Novel lantibiotics from microbial genomes

Dissertation
zur
Erlangung des Doktorgrades (Dr. rer. nat.)
der
Mathematisch-Naturwissenschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Diplom-Biologin
Jasmin Dischinger

aus
Freiburg

Bonn im März 2012

Die vorliegende Arbeit wurde mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn am Institut für Medizinische Mikrobiologie, Immunologie und Parasitologie (IMMIP) unter Leitung von Frau Prof. Dr. rer. nat. Gabriele Bierbaum angefertigt.

Erstgutachterin:	Prof. Dr. rer. nat. Gabriele Bierbaum
Zweitgutachter:	Prof. Dr. rer. nat. Hans-Georg Sahl
Fachnahes Mitglied:	Prof. Dr. rer. nat. Walter Witke
Fachangrenzendes Mitglied:	Prof. Dr. rer. nat. Gabriele M. König
Tag der Promotion:	11. Juni 2012
Erscheinungsjahr:	2012

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Abbreviations

For all proteinogenic amino acids the standard IUPAC three- or one-code abbreviations were used. For length, time and mass descriptions the standard IS units were used.

aa	amino acid(s)
a.dest.	*aqua destillatum*
ATCC	American Type Culture Collection, USA
bp	base pair
C55P	undecaprenyl phosphate / lipid carrier
°C	degree Celsius
Da	Dalton
Dha	didehydroalanine
Dhb	didehydrobutyrine
DMSO	dimethylsulfoxide
DNA	desoxy ribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DSMZ	German Research Centre for Microorganisms and Cell Cultures, Braunschweig, Germany
EDTA	ethylenediaminetetraacetic acid
FMN	riboflavin-5'-phosphate
GlcNAc	N-acetylglucosamine
HPLC	High Performance Liquid Chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
Lan	lanthionine
lipid II	GlcNAc-MurNAc-pentapeptide-pyrophosphoryl-undecaprenol

MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry
MeLan	methyllanthionine
MIC	minimal inhibitory concentration
MilliQ	ultrapure water
mRNA	messenger RNA
msc	multiple cloning site
MurNAc	N-acetylymuramic acid
MW	molecular weight
NADH	nicotinamide adenine dinucleotide
NCS	German Reference Centre for Staphylococci, Werningerode, Germany
NCTC	Health Protection Agency Culture Collections, Salisbury, UK
ORF	open reading frame
ORI	origin of replication
PEG	polyethylene glycol
^{r}	resistant
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)aminomethane
U	units
v/v	volume per volume
w/v	weight per volume

Introduction

The discovery of the first antibiotic penicillin was one of the most important milestones in medicine and in the fight against infectious disease (Fleming, 1929). Before antibiotics were discovered bacterially caused infectious diseases had been the most frequent cause of human death. The beginning of industrialized production and widespread use of penicillin in the 1940ies heralded the dawn of the golden era of antimicrobials. In the past 70 years the successful therapeutic use of penicillin and its derivatives led to discovery and development of a multitude of antibiotics, many of which are of bacterial or fungal origin. Today, more than 80% of anti-infective drugs are natural compounds or derivatives thereof (Newman and Cragg, 2007; Li and Vederas, 2009).

However, emergence and spread of antibiotic resistance among pathogens has been reported since antimicrobials were employed for medical purposes. In the past years, rapidly developing superbugs, i.e. human pathogens that are resistant to almost all commonly used antibiotics, have become an enormous problem. Among those are Gram-positive bacteria like methicillin-resistant *Staphylococcus aureus* strains (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE), as well as multiresistant Gram-negative pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and Enterobacteriaceae. This necessitates further search for new antibiotic substances and sources, especially for compounds with new MoA and novel targets (van Heel *et al*., 2011). To this end, bacteria and their huge potential to produce antimicrobial substances represent an inexhaustible source for the discovery of new antibiotics and so far neglected antibiotic classes (Newman and Cragg, 2007; Davies, 2011), especially since metagenomic data indicate that less than 1% of all existing microbes have been identified so far (Singh and MacDonald, 2010).

Bacterial peptide antibiotics

Production of peptide antibiotics is a common feature of bacteria and antibiotic producers are found in every major lineage. These substances can be divided into two main groups, non-ribosomally and ribosomally expressed polypeptide antimicrobials.

Non-ribosomal peptides are made by multi-modular enzyme complexes called non-ribosomal-peptide synthases (NRPS). Frequently, extreme modifications of these peptides result in formation of unusual aa, cyclic or branched structures, in introduction of lipid based

side chains or sugar substitutions. More than 300 different modifications, introduced by isomerization, methylation, acetylation, glycosylation and heterocyclic ring formation are known (Marahiel, 2009; Hancock and Chapple, 1999). Clinically established members are the glycopeptide vancomycin and the cyclic lipopeptide daptomycin.

Members of the class of **ribosomally produced peptide antibiotics** are structurally very heterogeneous and commonly are referred to as bacteriocins (Tagg *et al.*, 1976). Since 1925 bacteriocinogenic strains have been identified within the domain of Gram-negative and Gram-positive bacteria as well as within Archaea (**archaeacins**) (Gratia, 1925; Rodriguez-Valera *et al.*, 1982; O'Connor and Shand, 2002; Riley and Wertz, 2002).
Generally, **bacteriocins** exhibit an antimicrobial activity at extremely low concentrations and their involvement in colonization and defense of ecological niches is suggested (Riley and Wertz, 2002; Cotter *et al.*, 2005). Some bacteriocins, mostly those produced by Gram-positives, were found to function as peptide pheromones in quorum-sensing and cell-cell communication resulting in a co-ordinative regulation of competence, sporulation, virulence and biofilm production (Turovskiy *et al.*, 2007).
Characteristically, bacteriocins are gene-encoded and synthesized as precursors consisting of an N-terminal leader and a C-terminal propeptide sequence. The following hypotheses for leader functions are currently discussed in literature: 1. the leader resembles a secretion signal and mediates the transport through the cytoplasmatic membrane; 2. it serves as recognition signal for recruitment of the modification machinery; 3. a function as an intramolecular chaperon; 4. the leader is part of the self-immunity as it keeps the modified prepeptide inactive previous to export; and 5. the leader peptide enhances stability against proteolytic degradation resulting in a prolonged half-life of the precursor (Jack *et al*., 1995).
The propeptide is the inactive and unmodified precursor of the bacteriocin. In general, the propeptide undergoes several post-translational modifications during maturation, resulting in formation and introduction of unusual aa and intramolecular rings. These enzyme-mediated alterations occur on aa side chains and, less frequently, on the peptide backbone. They often mediate enhanced stability and are essential for the 3-dimensional structure and consequently, for the bioactivity of bacteriocins.

Accordingly to Klaenhammer (1988), nearly 99% of all existing bacteria at least make one bacteriocin and, indeed, a plethora of new bacteriocin classes and families has been identified by genome sequencing that led to an enormous progress in the field of ribosomally synthesized and post-translationally modified compounds. Interestingly, these novel bacteriocins are often widely distributed over all bacterial lineages (Schmidt, 2010).

A closer analysis of the previously falsely classified lantibiotic cypemycin revealed a new class, the **linearidins** (Claesen and Bibb, 2010). Members of this class are predicted to share a linear structure and a high amount of unusual aa, including didehydro-aa. Although didehydro-aa are also present in another bacteriocin class, lantibiotics, the respective modification enzymes share no significant sequence similarity and therefore, an alternative biosynthesis mechanism is discussed. Linearidins might rather be involved in extracellular signalling, since cypemycin only exhibits marginal antimicrobial activity. Bioinformatic analysis indicated a widespread occurrence of linearidins-like gene clusters within prokaryotic strains and even in certain Archaea.

