( S. gallinarum\_\text{\textregistered} \text{cluster}[\text{pCLUST2S}] \) and \( S. gallinarum\_\text{\textregistered} \text{cluster}[\text{pmADcTH}] \) cultures. The absence of \textit{aphIII} in \( S. gallinarum\_\text{\textregistered} \text{cluster}[\text{pCLUST2S}] \), conferring kanamycin resistance, might suggest that deletions could have occurred during replication of the plasmid and formation of HMW. Still, the possibility of illegitimate intermolecular recombination between production plasmids and chromosomal sequences cannot be excluded. Anyhow, we may assert that, even in the cluster-deleted mutant \( S. gallinarum\_\text{\textregistered} \text{cluster} \), recombination events are likely to have taken place, probably due to structural instability of the cloning vector.

Surprisingly, no documentation of such problems was available to us from previous genetic studies conducted on \( S. epidermidis \) and \( S. gallinarum \), where pT181- or pUB110-derived RC plasmids were used (12, 165, 166, 338, 341). However, to our knowledge, the only attempt to transform \( S. gallinarum \) was performed by Hille, who introduced two plasmids in this strain, the first carrying the epidermin gene \textit{epiQ}, and the second the gallidermin genes \textit{gdmTH} and \textit{gdmFEG} (165). In his work, Hille does not mention whether he could re-isolate the plasmids.

Production experiments with \( S. gallinarum\_\text{\textregistered} \text{cluster}[\text{pCLUST2S}] \) and \( S. gallinarum\_\text{\textregistered} \text{cluster}[\text{pmADcTH}] \) showed that if the former was able to produce pregallidermin only at very low concentrations, in the latter gallidermin production was restored to levels similar to the wild-type \( S. gallinarum \). In pCLUST2S, expression of \textit{gdmQ} is driven by the \textit{aphIII} promoter. As \textit{aphIII} seems no longer to be present in the recombinant \( S. gallinarum\_\text{\textregistered} \text{cluster}[\text{pCLUST2S}] \), we speculate that drastic decrease of pregallidermin production might be due to strongly reduced \textit{gdmQ} transcription, and thus to the absence of the transcriptional activator GdmQ, which is necessary for full activation of transcription of the cluster genes.
On the other hand, gallidermin production by \textit{S. gallinarum}\textsubscript{\text{\textregistered}cluster}[pmADcTH] is very surprising. The \textit{gdmP} gene, encoding the serine protease which catalyses the cleavage of pregallidermin into active gallidermin, is not supposed to be intact in \textit{S. gallinarum}\textsubscript{\textregistered}cluster. A speculation could be that production of a protease capable of activating pregallidermin might have been activated under the given growth conditions after insertion of the plasmid in proximity of this gene, for example by readthrough from one of the two strong \textit{S. aureus} promoters. It is known from the literature that unspecific proteases can activate precursor peptides by cleaving them at the exact site (10, 73).

In this study, two genetic strategies were investigated with the aim of further increasing pregallidermin production by \textit{S. gallinarum}\textsubscript{\textregistered}gdmP::aphIII or gallidermin production by \textit{S. gallinarum}. Even though some of them have already been proven useful for overproduction of other lantibiotics, they were not appropriate for overproduction of the lantibiotic gallidermin or its precursor pregallidermin, as no positive effect was observed on their production titers. Moreover, the experiments performed in this study were only modestly conclusive due to the apparent unsuitability of \textit{S. gallinarum} as a strain for genetic engineering studies and to the structural instability of the cloning vectors used throughout this study. The use of a more stable theta-replication plasmid, as the ones carrying multiple resistance determinants in \textit{S. aureus} (136) or a \textit{pAM}\textsubscript{\textbeta}1-derived plasmid (65), as well as strategies based on chromosomal integration may provide a valuable alternative to confront the instability issue.

6. Acknowledgements

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Chapter 4
Heterologous production of pregallidermin

1. Abstract

Heterologous production of bacteriocins of Gram-positive bacteria is an overproduction strategy that has been confirmed to be successful in the case of unmodified group IIa bacteriocins, but had only limited success for antimicrobial peptides such as the lantibiotics that contain non-canonical amino acids and require post-translational modifications. We investigated the overproduction of the inactive lantibiotic precursor pregallidermin in various heterologous hosts instead of its natural producer, *Staphylococcus gallinarum*. The Gram-negative host *Escherichia coli* and various Gram-positive bacterial strains, as *S. carmosus*, *S. aureus*, and *Bacillus subtilis*, were transformed with the production plasmid pCLUST2S, carrying most of the pregallidermin gene cluster, in particular the structural gene *gdmA* and the genes encoding the proteins responsible for pregallidermin modification, transport, and regulation of biosynthesis, but not the immunity genes.

Production of pregallidermin or gallidermin was investigated by HPLC, tricine-SDS-PAGE, and in bioassays against the sensitive strain *Kocuria rhizophila*. Only *S. aureus*[pCLUST2], which is phylogenetically closely related to *S. gallinarum*, produced pregallidermin in detectable quantities, and the peptide resulting from its tryptic cleavage showed antimicrobial activity. Under comparable conditions, pregallidermin titers by *S. aureus*[pCLUST2S] were 1.3-fold higher than by *S. gallinarum*Δ*gdmP::aphIII*, a knock-out mutant of the wild-type strain that lacks the protease responsible for the conversion of pregallidermin into active gallidermin.
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2. Introduction

Overproduction of antimicrobial peptides is a prerequisite for comprehensive studies on optimization of antimicrobial activity, product stability, mode of delivery and industrial-scale production (192). As many gene clusters encoding the required biosynthetic machinery have been identified (152, 154, 474), rational biocatalyst engineering appears to be an attractive option for overproduction. However, recombinant production of cationic antimicrobial peptides in heterologous systems is a challenging task, e.g. due to their toxicity against the host cell and their susceptibility to proteolytic degradation.