The classical bacteriocin classification scheme that divides bacteriocins into those produced by Gram-positives and Gram-negatives has been abolished recently. In this context, discovery of microcin B17-like peptides (figure 1.2), that had been thought to be unique to Gram-negatives, in the genomes of Gram-positive bacteria, led to the definition of a new class called **TOMMs (thiazole/oxazole-modified microcins)** (Lee *et al.*, 2008; Haft *et al.*, 2010). Microcins have a MW below 10 kDa and members of this class are highly modified, hydrophobic peptides. They are characterized by class-specific modifications, the heterocyclic oxazoles/thiazoles. TOMMs are encoded by short structural genes that code for peptides of 50-70 aa and that tend to be rich in Cys, Ser, Thr and Gly residues. These aa are often found in repetitive motifs and serve as precursor aa for the class-specific thiazoles and oxazoles (Melby *et al.*, 2011; Haft, 2009). They are introduced by the concerted action of a trimeric complex consisting of a cyclodehydratase (C), a dehydrogenase (B) and a docking scaffold protein (D) (Li *et al.*, 1996).

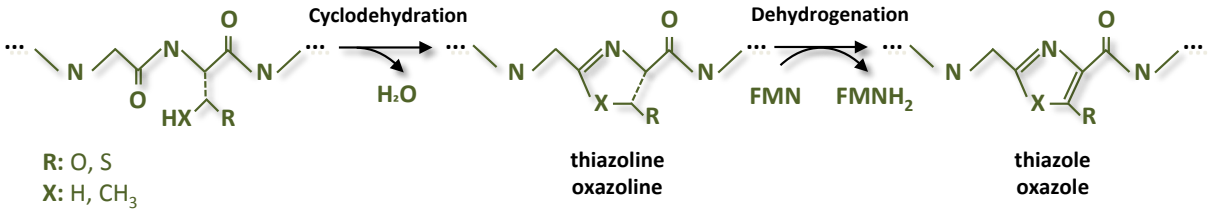

Figure 1.1: Formation of the heterocyclic aa thiazole and oxazole. Peptide backbone cyclodehydration of Cys, Ser or Thr results in thiazoline or (methyl)oxazoline rings. Subsequently, these are oxidized to thiazoles or (methyl)oxazoles.

The cyclodehydratase joins the Cys, Ser or Thr side chains to the carbonyl group (contributed by the peptide bond) of the most neighboring aa. This reaction results in elimination of a water molecule from the amide backbone and in formation of a heterocyclic structure named thiazoline (Cys) or oxazoline (hydroxy-aa) (figure 1.1). In general, these structures are immediately oxidized by a dehydrogenase to aromatic thiazole or oxazole residues (figure 1.1). The scaffolding protein is apparently important for complex assembly and regulation of the cyclodehydrogenase. Often, the docking protein is present as a C-terminally fused domain of the corresponding cyclodehydrogenase (Melby, 2011; Haft *et al.*, 2010).

Genomic data mining revealed that homologous gene clusters are widespread among prokaryotes (Lee *et al*., 2008; Haft *et al*., 2010; Brown *et al*., 2009)**.** Interestingly, these clusters contain additional biosynthetic enzymes, some of which possess significant similarity to modification enzymes of other bacteriocins, like the lantibiotic dehydrogenases (LanB, LanM). This fact might suggest that TOMMs undergo further, mainly peptide specific modifications. The cluster of the TOMM-peptide thiocillin IV (*B. cereus*) contains two LanB homologs which dehydrate Ser and Thr. These dehydrated aa are either involved in formation of the thiopeptide subclass-specific pyridine ring or remain as didehydro- aa in the active peptide (Brown *et al.*, 2009). Unusual for bacteriocins, the structural gene of thiocillin IV is situated far from the genes coding for the biosynthetic enzymes (Lee *et al.*, 2008).

In 2009 Haft postulated, based on a bioinformatic approach, a new family of TOMMs, the heterocycloanthracins (Haft, 2009). Precursors of this family are characterized by the presence of Gly and Cys rich repeats consisting of CGG, GCG or RCG triplets. In 2010, the author identified two further subfamilies, whose structural genes share strong aa similarity either to the α-subunit of a nitrile hydratase (nitrile hydratase-related leader peptides; NHLP) or to the cyanobacterial nitrogen-fixing proteins containing a conserved Nif11 domain (Nif11-related leader peptides; N11P). NHLP-encoding clusters lack the dehydratase, but in their neighborhood a docking-protein-cyclodehydrogenase fusion protein is found. N11P precursors are often clustered with homologs of the lantibiotic modification system, rather than being surrounded by genes having sequence similarity to those of the TOMM machinery (Haft *et al.*, 2010).

Recently, it has been shown that introduction of the heterocyclic aa oxazoline and thiazoline or of their oxidized derivatives, oxazole and thiazole, into peptides of other bacteriocin classes is based on the same biosynthetic pathway, employing enzymes that are homologous to those of the TOMM biosynthesis machinery (Melby *et al.*, 2011; Donia *et al.*, 2008).

This suggests that these bacteriocins, e.g. the cyclic cyanobactins produced by cyanobacteria, the streptolysin-like toxins and the thiopeptides, also are members of TOMMs.

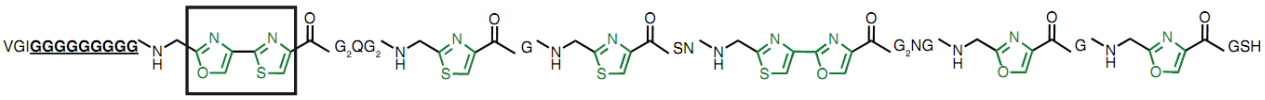

Figure 1.2: Structure of the linear TOMM peptide microcin B17. The class-typical oxazole and thiazole rings are highlighted in green (Melby *et al*., 2011).

The members of the TOMM subclasses comprise compounds with different bioactivities like antibacterial, antifungal, antimalarial, antitumor or multidrug reversing functions. Their MoA either depends on 1. membrane depolarization; 2. inhibition of the ribosome and protein biosynthesis; 3. degradation of nucleic acids by targeting enzymes involved in RNA/DNA structure maintenance and synthesis or 4. targeting biosynthetic enzymes (Melby *et al*., 2011; Lee *et al*., 2008). Additionally, an effect on the fungal cell wall has been reported for the antifungal thiopeptide cyclothiazomycin (Mizuhara *et al*., 2011).

A divers group of ribosomally produced bacterial peptide antibiotics: the lantibiotics

The term lantibiotics, introduced in 1988 by Schnell *et al.*, is an abbreviation for “lanthionine containing antibiotics” and highlights the importance of (methyl-)lanthionine to this group: all lantibiotics are structurally defined by the presence of these thioether-aa.

The non-proteinogenic amino acid lanthionine

In 1941, Horn *et al.* identified a non-proteinogenic aa in sodium carbonate treated wool consisting of two Ala residues linked by a thioether bridge and called this aa lanthionine (Lan). Lanthionine is widely distributed in nature, occurring not only in lantibiotics, but also in the cell wall of some *Fusobacteria* (Vasstrand *et al.*, 1979; Fredriksen *et al*., 1990), in human hair and in feathers. Furthermore, lanthionine-containing compounds, like ketimines, appear to play a role in signal transduction of mammalian vascular and nervous systems (Fontana *et al.*, 1990; Ricci *et al.*, 1989; Hensly *et al.*, 2010).

In lantibiotic and ketimine peptides, thioether formation is catalyzed by specific modification enzymes. The prokaryotic lantibiotic modifying enzymes and the eukaryotic ketimine biosynthesis machinery only share low aa sequence similarity, but have comparable overall folding structures including zinc ligand motifs and active sites (Zhang *et al.*, 2009). Formation of thioether-aa results in introduction of covalent, intramolecular junctions that are essential for peptide stability and structure.

The lantibiotic biosynthesis machinery and the current lantibiotic classification scheme

In lantibiotic production, the rare aa are introduced by post-translational modifications. Like other bacteriocins, lantibiotics are ribosomally synthesized, resulting in the expression of a precursor containing an N-terminal leader and C-terminal propeptide (Ingram, 1970; Banerjee and Hansen 1988). Extensive post-translational modifications as well as proteolytic removal of the leader convert the inactive propeptide into its active form (van de Meer *et al*., 1993). In general, the active peptide has a length of 18 up to 35 aa and a high content (24-47%) of Cys, Thr and Ser residues (Lawton *et al.*, 2007).

The most important modification results in the introduction of the typical thioether-aa **lanthionine (Lan) and methyllanthionine (MeLan)**. This modification is based on an enzymatically catalyzed two step reaction including a dehydratisation and a cyclization step (figure 1.3). Although spontaneous cyclization has been observed (Toogood, 1993), lanthionines of lantibiotics are cyclized by chemoselective and regioselective enzymes, that generate a specific bridging pattern and one favored isomer (Goto *et al.*, 2010; Xie and v. d. Donk, 2004).

Dehydration of Ser and Thr, respectively, leads to the formation of the didehydro-aa **didehydroalanine (Dha) and didehydrobutyrine (Dhb)**. Based on their location within the propeptide sequence, few hydroxy-aa specifically escape dehydration and remain unmodified within the active peptide. These are mainly residues that are located within the lanthionine ring structures. The majority of the didehydro-aa is subsequently involved in formation of the thioether-aa. In a nucleophilic Michael addition a sulfhydryl-group of a neighboring Cys residue is linked to the unsaturated double bound of the didehydro-aa resulting in the generation of lanthionine (Dha + Cys) and methyllanthionine (Dhb + Cys) (Ingram, 1969; 1970). In class I lantibiotics, the Cys is located C-terminally to the didehydro-aa, whereas this arrangement is not uniformly found in peptides of class II and III (Lawton *et al.*, 2007; Rink *et al.*, 2005).

Today four different modification enzymes are known to play a role in lanthionine formation: the enzymes LanB, LanC, LanM and LanL. Based on the enzyme types involved, on

the proteins used for transport and processing and on the presence of conserved leader structures, the lantibiotics are currently classified into three main classes (Willey and v. d. Donk, 2006; Pag and Sahl, 2002). This new classification scheme focuses on the biosynthesis of the prepeptides in contrast to the previous structure- and MoA-based schemes from Jung *et al.* (1991) and de Vos *et al.* (1995). Based on aa similarity, the members are additionally divided into subclasses that are named after the respective prototype lantibiotic (table 1.2).

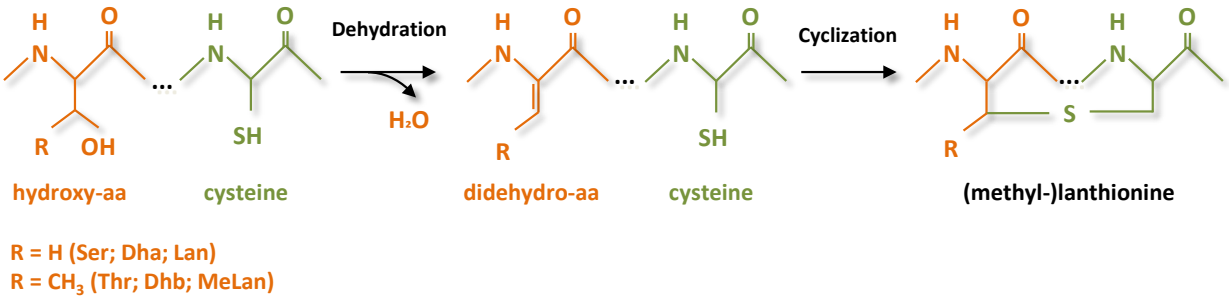

Figure 1.3: Formation of the thioether-aa lanthionine. Hydroxy-aa are enzymatically dehydrated to the corresponding didehydro-aa. Subsequently, in a nucleophilic Michael addition, a Cys derived SH group is connected to the didehydro-aa resulting in the formation of (methyl-)lanthionine.

Lantibiotics of the **class I** are modified by a membrane associated enzyme complex consisting of two individual enzymes, the dehydratase LanB and the cyclase LanC (Klein *et al.*, 1992; Schnell *et al.*, 1992; Engelke *et al.*, 1994; Siegers *et al.*, 1996), whereas peptides that are modified by the bifunctional enzyme LanM (Rincé *et al.*, 1994) are classified as **class II** lantibiotics. LanM enzymes have an aa sequence similar to that of LanC, but are distinct from LanB (Sahl *et al.,* 1995; Siezen *et al.*, 1996). Up to now, little is known about the reaction pathway leading to lantibiotic dehydration. Paul *et al.* (2007) demonstrated in *in vitro* dehydration assays with the lacticin 481 modifying LanM enzyme that ATP hydrolysis is absolutely required for this reaction. The authors suggested that a.) the phosphate group is used to phosphorylate Ser/Thr residues, turning the hydroxyl-group into a good leaving group, and that b.) phosphate elimination finally leads to the generation of the didehydro-aa. Interestingly, participation of ATP is usually not found in dehydratases that in general act via acid-base reactions and LanM enzymes miss typical ATP binding sites (Paul *et al.*, 2007).
LanM as well as LanC proteins contain three conserved motifs including characteristic zinc binding sites that are essential for their cyclase activity (Okeley *et al.*, 2003). Here, zinc ions are involved in the activation of the Cys derived SH-group into an ethiolate which

subsequently reacts with the didehydro-aa to form the thioether-aa (methyl-)lanthionine (Li *et al.*, 2006; Zhu *et al.*, 2003).

Additionally, class I and class II differ in the proteins involved in transport and processing, as well as in typical leader sequence motifs. Class I peptides are transported by an ABC transporter (LanT) and leader cleavage is catalyzed intracellularly or extracellularly by the protease LanP (v. d. Meer *et al*., 1993; Meyer *et al*., 1995) or another protease of the producer. Leaders of class I lantibiotics contain a common "F(N/D)L(E/N/D)" motif at position -20 to -15 to the cleavage site, as well as a conserved Pro at position -2, whose presence is not essential for the removal of the leader (v. d. Meer *et al*., 1994; Oman and v. d. Donk, 2010).

In contrast, class II peptides usually possess a double glycine cleavage site (GG) that is altered in some of these peptides to GA or GS (Chatterjee *et al.*, 2005) and a leader motif with the sequence ELXX(V/L/I)X (Patton *et al*., 2008). The leader cleavage occurs during the export of the modified prepeptide and is catalyzed by an N-terminal protease domain of the exporter (Pag and Sahl, 2002). Class II peptides are further divided into four subclasses based on the sequence similarity of the active lantibiotic. These subclasses are 1. the lacticin 481-like peptides, 2. the mersacidin-like peptides, 3. the two-peptide lantibiotics and 4. the cinnamycin-like peptides.

Members of the lacticin 481- and mersacidin-like peptide families share a common motif that consists of the following conserved aa sequence GxxxxTxs/tCd/eC(3-10)xC (Cotter *et al*., 2006). Interestingly, an elongated version (SxxxGNxGxxCTxTxECmxxC) of this motif also is present in the α–peptides of the two-peptide lantibiotics (Cotter *et al*., 2006). These lantibiotics consists of two peptides, the α- and the β-peptide that are separately modified by two distinct LanM enzymes (McAuliffe *et al*., 2000). Moreover, the α- as well as the β-peptides characteristically have related ring patterns and the β-peptides additionally harbor a conserved motif consisting of the aa sequence s/txxxxcps/tTxCs/TxxC (Cotter *et al*., 2006).

A third class (**class III**) is formed by lanthionine containing peptides (lantipeptides) that are devoid of a significant antimicrobial activity (Willey and v. d. Donk, 2007). Instead, these peptides exert morphogenetic and signalling functions e.g. SapB serve as biosurfactant facilitating the emergence of newly formed aerial hyphae in streptomycetes (Kodani *et al*., 2005). Generally, class III peptides are modified by LanM-like proteins, but these enzymes often do not possess zinc ligands indicating an alternative cyclization mechanism (Willey and v. d. Donk). The leaders often harbor GG protease sites and additionally, a conserved helical recognition region at their N-termini (Müller *et al*., 2011).