Klaenhammer (1993) defined four distinct classes of bacteriocins of Gram-positive bacteria: class I, modified bacteriocins (lanthionine-containing lantibiotics); class II, non-modified, heat-stable, membrane-active peptides; class III, large heat-labile proteins; and class IV, complex bacteriocins that contain essential lipid or carbohydrate moieties in addition to protein that may mimic the physiological activities of bacteriocins (hemolysins and muramidases) (224).

The simplest bacteriocins of Gram-positive bacteria that have been produced in heterologous systems are non-modified, heat-stable class IIa bacteriocins. The best studied member of this class is pediocin, which is naturally produced by Pediococcus acidilactici, and was synthesized in Saccharomyces cerevisiae, E. coli, Streptococcus thermophilus, Enterococcus faecalis, and Lactococcus lactis subsp. lactis if the pediocin gene cluster was under control of a streptococcal promoter (18, 71, 403). However, biosynthesis of class IIa bacteriocins does not entail post-translational modifications. Thus, their production does not require the action of modification enzymes and can be implemented even in evolutionarily distant bacteria, as the impact of corresponding bottlenecks such as different codon usage can be assumed to remain small for the expression of small genes such as the structural genes of class I and II bacteriocins.

In contrast, heterologous production of lantibiotics is complicated by their post-translational modification systems. Their antimicrobial activity has been shown to be dependent on the presence of post-translationally modified amino acids (248) which are introduced post-translationally by the concerted action of multiple enzymes (LanB, and LanC for class I lantibiotics, or LanM for class II lantibiotics), which are responsible for the formation of lanthionine and methylanthionine bridges. Little is
known about optimal condition for enzymatic reactions of LanB/C and LanM. Therefore, different cellular conditions, improper folding, and other, potentially unknown, factors in heterologous hosts might hamper the generation of functional modification enzymes or influence their activity (312). Consequently, synthesis of functional modification enzymes in phylogenetically distant hosts often failed. As an example, coexpression of the epidermin precursor gene epiA and the gene coding for the dedicated dehydratase epiB was achieved in E. coli, but modification of EpiA by EpiB could not be verified (253). Also, the cyclase EpiC has been purified but did not react with EpiA, with preproepidermin, or with oxidative decarboxylated peptides (254). Production of the homologous proteins NisC and SpaC involved in the production of nisin and subtilin was successful in E. coli only if cells were grown at the suboptimal temperature of 25°C, but its activity could not be tested due to the absence of a dehydrated peptide substrates (325). Moreover, heterologous production of a functional LctM, involved in lacticin 481 modification, was successful in E. coli at a temperature of 18°C (480). As a further example, production of nukacin IKS-1 prepeptide NukA and of a functional NukM in E. coli was detected only if cells were grown at 20°C, even though production of active NukA remained very low (312). These results strongly suggest that folding problems are likely to occur in E. coli that may lead to the formation of inclusion bodies. This issue has been faced in a study where SpaB was produced at 25°C in soluble form by co-production of the molecular chaperones GroEL/ES (72), whereas high level expression of spaB in the absence of GroEL/ES resulted in the recovery of SpaB in the insoluble fraction (478). However, attempts to detect dehydratase activity of purified SpaB in the presence of SpaS, SpaC, and a range of potential co-factors or metal ions were unsuccessful (478). Recently, functional NisC has been heterologously produced in E. coli, but no information on the high-level heterologous expression system used in this study was provided (264).
Tab.1: Classification of four representative microorganisms mentioned in this study.

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<th><strong>S. gallinarum</strong></th>
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<td>Streptococcaceae</td>
<td>Bacillaceae</td>
<td>Enterobacteriaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Staphylococcus</td>
<td>Lactococcus</td>
<td>Bacillus</td>
<td>Escherichia</td>
</tr>
<tr>
<td>Species</td>
<td>gallinarum</td>
<td>lactis</td>
<td>subtilis</td>
<td>coli</td>
</tr>
</tbody>
</table>

Heterologous production of lantibiotics has been carried out with more success in phylogenetically closer microorganisms. As examples, nisin from *Lactococcus lactis* was produced in the heterologous hosts *Enterococcus* sp. N12β (sharing the same order, Tab. 1) (265), epicidin 280 from *S. epidermidis* BN280 was produced in *S. carnosus* TM300 (same genus) (159). Moreover, heterologous expression of the genetic determinants of the two-component *L. lactis* DPC3147 lacticin 3147 in *E. faecalis* FA2-2 led to production of the active lantibiotic (378), and nukacin ISK-1 from *S. warneri* ISK-1 was produced in *L. lactis* (same class) (8). The lantibiotic epidermin from *S. epidermidis* Tü3298, which shares a very high degree of similarity with gallidermin (exchange of Ile by Leu at position 6; Ile6Leu-epidermin) (212), has been produced in *S. carnosus* (same genus) (12), and the gallidermin structural gene gdmA was successfully expressed in *S. epidermidis* Tü3298 (same genus) (10) but not in *S. carnosus* (same genus) (328).

From a technological perspective, it is important to note that even where heterologous synthesis of lantibiotics was successful, the yields of the in the heterologous hosts were always lower than in the native systems. As an example, heterologous expression of lacticin 3147 in *E. faecalis* FA2-2 led to lower production levels in the heterologous host (378). Moreover, production of nisin in *Enterococcus* sp. strain N12β led to 75 % lower titers than in the wild-type producer *L. lactis* (20 U mL⁻¹ vs. 120 U mL⁻¹) (265).