In 2010, a further lantibiotic family was identified, the labyrinthopeptins (Meindl *et al.*, 2010, Müller *et al.*, 2010). Peptides of this family are characterized by the presence of a novel carbacyclic aa called labionin that is assembled from two Dha and one Cys in two consecutive Michael additions. This unusual aa is introduced by post-translational modifications catalyzed by the biosynthetic enzyme LabKC which has a sequence similar to that of the class III peptide SapB modifying enzyme RamC. Therefore, labyrinthopeptins were assigned to the type-III lantibiotics (Müller *et al.*, 2010). Interestingly, LabKC enzymes show only little sequence similarity to the LanC-like domain and important active sites, such as zinc binding motifs, are missing. In addition, also the N-terminus does not show sequence similarity to lantibiotic dehydrases. Instead, it exhibits similarity to eukaryotic Ser/Thr kinases. As for LanM enzymes, a phosphorylation of Thr and Ser residues, resulting in the formation of the didehydro-aa, is postulated, but in contrast to LanM enzymes, LabKC were shown to require GTP, instead of ATP, for this reaction (Müller *et al*., 2010).

Also in 2010, another lantibiotic modification enzyme was discovered (Goto *et al.*, 2010). The corresponding lantibiotic peptide (venezuelin) lacks antimicrobial activity and is modified by the bifunctional lantibiotic modification enzyme, LanL, via an alternative biosynthetic route. Similarly to the previously described enzymes of the LanM/C type, this enzyme contains a classical LanC-like domain in its C-terminal part, including all active and zinc binding sites. However, there is no dehydratase-like domain and, instead, two regions resembling a Ser/Thr kinase and lyase domain, like in the LabKC enzyme, are present at the N-terminus and these are essential for the dehydration. Based on sequence alignment analysis, Goto and co-workers (2010) postulated a new biosynthetic pathway for lanthionine formation by this enzyme including 1. phosphorylation of Thr/Ser residues by the kinase domain; 2. a β-elimination of these phosphate groups that generates Dha and Dhb as a result of the lyase activity and 3. cyclization by the LanC-like domain resulting in the formation of the thioether-aa by the same strategy as described for LanC and LanM enzymes.
Although the domains responsible for the dehydration step have dissimilar aa sequences in LanB, LanM and LanL/LabKC, the underlying reaction chemistry appears to be quite similar, as phosphorylation and elimination of the phosphate is used to generate the didehydro-aa in all three dehydration reactions (Chatterjee *et al.*, 2005; Paul *et al.*, 2007; Goto *et al.*, 2010). Lantibiotics are characterized by a high proportion of unusual aa. About one-third of the aa within the propeptide undergo structural changes of their side chains by post-translational modifications (Chatterjee *et al.*, 2005). Introduction of the thioether rings converts the linear peptide chain into a polycyclic structure, which confers enhanced peptide rigidity, proteolytic resistance (Bierbaum *et al.*, 1996), and resistance to thermal inactivation as well

as increased tolerance to oxidation (Sahl *et al.*, 1995). Furthermore, since some of these ring structures are involved in target binding by forming specific binding pockets (Hsu *et al.*, 2004), a structure-function relationship becomes evident for several lantibiotics (Ross and Vanderas, 2011).

Beside the typical lantibiotic modifications, some lantibiotics harbor **additional** less frequently encountered **modifications** that are often confined to a single lantibiotic or to related lantibiotics (Sahl and Bierbaum; 1998; Willey and v.d. Donk, 2006), but also contribute to activity and stability of peptides (McAuliffe *et al.*, 2001). So far, a total of 20 modifications have been described (table 1.1).

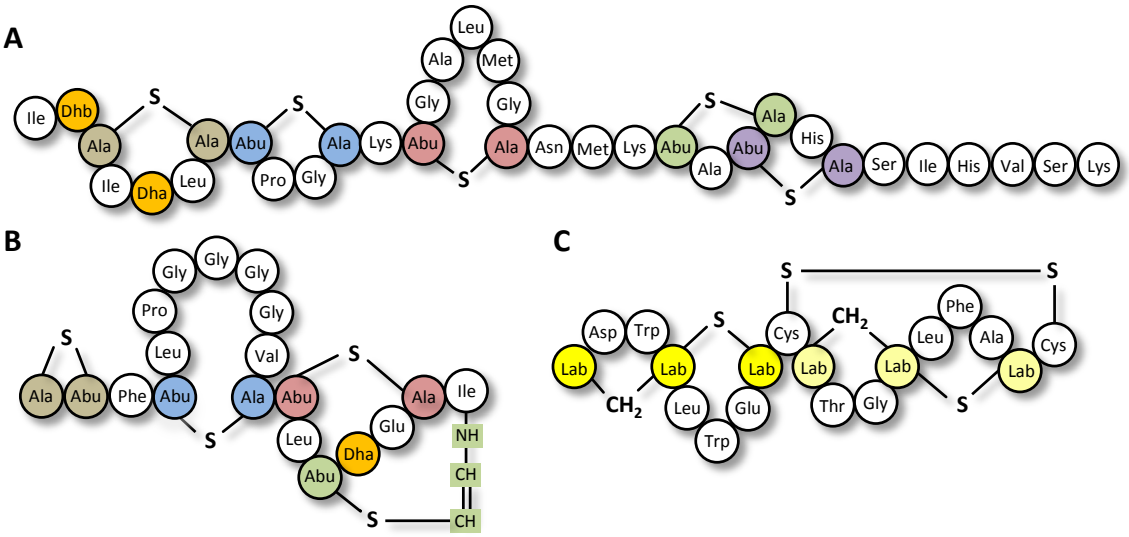

Figure 1.4: Structures of (A) nisin (class I), (B) mersacidin (class II) und C) labyrinthopeptin A2 (class III). (Me)Lan and labionin rings are highlighted by colors, whereas residues of the same thioether-aa are marked in same colors. Didehydro-aa are designated in orange.

As a consequence of various modifications, structure prediction of active lantibiotic peptides based on the primary sequence is almost impossible. Even the prediction of the typical lantibiotic aa lanthionine and didehydro-aa is difficult, since some hydroxy-aa, didehydro-aa and Cys prevent further modifications and remain unmodified in the active peptide. Based on an *in silico* analysis of all known lantibiotic peptides, Rink *et al.* suggested universal rules

Table 1.1: Overview over all post-translationally introduced aa modifications found in lantibiotic peptides.

Name		Description	Enzymes	Mechanism	Example
Didehydroalanine	Dha	unsaturated didehydro-aa	LanM, LanB, LanL, LabKC	Dehydration of Ser	all lantibiotics
Didehydrobutyrine	Dhb			Dehydration of Thr	
Lanthionine	Lan	thioether bridged di-carboxy-di-aa	LanM, LanC, LanL	Nucleophilic addition of Cys to Dha	
Methyllanthionine	MeLan			Nucleophilic addition of Cys to Dhb	
Lanthionine sulphoxide	SO-Lan	oxidized Lan	?	Oxidation of a C-terminal Lan	actagardine
Labionin	Lab	carbacyclic tri-aa with a methylene and a thioether bridge	LabKC	Two consecutive nucleophilic Michael additions of two Dha and one Cys	labyrintho-peptins
2-Aminovinyl-D-cysteine	AviCys	thioether cross-linked Ser-Cys derived di-aa	LanD	Oxidation and decarboxylation of a C-terminal Cys followed by an addition to Dha; elimination of the free COO^-	epidermin
2-Aminovinyl-methyl-D-cysteine	AviMeCys	thioether cross-linked Thr-Cys derived di-aa	LanD	Oxidation and decarboxylation of a C-terminal Cys followed by an addition to Dhb; elimination of the free COO^-	mersacidin
Lysinoalanine	LysN-Ala	cross-linked Lys-Dha derived di-aa	CinOrf7	Alcylation of a C-terminal Lys to Dha	cinnamycin
Disulfide bridge	S-S	cross-linkage of two Cys	spontaneous	Oxidation of two Cys derived sulfhydryl groups	haloduracin α
D-Alanine	D-Ala	D-isomer of Ala	LanJ	Hydrogenation of Dha	lacticin 3147
2-Oxopropionate	OPr	α-ketoalkanate	spontaneous	Hydratisation and desamination of N-terminal Dha; elimination of free NH_3^+	lactocin S
2-Oxobutyrate	Obu	α-ketoalkanate	spontaneous	Hydratisation and desamination of N-terminal Dhb; elimination of free NH_3^+	Pep5
2-Hydroxypropionate	HPr	hydroxylated propionate	?	Reduction of OPr	epicidin 280
Erythro-3-hydroxy-aspartate	HyAsp	hydroxylated Asp	CinX	Hydroxylation of Asp	cinnamycin
Nα-Succinyl-tryptophan	Suc-Trp	succinylated Trp	?	Succinylation of a Trp	subtilin variant
5-Chlorotryptophan	ClTrp	chlorinated Trp	MibH	Chloronation of Trp	microbispori-cines
4-Dihydroxyproline	OH-OHPro	two-fold hydoxylated Pro	MibO	Di-Hydroxylation of Pro	
Hydroxyproline	OHPro	hydroxylated Pro	MibO	Hydroxylation of Pro	
D-Lactate	D-Lac	reduced and hydrolyzed Dha	ElxB + ElxO	Reduction of the pyruvate that is based on the hydrogenation of an N-terminal Dha to lactate; NADPH dependent	epilancin 15X

for the occurrence of modifications in lantibiotic precursors (Rick *et al.*, 2005; Moll *et al*., 2010). According to these rules, the extent of dehydratisation is controlled by the aa context of flanking regions near to the dehydratable residues. Ser residues are predicted to escape dehydration more often than Thr. Hydroxy-aa that undergo dehydratisation more often are flanked by non-aromatic hydrophobic rather than by hydrophilic aa. Furthermore, the ring patterns differ in various lantibiotics, even in peptides of the same class.

Mode of action of lantibiotic peptides (MoA)

Except for class III peptides, the overwhelming majority of lantibiotics displays an antibacterial activity that mainly is restricted to Gram-positive bacteria. Although sometimes reported, Gram-negatives are generally not affected due to the protective effect of their outer membrane that prevents access to the main target. Consequently, treatment of bacterial cells with lantibiotics in concert with an outer membrane (OM) destroying agent like EDTA showed an antibiotic effect even against Gram-negatives e. g. *Salmonella* strains (Kordel and Sahl, 1986; Stevens *et al.*, 1991). Some lantibiotics (e.g. nisin Z) can affect *E. coli* and other Gram-negatives like *Helicobacter pylori* or *Neisseria* at higher concentrations, probably due to either a self-promoted uptake or a lantibiotic-based destabilization of the OM by its binding to lipopolysaccharides (Nagao *et al*., 2009).

Initial binding to the cell surface is driven by charge and based on the electrostatic attraction of the peptide to the membrane. Thereby, the cationic peptides interact with acidic polymers, such as the cell wall associated teichoic acids, as well as with acidic phospholipids e.g. phosphatidylglycerol and cardiolipin (Matsuzaki *et al*., 1999). This binding induces a conformational change that results in a rearrangement of hydrophilic and hydrophobic aa into an amphipathic helix and, thus in formation of a 3-dimensional structure that allows membrane insertion (Jung and Sahl, 1991).

The antibacterial effect generally depends on the **inhibition of cell wall biosynthesis** or the disruption of membrane integrity by pore formation. Inhibition of cell wall biosynthesis is achieved by binding of active peptides to the membrane-bound cell wall precursors lipid I and lipid II. Lipid I is assembled on the cytoplasmic side of the membrane by transfer of the UDP-activated amino-sugar N-acetyl-muramic acid (MurNAc) that carries the pentapeptide side chain to the membrane-bound lipid carrier undecaprenyl-pyrophosphate (C55PP). Coupling of the second UDP-activated amino-sugar, N-acetyl-glucosamine (GlucNAc) to lipid I generates lipid II and is followed by transport of the hydrophilic cell wall building block through the hydrophobic environment of the membrane (Bouhhs *et al.*, 2008). Subsequently, by transglycosylation and transpeptidation, the cell wall building blocks are

coupled to growing peptidoglycan chains of a nascent cell wall. The lipid carrier is recycled and returns to the cytosolic side where it is dephosphorylated to C55P (Bauer and Dicks, 2005; Schneider and Sahl, 2010 a,b).

Nisin, the most intensively studied lantibiotic, acts by a dual MoA including both mechanisms a) cell wall biosynthesis inhibition via complexing and dislocalization of lipid I and lipid II and b) disruption of the membrane integrity caused by the formation of short-lived membrane spanning pores (Wiedemann *et al.*, 2001 and 2004).
Both MoA are assigned to specific regions of the active peptide. The N-terminal rings A and B form a binding pocket, called the pyrophosphate cage, that allows the specific interaction and binding of nisin to the pyrophosphate moiety of lipid I/ II. As demonstrated in NMR spectra, this binding depends on five hydrogen bonds between the backbone amides of nisin and the pyrophosphate linkage group of lipid II (Hsu *et al.*, 2004). Since nisin is able to bind to both cell wall precursors, but not to C55P alone, the monosaccharide MurNAc-pentapeptide is also involved in target binding. Binding and complexing of lipid I/II result in delocalization of the cell wall precursors and, hence, in their abduction from the sites of nascent cell wall biosynthesis. For nisin-treated cells an accumulation of delocalized lipid II molecules has been demonstrated (Brötz *et al*., 1998). This is most likely accompanied by delocalization of proteins involved in cell division. As a consequence of delocalization of the cell wall biosynthesis and cell division machinery, formation of the divisom might also be affected (Hyde *et al*., 2005; Errington *et al*., 2003; Henrichfreise *et al*., 2009).
Moreover, nisin causes formation of short-lived (6 s) **membrane spanning** and potential-dependent **pores**. In lipophilic environments, an amphipathic helix with a central and flexible hinge region is formed that enables the peptide to insert into the membrane. This subsequently results in temporary membrane perturbations and assembly of a membrane spanning pore (van Heusden *et al.*, 2002). Within the pore, hydrophobic aa side chains are immersed into the core of the membrane, whereas hydrophilic groups, like the positively charged aa His and Lys, face the water-filled channel or interact with the phospholipid head groups (v. d. Hooven *et al.*, 1996; van Heusden *et al.*, 2002). Pore formation (2-2.5 nm in diameter) allows small molecules, like aa and ions (e.g. K^+) to leak from the cell, resulting in disruption of the barrier function of the membrane and, consequently, in dissipation of the membrane potential. Finally, this results in the abrupt arrest of cellular biosynthesis processes and in cell death (Sahl and Brandis, 1982; Wiedemann *et al.*, 2001). Although nisin mutants incapable to form pores retained considerable antimicrobial activity, the capacity for pore formation appears to be the primary effect of its antimicrobial activity. This is based on the fact that pore formation results in a rapid killing so that inhibition of peptidoglycan

biosynthesis does not come into effect (Brötz *et al.*, 1998; Wiedemann *et al.*, 2001). Nevertheless, lipid II binding is essential for nisin activity, as nisin had enhanced binding efficacy to lipid II-containing model membranes. This indicates that lipid II serves as docking molecule and mediates a 'targeted' pore formation (Brötz *et al.*, 1998). Binding of lipid II appears to enable the peptide to integrate into the membrane by causing a conformational change resulting in a transmembrane orientation of the lantibiotic (van Heusden *et al.*, 2002). Furthermore, lipid II appears to stabilize the resulting pores (Wiedemann *et al.*, 2004; Breukink *et al.*, 1999). The essential function of lipid II in pore formation is underlined by the fact that nisin pores exist of eight nisin and four lipid II molecules (Hasper *et al.*, 2004).