In *S. gallinarum*::gdmP::aphIII (Chapter 3), pregallidermin formation starts with ribosomal synthesis of a prepropeptide encoded by the structural gene gdmA, which is 30 amino acids longer than the final product gallidermin. This is post-translationally
modified by the gene products GdB, GdC, and GdD which introduce lanthionines, methyllantionines and a S-aminovinyl–D-cysteine ring (23, 454) to generate the fully modified prepeptide. GdT and GdH, which show high homology to ATP binding cassette (ABC) transporters, should be responsible for active translocation of the prepeptide out of the cell (166, 341, 346). The regulator GdQ shows high homology to EpiQ and is very likely to activate the expression of every gene encoded in the pregallidermin gene cluster (165, 339). Transcription of gdmQ is driven by P_{aphIII}, the promoter of the aminophosphotransferase gene aphIII. A part of the pregallidermin gene cluster including the aforementioned genes, was isolated and cloned into the E. coli–S. aureus shuttle vector pALCNotl (Chapter 3). The immunity genes were not included in the isolated fragment, since no antibiotic activity of pregallidermin against S. gallinarum or the sensitive strain K. rhizophila has been detected so far (Chapter 3).

In order to identify a potentially suitable recombinant host, we selected a set of strains that cover a large spectrum of phylogenetic distance from the original producer S. gallinarum (95) (Tab. 1). S. arlettae (sharing the same genus) (396) was selected as a potential host due to its evolutionary proximity based on 16S rRNA gene sequence analysis (436) and because of its risk-class I status (non-pathogenic). It is the closest relative to S. gallinarum in this study. S. xylosus (same genus) (397) is widely used in the food industry as a fermenting agent in the production of meat and milk products and was successfully used as epidermin production host (10, 400). S. carnosus (same genus) and B. subtilis (same order) have been selected because of their important role in industrial bioprocesses and for their “generally recognized as safe” (GRAS) status (130, 409). S. carnosus TM300 (395) is widely used in the ripening process of dry sausages and as a starter culture for the fermentation of meat and fish products (131, 267). Moreover, as it shows low level of extracellular proteolytic activity, it might be considered as a suitable host organism for recombinant protein production (131). B. subtilis is an important source of industrial enzymes and its biochemistry, physiology and genetics have been intensely studied for more than 40 years (468). Moreover, it has long been considered as a promising organism for production of recombinant biopharmaceuticals (333, 468). In this study, B. subtilis DB104 (his nprR2 nprE18 Δapr3) was chosen, a strain lacking the extracellular neutral protease NprE and the serine protease Apr3, in order to minimize proteolytic degradation of pregallidermin (211).
strain that was selected was *S. aureus* RN4220 (same *genus*), a risk-class II strain, and was included in this study as a control to verify the proper functioning of the pregallidermin genetic determinants used. Moreover, since Nagao and coworkers reported the successful lanthionine introduction into the nukacin ISK-1 prepeptide by the modification enzyme NuKM in the Gram-negative *E. coli* (same *kingdom*) (312), we included also this phylogenetically distant strain in the list of candidate hosts for heterologous production of pregallidermin.

3. **Material and methods**

3.1. **Bacterial strains and growth conditions**

Bacterial strains and plasmids are listed in Tab. 2. *Staphylococci* and *B. subtilis* strains were purchased from DSMZ. *E. coli* strains were routinely grown in Luria-Bertani broth (387), whereas staphylococci and *B. subtilis* DB104 were grown in Brain Heart Infusion broth (BHI, Becton Dickinson). For production of gallidermin, staphylococci were grown in medium 21 (Chapter 3) or BHI broth, *B. subtilis* and *E. coli* in BHI broth. Media were supplemented when appropriate with ampicillin (100 µg mL⁻¹), chloramphenicol (10 µg mL⁻¹), kanamycin (50 µg mL⁻¹), or tetracycline (10 µg mL⁻¹), unless otherwise noted. All cultures were grown at 37°C and 190 rpm in a shaking incubator with an amplitude of 25 mm, unless otherwise noted. Shake flasks experiments were repeated at least three times and were carried out with plasmid-containing strains and wild-type strains in one-baffled 500 mL Erlenmeyer flasks with in 100 mL of the suitable production broth.

3.2. **Genetic procedures**

Standard genetic procedures were performed as reported elsewhere (Chapter 3). Staphylococcal chromosomal DNA was isolated using Qiagen Genomic Tips (Qiagen, Hilden, Germany) according to the protocol supplied by the manufacturer, except that prior to extraction cells were incubated with 20 µg mL⁻¹ of lysostaphin at 37 °C for 1 h. Low-copy plasmid isolation from staphylococci was performed as described by Kies (218). In our hands, only staphylococcal plasmid DNA could be introduced into other staphylococci, therefore plasmid DNA prepared from *E. coli* had to be first electroporated into the restriction negative *S. aureus* RN4220 as described
Chapter 4 – Heterologous production of pregallidermin

elsewhere (393). The heterologous production plasmid pCLUST2S was constructed as described previously (Chapter 3).

*B. subtilis* transformation was performed as described by Yasbin (481). Preparation of *S. carnosus* electrocompetent cells was carried out with a modified version of a previously described protocol (273). Briefly, a 500 mL culture was grown in medium B2 (393) in a 3 L Erlenmeyer flask with four baffles until an OD$_{600}$ of 0.6. Cells harvested by centrifugation at 3500*g, 4°C and four washing steps were performed with 500 mL, 250 mL, 20 mL, and 5 mL of ddH$_2$O at 4°C. The pellet was resuspended in 1.5 mL of 10% glycerol and aliquots were stored at -80°C. For electroporation, cells were thawed on ice, then centrifuged at 4000*g, 4°C, washed once with 200 µL of 0.5 M sucrose, and finally resuspended in 50 µL of 0.5M sucrose. The cells were incubated at room temperature (RT) for 30 min with approximately 2 µg of plasmid DNA. Electroporation was performed in 0.1 cm cuvettes at 2.2 kV, 100 Ω, and 25 µF. An aliquot of 950 µL of B2 medium was immediately added, the bacteria were incubated at 37°C, 225 rpm for two hours, and then spread on selective plates.