Lantibiotics like mersacidin/epidermin/gallidermin and Pep5 only act by one of these two mechanisms. Due to its elongated structure **Pep5** apparently binds the membrane and acts via dissipation of membrane integrity by pore formation (Sahl and Brandis, 1982; Pag, phD thesis, 1998). In contrast, epidermin and gallidermin preserve a nisin-like lipid II binding motif, but since they are shorter and lack the C-terminal amphiphilic tail of nisin, they are incapable to perform pore formation and solely act on lipid II and the cell wall biosynthesis (Bonelli *et al.*, 2006). This also was shown for the globular lantibiotics of the **mersacidin-like** subgroup. In contrast to nisin, mersacidin has a lower affinity to lipid II and the N-acetyl-glucosamine of the cell wall precursor takes part in the complex, since lipid I is not bound by those peptides. All mersacidin-like lantibiotics share a conserved TxS/TxEC motif (Böttiger *et al*., 2009; Cotter *et al*., 2006) within their essential C-ring which is involved in lipid II binding although it differs from the lipid II binding pocket of nisin. Interestingly, this essential C-ring is similar to the A-ring of lacticin 481 subfamily peptides (Szekat *et al*., 2003). Consequently, lipid II complexing here results in accumulation and dislocation of lipid II as well as in prevention of transglycosylation by steric hindrance (Brötz *et al*., 1997).
Divalent ions like calcium increase the activity of some lantibiotics that contain the lipid II motif of mersacidin (Barrett *et al*., 1992). By NRM studies, Kärcher and coworkers observed that a negatively charged and conserved E residue is involved in Ca^{2+} binding of actagardine (Schneider *et al*., 2000). Depending on the net charge and presence and localization of positively charged aa within the lantibiotic peptide, Ca^{2+} ions appear to contribute to the antimicrobial activity of these peptide in many ways (Böttiger *et al.,* 2009): 1. binding of calcium confers a positive net charge to neutral peptides like mersacidin, which might result in an enhanced membrane interaction and, consequently, in a deeper membrane insertion; 2. for positively charged peptides, e.g. plantaricin C , calcium might stabilize a conformation that promotes membrane insertion.

An ion independent MoA was demonstrated for the uncharged lacticin 481. In contrast to other neutral peptides, it contains a positively charged aa on its N-terminus that might be sufficient for membrane interaction and formation of complexes with lipid II (Böttiger *et al*., 2009).

Two-peptide lantibiotics have a dual MoA like nisin, but here it depends on two synergistically acting peptides (Morgan *et al*., 2005). While lipid II binding is mediated by the α-peptides that show sequence similarity to mersacidin-like lantibiotics, the elongated β-peptide acts on the membrane. The individual peptides are separately modified by two LanM enzymes (McAuliffe *et al*., 2000) and the peptides themselves are inactive or display only little activity (Holo *et al.*, 2001; Navaratna *et al.*, 1999).
For lacticin 3147 it has been demonstrated that lipid II binding results in a conformational change within the α-peptide leading to enhanced complex formation with the second peptide (Wiedemann *et al.*, 2006). A proline residue within the β-peptide motif is apparently involved in complex formation, since mutations in this site strongly affect the peptide:peptide synergism (Lawton *et al.*, 2007). Complex formation itself enhances the affinity of the α-peptide to lipid II and, therefore, results in a deeper insertion of the complex into the membrane. This complex arrangement allows the β-peptide to adopt a transbilayer orientation and, thereby, results in pore formation. These pores were found to be significantly smaller (0.6 nm) than those formed by nisin (Bonelli *et al.*, 2006). Since all members of the two-peptide lantibiotics are quite homologous, it is hypothesized that they, with exception of the cytotoxic cytolysin, share a similar MoA (Willey and v. d. Donk, 2006).

Cinnamycin-like peptides display a different MoA and act by the disruption of enzymatic functions. These peptides indirectly inhibit the phospholipase A2 by forming a complex with its lipid substrate phosphatidylethanolamine (PE) in a 1:1 stoichiometry. Subsequently, this results in a transbilayer lipid movement and reorganization of the membrane (Hosoda *et al.*, 1996; Iwamoto *et al.*, 2007; Makino *et al*., 2003).

Class III lantibiotics like SapT and SapB exhibit a morphogenetic rather than an antibacterial effect. If at all, these peptides have low antimicrobial activities. Based on their amphiphilic structure, they serve as biosurfactants e.g. during the emergence of aerial hyphae of *streptomyces* strains (Kodani *et al.*, 2005 and 2004).
For some lantibiotics (e.g. subtilin) an inhibiting effect on the outgrowth of endospores is reported (Oman and v. d. Donk, 2009). A recent publication dealing with the molecular basis of the antisporal effect of nisin demonstrated that this effect also depends on the

membrane and lipid II binding activity of active peptides. By preventing the establishment of a membrane potential and cell wall biosynthesis, the spore outgrowth is inhibited at the stage of spore germination (Gut *et al.*, 2011).

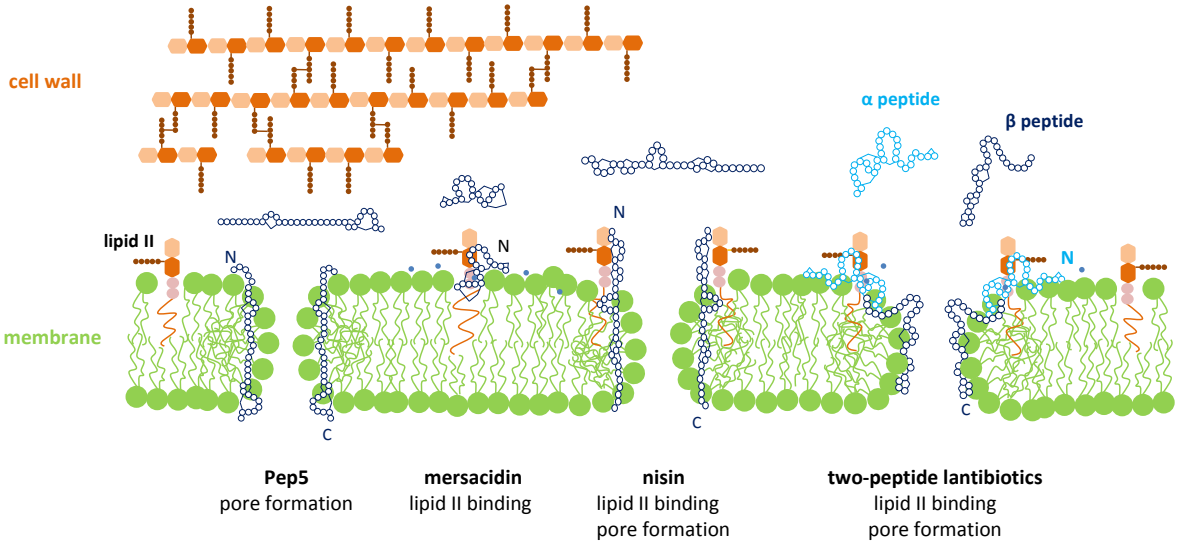

Figure 1.5: Overview over the MoA of lantibiotics: Almost all lantibiotics act either by inhibition of cell wall biosynthesis by lipid II binding and or disruption of the membrane.

Producer self-protection of lantibiotic producers

Production of antimicrobials targeting classical and widely distributed cell structures, like lipid II or the prokaryotic membrane, necessitates development of a dedicated producer self-protection machinery in order to avoid the risk of self-destruction. Lantibiotic producers are highly resistant against their own lantibiotic and this resistance is very specific and restricted to their own lantibiotics or very closely related variants thereof (Jack, Bierbaum and Sahl, 1998; Heidrich *et al*., 1998).

In general, two strategies have evolved. The most widely distributed mechanism relies on an ABC-transporter, LanEFG (Klein and Entian, 1993; Siegers and Entian, 1995), that removes the membrane-bound lantibiotic by pumping it back into the extracellular space without degradation (Peschel and Götz, 1996; Otto *et al*., 1998; Stein *et al*., 2003). Thereby, the amount of membrane-bound peptide is kept below a critical level. Energy for transport is provided by ATP hydrolysis (Okuda *et al*., 2010; Okuda and Sonomoto, 2011). These immunity-transporters consist of two heterodimeric membrane spanning proteins (LanEG) and two homodimeric ATP-binding proteins (LanF). The presence of an accessory factor (LanH) which acts as an ancillary protein of the transporter has been described for some

lantibiotic producers, e.g. epidermin, gallidermin (Hille *et al*., 2001; Peschel *et al*., 1997) and nukacin ISK-1 (Aso *et al*., 2008) producing strains. For the gallidermin producer, the presence of a LanH, which acts as an ancillary protein for the assembly of the LanFEG transporter, has been reported (Peschel *et al*., 1997). The NukH protein was shown to cooperatively contribute to immunity as a lantibiotic binding protein that inactivates membrane-bound nukacin prior its export by NukEFG (Okuda *et al*., 2005).

The second mechanism is based on the expression of LanI immunity peptides or proteins, which belong to three classes, 1. the lipoproteins; 2. a group of small peptides and 3. transmembrane proteins. LanI are found, for example, in Pep5 and Epicidin 280 as well as in nisin and subtilin and lacticin 3147 producers.

PepI and EciI peptides are very small (about 70 aa), share a highly hydrophobic N-terminus and a hydrophilic C-terminus (Reis *et al*., 1994). They are membrane-associated and localized at the interface between cell membrane and cell wall (Hoffmann *et al*., 2004). In contrast, NisI (245 a) and SpaI (165 aa) are larger and possess a consensus lipopeptide signal sequence. These proteins are attached to the outside of the cytoplasmic membrane via a lipid anchor (Klein and Entian, 1994). Transmembrane spanning LanI proteins contain at least three transmembrane domains and are found in the gene clusters of lacticin 3147 and cytolysin (McAuliffe *et al*., 2000; Coburn *et al*., 2009).

LanI proteins and peptides act *via* 1. a direct interaction with the lantibiotic, resulting in its antagonization by sequestering the peptide (nisin) or 2. a membrane interaction generating an environment unsuitable for the function of the lantibiotic, or 3. a direct interaction with the target has been proposed (Pep5).

Studies on nisin and lacticin 3147 indicate that their immunity peptides directly interact with the corresponding lantibiotic resulting in the formation of an insoluble complex (Stein *et al*., 2003; Draper *et al*., 2009), whereas PepI appears to be located in the membrane cell wall interface (Hoffmann *et al*., 2004). An additional feature of LanI was discovered, when it was found, that secreted but lipid anchor-free NisI proteins aggregated nisin extracellularly (Koponen *et al*., 2004). In general, LanI proteins and peptides are highly specific and so they have no or only very little sequence similarity (Stein *et al*., 2002; Draper *et al.*, 2008).

Interestingly, some lantibiotic producers such as the producers of nisin, subtilin and lacticin 3147, developed both strategies (Siegers and Entian, 1995; Klein and Entian, 1993; Draper *et al*., 2009) and in these cases, although both systems are required for the full immunity, the larger part is mediated by the LanI system (Pag *et al*., 1999; Stein *et al*., 2002; McAuliffe *et al*., 2000).

Lantibiotic gene clusters and their regulation

Genes associated with lantibiotic biosynthesis are almost always located adjacent to the structural gene and together they form the lantibiotic gene cluster (Siezen *et al*., 1996). One exception, in the cyanobacterium *Prochlorococcus,* has been described so far. This lantipeptide producing strain harbors 29 putative *lanA* that are widely distributed within the genome. These prepeptides share quite similar aa sequences in their leaders and six of them were shown to be modified *in vitro* by the same LanM (Shi *et al.*, 2010).

In addition to the genetic information for the prepeptide (*lanA*), the gene clusters encode for proteins essential for lantibiotic modification (mainly *lanB,C,M*), regulation (*lanR,K*), export (*lanT*), processing (*lanP*) and immunity (*lanEFG,H,I*). In this nomenclature the generic symbol *lan* replaces a specific designation, that is derived from the name of the peptide (Jack *et al.*, 1995). Usually, at least parts of the cluster are organized in operons that allow the co-expression of proteins that are involved in same functions. L*anA* often is clustered with genes of modification enzymes in a biosynthesis operon, in which it usually is the first ORF. Besides this, there is no uniform gene order in individual clusters (Siezen *et al.*, 1996; Willey and v. d. Donk, 2006).

Clusters are often found on plasmids (Pep5) or, as far as they are chromosomally encoded, associated with mobile elements like IS elements or transposons (Nisin; Schnell *et al*., 1988; Horn *et al*., 1991). This finding together with the fact that GC contents of many gene clusters differ from the overall GC content of the producer, indicates that clusters might be spread through horizontal gene transfer (Willey and v. d. Donk, 2006).

Production of a lantibiotic is energy-intensive and therefore, this process has to be tightly regulated. For many clusters, a **specific regulation** was demonstrated**,** that is most frequently based on dedicated two-component response regulatory systems. This system consists of a membrane-bound histidine kinase (LanK) which responds to an external stimulus by autophosphorylation of a conserved His residue. As shown in detail for nisin (Kuipers *et al.*, 1995) and mersacidin (Schmitz *et al.*, 2006), triggering agents are the active peptides themselves. Nisin, that had been rendered antimicrobially inactive, still retained its induction capacity, indicating that induction is not based on pore formation and might be due to a direct interaction of the peptides with the kinase (Kuipers *et al.*, 1995; Kleerebezem, 2004). Subsequently, the phosphate group is transferred to an Asp residue of the appropriate response regulator (LanR) that often functions as a transcriptional activator (Kraaij *et al.*, 1999). In the following, the response regulator undergoes a conformational change that enables it to bind to specific operator structures, thereby affecting transcription of the target genes (Willey and v. d. Donk, 2006; Yonezawa and Kuramitsu; 2005).

Certain gene clusters e.g. in mersacidin and cinnamycin producer strains, contain additional LanR proteins, indicating a more complex regulation. In the mersacidin cluster the two-component system consists of MrsK2 and MrsR2 that control producer self-protection in an auto-regulated manner, whereas mersacidin production is under control of the MrsK2 independent, orphan response regulator MrsR1 (Guder *et al.*, 2002). Other lantibiotic gene clusters, like the lacticin 3147 cluster, are controlled by repressor proteins.
Even uninduced cells produce a basal level of the bacteriocin, so that a subinhibitory concentration accumulates in the surrounding. These peptides serve as signal molecules and, once a threshold concentration is reached, induce their own biosynthesis (Kuipers et al., 1995). Such auto-inducing mechanisms strongly underline the signalling function of lantibiotics and enable the producer to communicate with other cells in order to achieve high level production rates and a rapid build-up of the self-immunity in a concerted action (Kuipers *et al.*, 1995).

Furthermore, lantibiotic production and other cellular processes, such as competence and sporulation, usually are co-regulated and appear to be part of a complex stress response. For example, in *B. subtilis*, transcription of *spaR* and *spaK* is controlled by the alternative sigma factor σ^H (Stein *et al*., 2002). Additionally, production of the two-peptide lantibiotic Smb depends on induction by the competence stimulation peptide (CSP) (Yonezawa and Kuramitsu, 2005). As a consequence, lantibiotic production and competence development occur simultaneously and might provide a mechanism to ensure a pool of heterologous DNA for transformation and uptake (v. d. Ploeg, 2005; Yonezawa and Kuramitsu, 2005).

Current and future applications of lantibiotics and associated proteins

Nowadays, over 80 lantibiotics have been identified (table 1.2), several of which exhibit high potential to inhibit clinically significant pathogens. In consideration of the fact that the potential to act as antimicrobials is combined with a low tendency to generate resistance, makes lantibiotics highly attractive for medical applications (v. Heel *et al.*, 2011; Wilson-Standford and Smith, 2011). The majority of lantibiotics acts on targets (e.g. lipid II) that are known from other clinically used antibiotics, but in contrast to these, they are orders of magnitude more potent and bind to different target sites. Thus, they might overcome the problem of pre-existing resistance mechanisms.
In general, lantibiotics have many characteristics, like their low MW, lack of toxicity, broad range of activity and low immunogenicity which make them suitable for applications in many areas, mainly in **food industry**. Nevertheless, particularly for **medical applications**, some

hurdles have to be overcome. For nisin, these are the low solubility, lack of stability and activity at higher pH as well as their tendency to interact with blood components.