The preparation of competent cells and electroporation of *S. xylosus* and *S. arlettae* were carried out following various previously published protocols (11, 45, 165, 273, 451).

### 3.3. Curing of plasmid pTE15 from *S. carnosus* DSMZ 4602

Plasmid pTE15 (Tet$, Em$) was cured from *S. carnosus* DSMZ 4602 through non-selective loss by repeated overnight growth of the plasmid-containing strain in non-selective medium, followed by replica plating of isolated colonies onto both selective (10 µg mL$^{-1}$ tetracycline, 5 µg mL$^{-1}$ erythromycin) and non-selective media. Colonies sensitive to tetracycline and erythromycin were considered to be plasmid-free *S. carnosus* TM300.

### 3.4. Gallidermin and pregallidermin analytics

Detection of gallidermin and pregallidermin and bioassays with the gallidermin sensitive strain *K. rhizophila* were performed as described earlier (Chapter 3). Activation of pregallidermin was performed by tryptic cleavage: The pH of potentially pregallidermin-containing supernatants was adjusted to 6.0 by addition of 1 M HCl. Trypsin (20 U mL$^{-1}$, American Laboratories Inc., Omaha, NE, USA) was added to a
final concentration of 100 mg L\(^{-1}\), and proteolytic cleavage was carried out at 37°C for 30 min. In coelution experiments, purified gallidermin or pregallidermin were added to supernatants to ensure that peaks indeed eluted at exactly the same time. Detection limits of HPLC analysis and bioassays were investigated by analyzing a dilution row of purified pregallidermin/gallidermin in BHI.

3.5. Tricine-SDS-PAGE analysis
Tricine-SDS-PAGE was performed as described previously (240, 391). Blotting of the gels was performed as described in Chapter 3, and silver staining of the membranes was performed as described elsewhere (111). The detection limit of tricine-SDS-PAGE was investigated by analyzing a dilution row of purified pregallidermin/gallidermin in BHI.
Tab. 2: Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Bacterial strains and plasmids</th>
<th>Genotype/ relevant characteristic(s)</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td><em>E. coli</em> cloning host strain [F&lt;sup&gt;−&lt;/sup&gt; mcrAΔmrr-hsdRMS-mcrBC) φ80lacZΔM15ΔlacX74 recA1 araD139Δ(araleu)7697galU galK rpsL (Str&lt;sup&gt;r&lt;/sup&gt;) endA1nupG]</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>S. aureus RN4220</td>
<td>Restriction negative mutant of <em>S. aureus</em> 8325-4 that accepts foreign DNA</td>
<td>(243)</td>
</tr>
<tr>
<td><em>S. gallinarum</em>ΔgdmP::aphIII;</td>
<td>Pregallidermin producer, Tet&lt;sup&gt;r&lt;/sup&gt; Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study, Chapter 3</td>
</tr>
<tr>
<td><em>S. xylosus</em> DSMZ 2066</td>
<td></td>
<td>(397); DSMZ, Braunschweig, Germany</td>
</tr>
<tr>
<td><em>S. carnosus</em> DSMZ 4602</td>
<td><em>S. carnosus</em> TM300[pTE15]</td>
<td>(395); DSMZ, Braunschweig, Germany</td>
</tr>
<tr>
<td><em>S. carnosus</em> TM300</td>
<td>Obtained from <em>S. carnosus</em> TM300[pTE15] by plasmid curing</td>
<td>(395), this study</td>
</tr>
<tr>
<td><em>S. arlettae</em> DSMZ 20672</td>
<td></td>
<td>(396); DSMZ, Braunschweig, Germany</td>
</tr>
<tr>
<td><em>B. subtilis</em> DB104</td>
<td><em>his nprE ΔaprE</em></td>
<td>(211)</td>
</tr>
<tr>
<td><em>K. rhizophila</em> ATCC 9341</td>
<td>Gallidermin-sensitive indicator strain, previously known as <em>Micrococcus luteus</em></td>
<td>(238); ATCC</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCLUST2S</td>
<td>pALCNotf containing the 10.5 kb Notf fragment from pCLUST1; Cm&lt;sup&gt;r&lt;/sup&gt;, Kan&lt;sup&gt;r&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study, Chapter 3</td>
</tr>
</tbody>
</table>
4. Results

4.1. Generation of heterologous hosts

In order to perform heterologous production experiments we selected the plasmid pCLUST2S (Chapter 3). The strains chosen as heterologous hosts were transformed with this vector, which carries all the genes encoding proteins responsible for pregallidermin biosynthesis and transport, and in addition gdmQ under control of the constitutive promoter of the S. faecalis aminoglycoside phosphotransferase gene aphIII (147). The genes encoding for aminoglycoside-modifying enzymes in certain Gram-positive bacteria (e.g. Bacillus, Streptococcus, and Staphylococcus) are phenotypically fully expressed in Gram-negative organisms (442), and aphIII has been shown to confer resistance to kanamycin/neomycin in B. subtilis (147), S. aureus (424, 451), L. lactis (144), and E. coli (144, 404, 443). Therefore, we assumed that this promoter would be suitable for expression of the pregallidermin genes in the microorganisms investigated in this study.

While B. subtilis[pCLUST2S], S. carnosus[pCLUST2S], S. aureus[pCLUST2S], and E. coli[pCLUST2S] could be easily generated, no transformants could be produced with pCLUST2S for S. xylosus and S. arlettae even after five different methods for preparation of competent cells and electroporation (11, 45, 165, 273, 451) had been applied. However, transformants of S. xylosus with the control plasmid pALCNotI (5 kb) could be generated at a very low efficiency by means of electroporation performed as in Brückner (45), suggesting that in the case of S. xylosus, specifically transformation with pCLUST2S seems to be problematic, either as a consequence of its size (16 kb) or of a toxic effect due to the overexpression of (one of) the pregallidermin cluster genes. Nevertheless, as transformation represented no issue for all the others candidate hosts, a toxic effect of the expressed genes seems unlikely, and the problem is very likely to be attributable to a combination of bad transformation efficiency and, probably, restriction/modification problems.

Regarding S. arlettae, the phylogenetically closest strain to S. gallinarum, it appears to be recalcitrant to genetic engineering as it could not be transformed neither with the production nor with the control plasmid, either as a consequence of inappropriate transformation protocols or of restriction barriers.
4.2. Pregallidermin production experiments

Supernatants from shake flask experiments with the heterologous hosts and the corresponding wild type strains were analyzed by HPLC, tricine-SDS-PAGE, and with bioassays for the presence of gallidermin or any precursor peptides. HPLC analysis permitted detection of peptides with similar structure as pregallidermin or gallidermin from culture supernatants even at low concentrations (detection limit: 100 ng with an injection volume of 10 µL (10 mg L⁻¹), whereas silver staining of blotted tricine-SDS-PAGE is a more sensitive but also more laborious detection method (392), which in addition enables estimation of the molecular mass of the produced peptides. For pregallidermin, the detection limit was 5 ng in a sample volume of 10 µL (0.5 mg L⁻¹). Finally, bioassays allow the detection of antimicrobial activity in supernatants of the producing strains, and are thus necessary for verification of (sufficient) structural integrity of the produced peptides. The detection limit of bioassays for gallidermin was approximately 60 mg L⁻¹. Detection limits for HPLC, tricine-SDS-page, and bioassays were detected by analyzing dilution rows of known concentrations of purified gallidermin or pregallidermin in production medium (BHI).

4.3. HPLC analysis of cultures of the heterologous hosts

By comparison of HPLC data of supernatants of culture of the heterologous hosts with data from S. gallinarum and S. gallinarumΔgdmP::aphIII, high production of pregallidermin by S. aureus[pCLUST2S] was observed, which seemed to produce 1.3-fold higher amounts of pregallidermin than S. gallinarumΔgdmP::aphIII (Tab. 3). A peak that coeluted with gallidermin was detected in supernatants of E. coli[pCLUST2S], whereas no peak was detected in supernatants of the E. coli[pALCNotI] control strain. The peak area would amount half the area of the peak obtained from wild-type S. gallinarum supernatants (Tab. 3). In the case of B. subtilis[pCLUST2S], no pregallidermin or gallidermin peaks were observed in HPLC chromatograms (data not shown). Pregallidermin seemed also not to be produced by S. carnosus[pCLUST2S]. However, during HPLC analysis of its supernatants, a peak at a similar retention time as gallidermin (but which did not coelute with gallidermin) was observed that was missing in supernatants of the control strain S. carnosus, whose area increased with increasing incubation time.
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Tab. 3: HPLC analysis of supernatants from 14 h BHI cultures of the heterologous strains. Pregallidermin and gallidermin quantification were performed as described in Chapter 3. Detection limit: 10 mg L\(^{-1}\).

\(^{a}\) Concentration of the peak eluting with a retention time similar, but not identical, to gallidermin.

<table>
<thead>
<tr>
<th>strain</th>
<th>Peak present (corresponding to concentration (mg L(^{-1})))</th>
<th>Coeluting with gallidermin</th>
<th>Coeluting with pregallidermin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. gallinarum</td>
<td></td>
<td>55</td>
<td>12</td>
</tr>
<tr>
<td>S. gallinarum(\Delta gdmP::aphIII)</td>
<td></td>
<td>&lt; 10</td>
<td>85</td>
</tr>
<tr>
<td>S. aureus[pALCNotI]</td>
<td></td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>S. aureus[pCLUST2S]</td>
<td></td>
<td>&lt; 10</td>
<td>110</td>
</tr>
<tr>
<td>S. carnosus</td>
<td></td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>S. carnosus [pCLUST2S]</td>
<td></td>
<td>&lt; 10 (43)(^{a})</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>E. coli[pALCNotI]</td>
<td></td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>E. coli[pCLUST2S]</td>
<td></td>
<td>22</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

4.4. Tricine-SDS-PAGE

Gallidermin and pregallidermin are detected as a 2165 Da and an approximately 4000 Da band, respectively, on silver stained blots of tricine-SDS-polyacrylamide gels of supernatants of S. gallinarum and S. gallinarum\(\Delta gdmP::aphIII\) (Chapter 3).

Silver stained blots of tricine-SDS-polyacrylamide gels of supernatants of early stationary phase (14 h) cultures of S. aureus[pCLUST2S] showed the presence of an approximately 4 kDa peptide (Fig. 1), whereas no bands of a similar size as pregallidermin or gallidermin were detected in the supernatants of S. aureus[pALCNotI] (the negative control strain). Analysis of the supernatants of 14h cultures of S. carnosus[pCLUST2S], and E.coli[pCLUST2S] did not reveal the presence of either gallidermin or pregallidermin (data not shown). However, a peptide of roughly the same size as pregallidermin was present in the supernatant of B. subtilis[pCLUST2S]. Unfortunately, the corresponding band on silver stained blots of tricine-SDS-polyacrylamide gels was difficult to reproduce at a good resolution (data not shown).
4.5. Bioassays

In order to confirm the presence of fully modified pregallidermin in supernatants of the heterologous production strains, we performed proteolytic activation with trypsin under suboptimal conditions to avoid unspecific degradation of gallidermin but still allow for complete conversion. The pH of the supernatants was adjusted to a slightly acidic value, and cleavage was carried out for a shorter time at the optimal temperature and the solutions were then tested for the presence of active gallidermin on a hypersensitive indicator strain (K. rhizophila). Only supernatants of S. aureus[pCLUST2S] cultures displayed the expected activity in the bioassay (Fig. 2), whereas neither supernatants from cultures of E. coli[pCLUST2S] nor those from B. subtilis[pCLUST2S] showed antimicrobial activity on such plates even before treatment with trypsin (data not shown). Supernatants from cultures of S. carnosus[pCLUST2S] showed very small, turbid inhibition zones which lead us to classify the results as inconclusive (data not shown).
Fig. 2: Bioassay of trypsin-treated (T) and -untreated (NT) supernatants of a S. aureus[pCLUST2S] culture in medium #21 after 10h, 12h, 15h, 17h, and 21h. As controls, supernatants of S. gallinarum, S. gallinarumΔgdmP::aphII and S. aureus[pALCNotl] were also spotted.

### 4.6. Summary

An overview of the results is presented in Tab. 4. Production of fully modified pregallidermin that led to active gallidermin upon tryptic treatment was only demonstrated for S. aureus[pCLUST2S]. In two cases, E. coli[pCLUST2S] and S. carnosus[pCLUST2S], we could observe peaks of identical or similar retention times as gallidermin but did not detect the corresponding bands by protein gel electrophoresis, even though this analysis is supposed to be more sensitive. Several studies have reported aberrant lantibiotic migration patterns in tricine-SDS-PAGE. As an example, mutacin 1140 has been shown to migrate slower than expected based on its known molecular weight, due to the rigid, rod-like structure that is characteristic of lantibiotics (168). In contrast, the molecular size of lacticin 481 was reported to be 1.7 kDa by SDS-PAGE, whereas 1.3 kDa by gel filtration and 2.7 kDa by amino acid composition analysis (347). This discrepancy was also explained to result from its hydrophobicity and from the presence of thioether crosslinkages (308). From the information present in the literature it is hence difficult to generalize on the extent of a (possible) shift in mobility of lantibiotic (pre)peptides caused by incomplete or missing modifications. However, we might speculate that E. coli[pCLUST2S] and
S. carnosus[pCLUST2S] might produce partially-, non-, or mis-modified propeptides, which might have migrated faster than the modified prepeptides, and that the corresponding bands would have run too far to be detected in the gel.

On the other hand, the peaks in the chromatograms of E. coli[pCLUST2S] and S. carnosus[pCLUST2S] might also represent smaller fragments of (pre)gallidermin/(pre)progallidermin generated by unspecific cleavage by host-encoded proteases which nevertheless retain sufficiently similar physicochemical properties to (pre)gallidermin to have similar adsorption characteristics. A previous study described that partially- or unmodified epidermin and gallidermin prepeptides were more subject to proteolysis than fully modified ones (328). Therefore, we might speculate that if partially- or unmodified peptides are produced by the aforementioned strains, proteolysis might have taken place and would explain the absence of the expected peptides in tricine-SDS-PAGE at the size corresponding to (pre)gallidermin and the absence of antibiotic activity in bioassays with E. coli[pCLUST2S] or the ambiguous results of bioassays with S. carnosus[pCLUST2S]. Moreover, in the case of S. carnosus, results from a previous study seem to corroborate the proteolysis hypothesis. The gallidermin structural gene gdmA was expressed together with the immunity genes (gdmEFG), the regulatory gene gdmQ and the transport genes gdmT and gdmH in S. carnosus TM300, but no pregallidermin could be detected in silver-stained blots of tricine-SDS-PAGE of heterologous cultures (328). This finding was explained by the presence of a chromosomally encoded protease in S. carnosus which was reported to be responsible for unspecific gallidermin degradation (328).

Finally, in the case of B. subtilis[pCLUST2S], we observed a band corresponding to the size of pregallidermin in tricine-SDS-PAGE analysis, but not in the HPLC analysis. This result can be explained by the fact that the former analysis is more sensitive than the latter. We cannot exclude that it could be the result of different retention properties of the produced peptide due to partial, absent, or incorrect post-translational modifications. The absence of antimicrobial activity observed in bioassays might also result from the reduced sensitivity of the assay or from the absence of post-translational modification features that are essential for bioactivity.

To conclude, we could demonstrate that fully modified pregallidermin is produced by S. aureus transformants. On the contrary, S. carnosus and E. coli probably produced peptides with significantly different retention- and migration
Chapter 4 – Heterologous production of pregallidermin

properties. B. subtilis might produce the desired peptide, but further investigations are necessary to assess its suitability as an heterologous strain for pregallidermin production.

<table>
<thead>
<tr>
<th>heterologous strain</th>
<th>test</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli[pCLUST2S]</td>
<td>(+)</td>
</tr>
<tr>
<td>B. subtilis[pCLUST2S]</td>
<td>-</td>
</tr>
<tr>
<td>S. carnosus[pCLUST2S]</td>
<td>(+)</td>
</tr>
<tr>
<td>S. aureus[pCLUST2S]</td>
<td>+</td>
</tr>
</tbody>
</table>

HPLC tricine-SDS-PAGE bioassay

E. coli[pALCN] (−) a − −
B. subtilis[pCLUST2S] − + −
S. carnosus[pCLUST2S] (+) b − (−) c
S. aureus[pCLUST2S] + + +

a A peak was that coeluted with gallidermin and was missing in supernatant of the control strain E. coli[pALCN].
b A peak was detected at a retention time similar but not identical to gallidermin, whose area increased with increasing incubation time. This peak was missing in supernatant of the control strain S. carnosusTM300.
c Supernatants from cultures of S. carnosus[pCLUST2S] showed very small, turbid inhibition zones.

Table 3: Summary on the heterologous hosts used in this study and on the tests performed to investigate on gallidermin or pregallidermin production.

5. Discussion

Antimicrobial peptides might represent a valuable resource in the light of an increasing need for new antibiotics. Therefore, over the last 20 years, considerable pharmaceutical effort has been devoted to the development of peptide antibiotics as human therapeutics (124, 302, 319). However, their success has been often compromised by a limited production of the peptides by the native producers. Therefore, the development of heterologous expression systems for such antimicrobial compounds may offer a number of advantages over native systems, such as improved control of gene expression and higher production levels. Nevertheless, recombinant overproduction is not always possible for any of the following factors or a combination thereof:

[i] Genetic intractability of the selected host strain, e.g. due to the presence of restriction barriers and modification systems or to the absence of successful transformation procedures and suitable expression plasmids;

[ii] Failure on the transcriptional level, due to a lack of effective transcriptional signals or to differences in promoter sequences;

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[iii] Failure on the translational level, due e.g. to stability and translational efficiency of mRNA, differences in codon usage, inefficient folding procedures, or toxicity of the gene products;

[iv] Problems with post-translational processing, due to, e.g. inappropriate cellular conditions such as redox milieu, proteolytic degradation of the peptide, secretion bottleneck (wrong signal sequences), stress caused by high expression, toxicity to the heterologous host, or failure to carry out specific post-translational steps that would have been catalyzed by another recombinant protein whose production failed due to one of the reasons mentioned under [i] to [iii].

Nevertheless, production of several non-modified bacteriocins of Gram-positive bacteria has been successful in heterologous hosts (71, 149, 403). On the contrary, heterologous production of modified bacteriocins as the lantibiotics is still a challenging task, requiring synthesis of functional modification-, export-, and immunity-proteins. From an evolutionary point of view, the likelihood of differences in cellular conditions, promoter sequences, transcriptional (co)factors, codon usage, and folding (co)factors which might hamper the generation of functional modification enzymes or influence their activity, increases with increasing phylogenetic distance between microorganisms. Hence, heterologous production of lantibiotics will supposedly become increasingly difficult with increasing phylogenetic distance between the heterologous host and the native producer. Recombinant overproduction of gallidermin has been attempted in a previous study, where gallidermin was shown to be produced by the evolutionary very close S. epidermidis that carried the structural gene gdmA (10).

In this study, we attempted to enhance pregallidermin production titers by heterologous expression of the pregallidermin genetic determinants in several hosts. Heterologous production in the phylogenetically closest strains S. arlettae and S. xylosus was not possible due to their genetic intractability ([i]). However, even where direct transformation of the remaining potential heterologous hosts was successful, production of pregallidermin was not straightforward. In the case of S. aureus[pCLUST2S], the next evolutionarily closest strain, pregallidermin production has been confirmed and was higher than the production by S. gallinarum∆gdmP::aphIII. This increase in productivity could be due to an increase in gene dosage due to the fact that in S. aureus the synthesis cluster is located on a
plasmid with 15 copies per cell (4). However, to be able to draw solid conclusions, a single copy experiment should be performed.

Even though heterologous production of the highly similar lantibiotic epidermin in S. carnosus TM300 has been demonstrated before (12, 339), the occurrence of pregallidermin or gallidermin in supernatants of S. carnosus[pCLUST2S] could not be verified. Results suggest that a peptide with structural modifications might be produced, either as a result of incomplete post-translational modifications or of partial proteolytic degradation ([iv]). As S. carnosus is phylogenetically relatively close to S. gallinarum, and as post-translational modification of epidermin in S. carnosus has been demonstrated before (12, 328, 339), the first hypothesis seems unlikely. Moreover, we cannot fully exclude that treated supernatants of S. carnosus[pCLUST2S] showed some activity in the bioassays, which might be compatible with the hypothesis of proteolytic degradation of gallidermin to a less active molecule. In addition, results from a previous study on gallidermin production in S. carnosus corroborate the proteolysis hypothesis (328).

Experiments with B. subtilis[pCLUST2S] strongly suggest that failure of production of active gallidermin should not be due to factors belonging to the categories [i]-[iii]. Our results hint at production of an unmodified prepropeptide, possibly due to problems with post-translational processing ([iv]). Inappropriate cellular conditions such as redox milieu, as well as the improper folding of the modification enzymes GdmB, GdmC, and GdmD, might hamper the formation of thioether crosslinkages and thus functional post-translational modifications. A recent study might support the hypothesis of mislaid post-translational modifications during heterologous production in B. subtilis: Yuksel and coworkers showed that no active nisin could be detected in culture supernatants of B. subtilis 168 that carried all the genetic determinants required for nisin production, even though transcription of the cluster genes was confirmed. Instead, a peptide with a higher molecular weight than nisin was produced, which reacted with anti-nisin antibodies even though no nisin or nisin-like peptide could be detected by mass spectrometry (486).

Finally, heterologous production probably failed in the phylogenetically most distant microorganism, E. coli, likely due to factors belonging to the last category ([iv]). Production at lower temperatures, and co-production of molecular chaperones to enhance solubility of the peptide and its modification enzymes have been shown to have a positive impact on heterologous production in E. coli (312, 325, 478, 480) and
might be taken into consideration. However, as detection of EpiA in *E. coli* was only achieved upon production of an MBP-EpiA fusion protein that could be purified by affinity chromatography on amylase columns (253, 257), and as nukacin ISK-1 prepeptide was produced in *E. coli* as an hexa-histidine-tagged (His-tagged) NukA to enable purification and concentration of the prepeptide (312), we might also be confronted with a detection problem, attributable to the low production titers. These problems suggest that many efforts are still required for generation of a suitable heterologous production strategy in *E. coli*.

In conclusion, heterologous production of fully post-translationally modified pregallidermin could be confirmed only in *S. aureus*, the evolutionarily closest strain to *S. gallinarum* among the hosts that could be tested in this study. As assumed, production seems to be increasingly difficult with increasing phylogenetic distance between the host and *S. gallinarum*. Therefore, extensive efforts will be required for a deeper understanding of the several hurdles that still lie across the path leading to the implementation of pregallidermin overproduction in heterologous hosts other than *S. aureus*. 
Chapter 5  
Summary and Conclusions

In this thesis, various genetic engineering strategies aiming at the improvement of the production of the lantibiotic gallidermin by *Staphylococcus gallinarum* have been examined. Considerable effort has been previously devoted to the development of a production process for gallidermin with the wild-type strain, with a focus on bioprocess optimization. However, presumably due to the self-toxicity displayed by gallidermin on the native producer, titers in fermentations have generally remained low, i.e. in the order of 250 to 330 mg L\(^{-1}\). This thesis aimed at:

a) Removing the fundamental impediment to titer increases by developing a novel production strain that would produce a non-toxic gallidermin derivative instead of gallidermin itself; and

b) Using this strain – without product toxicity – for the implementation of overproduction strategies.

The second chapter describes the successful development of a novel two-step production strategy which is based on the overproduction of a non-toxic precursor of gallidermin, pregallidermin, followed by its enzymatic activation. Pregallidermin resulted to be not toxic against *S. gallinarum* up to a concentration of 8 g L\(^{-1}\), and the mutant produced the precursor to 50% higher molar titers than the wild-type in non-optimized fermentations, suggesting that the absence of self-toxicity alone has already a beneficial effect on the production.

In order to improve further the pregallidermin production capacity of the mutant and wild-type *S. gallinarum* strains, we attempted to boost the mRNA template concentration of the pregallidermin genes, by means of an increase of the gene dosage and by substitution of the natural promoters with the strong *S. aureus* \(P_{cap1A}\) and \(P_{mecA}\) as discussed in chapter 3. Production plasmids were generated, which consisted i) of the whole pregallidermin gene cluster, whose transcription was under indirect control of the promoter \(P_{aphIII}\), and ii) of the minimal pregallidermin genetic determinants (the biosynthesis and transport genes) under control of \(P_{cap1A}\) and \(P_{mecA}\). However, these attempts did not lead to a significant enhancement of (pre)gallidermin production and have been significantly complicated by the instability and recombination of the production vectors. These difficulties prompted us to generate a
novel mutant, \textit{S. gallinarum\_cluster}, which lacked the entire pregallidermin gene cluster. Nevertheless, the problems with vector instability persisted. Thus, we concluded that \textit{S. gallinarum} is not yet a suitable host for the straightforward application of standard genetic engineering manipulations. The use of more stable production plasmids or the chromosomal integration of the pregallidermin genes under control of stronger promoters may represent an alternative strategy that might be pursued to confront the instability issue.

As pregallidermin production in the wild-type producer and the mutants thereof resulted to be problematic, we investigated heterologous expression of the pregallidermin genetic determinants in several hosts, as described in chapter 4. Heterologous production of fully post-translationally modified pregallidermin resulted to be increasingly difficult with increasing phylogenetic distance between the host and the native producer, and could be confirmed only in \textit{S. aureus}, the evolutionarily closest strain to \textit{S. gallinarum} among the hosts that could be tested in this study. Even tough this host produced pregallidermin to 1.3-fold higher titers than the mutant \textit{S. gallinarum\_gdmP::aphIII}, its consideration for a pregallidermin production process on an industrial-scale has to be excluded due to its pathogenicity. Therefore, extensive efforts are still required for the implementation of pregallidermin overproduction in non-pathogenic strains, such as \textit{B. subtilis} and \textit{S. carnosus}.

This thesis has contributed with the development of a suitable strategy for pregallidermin overproduction, which should be further improved in combination with bioprocess optimization (medium, fermentation and downstream processing optimization) and might thereby lead to a process suitable to an industrial context. As the biosynthesis of class I lantibiotics is highly conserved, this strategy could be applied to the production of various potentially clinically relevant antimicrobial peptides. Furthermore, the presented results represent a clear motivation to either improve the genetic tractability of \textit{S. gallinarum} and other bacteria or to establish pregallidermin production in heterologous hosts.


the NisI immunity protein and enhancement of nisin activity by the lipid-free NisI. FEMS
antimicrobial peptides in
of D-alanine in teichoic acids in Gram-positive bacteria, confers resistance to cationic
and R. Brückner. 2006. A functional
Kovács, M., A. Halfmann, I. Fedtke, M. Heintz, A. Peschel, W. Vollmer, R. Hakenbeck,


337. Peel, J. E., and B. Suri. 1998. Use of gallidermin and epidermin for preventing or treating mastitis and for reducing withholding time of milk. US patent 5710124-A.


is required for assembly but not transport of CS1 pilin. Molecular Microbiology


lantibiotic lacticin 3147 - a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. Molecular Microbiology 61:285-296.


Curriculum vitae

Giorgia Valsesia

Date of birth: 18 January 1977
Place of birth: Locarno, Ticino, Switzerland
Citizenship: Swiss and Italian
Marital status: single

1983-1988 Primary School, Giubiasco (TI), Switzerland.
1992-1996 High School, Bellinzona (TI), Switzerland.
1996-2002 Study of Biology (MSc), ETH Zurich, Switzerland.
05/2002-09/2003 Research Scientist, Plant Pathology Group, Department of Agriculture and Food Science, ETH Zurich, Switzerland
10/2003-03/2008 Doctoral Student, Bioprocess Laboratory, Institute of Process Engineering, ETH Zurich, Switzerland.
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