The first and only lantibiotic in commercial use is nisin. Since it exhibits an antimicrobial activity against Gram-positives, including spoilage causing bacteria like *Listeria monocytogenes*, it has been adopted as a food preservative (E234) in processed dairy products, canned fruits and vegetables (Cotter *et al.*, 2005, McAuliffe *et al.*, 2001). Importantly, development of significant resistance levels has rarely been observed. Nisin also exhibits a high efficacy against clinically relevant human pathogens, like *Helicobacter pylori,* making it a putative agent for treatment of peptic ulcers (Nascimento *et al.*, 2006). Another application in veterinary medicine for treatment of bovine mastitis also has been reported (Broadbent *et al.*, 1989).

Besides nisin, other lantibiotics have been investigated regarding their potential application as antimicrobials (Des Field *et al.*, 2010). Many lantibiotics were demonstrated to be efficient *in vitro* and *in vivo* against multiresistant superbugs. Regarding this, mersacidin emerged as an effective agent to treat MRSA infections in a murine model (Limbert *et al*., 1991; Kruszewska *et al.*, 2004).

Besides the classical antibiotic use, lantibiotics could find application in further medical settings and might serve as **additives** and **probiotics** in order to prevent infections or bacterial colonization. Epidermin and lacticin 3147 are active against the acne causing bacterium *Propionibacterium acnes,* thus providing the opportunity to use these substances as additives in cosmetics and personal-care products (Kellner *et al.*, 1988; Lawton *et al.*, 2007). In an *in vitro* colonization experiment, Pep5 and epidermin prevented adhesion of coagulase-negative staphylococci, specifically of *S. epidermidis,* to siliconisated catheters (Fontana *et al.*, 2006). The coating of medical devices would be an elegant strategy to reduce catheter-related infections.

Lacticin 3147 exerts an antimicrobial effect on different cariogenic *Streptococcus mutant* strains and, therefore, its use as additive in dental applications is discussed (Suda *et al.*, 2011). Salivaricins have interesting effects against *Streptococcus pyogenes* strains. Therefore, the salivaricin A producer was supplemented as a probiotic to milk drinks and was demonstrated to persist in the oral cavity. Thus, *S. pyogenes* infections were prevented by a probiotic bacterial replacement strategy using a strain of the indigenous oral tongue microbiotia that is known for lantibiotic production (Dierksen *et al.*, 2007). Moreover, chewing gums and lozenges, that include salivaricin-producing strains, have been developed (BLIS Technologies). Some lantibiotics have additional properties that are interesting for medical applications other than the use as antimicrobials. A **contraceptive effect** due to spermicidal activities has been reported for nisin *in vitro* and *in vivo.* After intravaginal

application, nisin appears to completely impede sperm motility and, hence, block conception. The exact underlying mechanism has not been elucidated yet. Like bacterial membranes, the membrane of the sperms carries negatively charged phospholipids on its outside suggesting that a membrane-damaging effect could be responsible for sperm immobilization. As a consequence of its spermicidal activity, nisin could be employed as a safe and potent vaginal contraceptive. As a side effect, based on its antimicrobial activity, nisin would further inhibit growth of bacteria causing sexually transmitted diseases (Aranha *et al.*, 2004; Reddy *et al.*, 2004 a, b).

Furthermore, potential **protein chemistry applications** are discussed for lantibiotic biosynthesis enzymes. Usage of the biosynthesis machinery in *in vivo* and *in vitro* bioengineering approaches is a promising technology to design novel antimicrobial compounds. Novel antibiotics could be designed on the basis of either the generation of improved lantibiotic analogs or the introduction of thioether rings into existing therapeutics (Rink *et al.*, 2005; Kluskens *et al*., 2005). Since lantibiotics are ribosomally synthesized these peptides are amenable to rational drug design. Here, known lantibiotics structures serve as blueprints for the expression of mutated peptides. In this context, an *in vitro* mutasynthesis approach has been established by Leevengood *et al.* (2009) as a method to improve the class II lantibiotic lacticin 481 by introduction of unnatural aa side chain substitutions.

The promiscuity of the lantibiotic modification enzymes and their ability to display activity *in vitro*, allow introduction of lantibiotic modifications even into synthetic analogs (Chatterjee *et al*., 2006). Introduction of additional non-usual aa might be useful to enhance peptide efficacy, solubility and stability as well as to improve their pharmacodynamics properties. Successful improvement of therapeutically used peptide variants of enkephalin and somatostatin by chemical introduction of thioether bridges has been previously reported (Ösapay *et al.*, 1997; Rew *et al.*, 2002). To this end, Kuipers and coworkers established an *in vivo* system that is based on the nisin biosynthesis and transport machinery (Kuipers *et al.*, 2005; Bosma *et al*., 2011) and introduces didehydro- as well as thioether-aa into non-lantibiotic peptides. Using this system they successfully produced lanthionine-bridged variants of vasopressin, enkephalin and angiotensin.

In conclusion, lantibiotics hold a considerable promise as alternatives for traditional therapeutics, as probiotics, preservatives and additives in agro-food- or cosmetic-industry and veterinary and human medicine. Even the enzymes of the lantibiotic biosynthesis apparatus have a high potential for biotechnological use because of their ability to modify non-lantibiotic therapeutics and pharmaceuticals.

Table 1.2: Overview of all lantibiotics (01/2012). Classification according to Willey and v. d. Donk (2006). Residues involved in (Me)Lan bridges are highlighted in colored boxes, whereas subsidiary residues of one thioether aa are marked in same colors. The positions (x) of additional modifications are marked by **black**, bold letters. Ser and Thr residues that are found to be dehydrated to Dha and Dhb are designated in **orange**. Conserved leader structures are **underlined**. The overall net charge of the peptides was calculated at pH7.

Name	Producer	MW [Da]	aa	Charge	Amino acid sequence		References
					Leader sequence	Prepeptide sequence	
Class I lantibiotics (LanBC modified, elongated peptides)							
Nisin-like lantibiotics							
Nisin A	*Lactococcus lactis* ATCC 11454	3353	34	+3	MSTKD**FNLD**LVSVSKKDSG**A**S**P**R	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK	Gross and Morell, 1971
Nisin Q	*Lactococcus lactis* 61-14	3327	34	+3	MSTKD**FNLD**LVSVSKTDSG**A**STR	ITSISLCTPGCKTGVLMGCNLKTATCNCSVHVSK	Zendo *et al.*, 2003
Nisin Z	*Lactococcus lactis* N8, NIZO22186	3330	34	+3	MSTKD**FNLD**LVSVSKKDSG**A**S**P**R	ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK	Mulders *et al.*, 1991
Nisin F	*Lactococcus lactis F10*	3457	34	+3	MSTKD**FNLD**LVSVSKKDSG**A**S**P**R	ITSISLCTPGCKTGALMGCNMKTATCNCSVHVSK	De Kwaadsteniet *et al.*, 2008
Nisin U	*Streptococcus uberis 42*	3029	31	+3	MNNED**FNLD**LIKISKENNSG**A**S**P**R	ITSKSLCTPGCKTGILMTCPLKTATCGCHFG three variants (U, U2, U3), differ in 1-2 aa, sequences of nisin U2 and U3 haven't been published, yet.	Wirawan *et al.*, 2006 and 2007
Subtilin	*Bacillus subtilis*	3317	32	+2	MSKFDD**FDLD**VVKVSKQDSKIT**P**Q	WKSESLCTPGCVTGALQTCFLQTLTCNCKISK	Gross *et al.*, 1973
Entianin	*Bacillus subtilis* DSM 15029T	3348	32	+2	not annotated	WKSESVCTPGCVTGLLQTCFLQTITCNCKISK	Fuchs *et al.*, 2011
Ericin S	*Bacillus subtilis* A1/3	3442	32	+1	MSKFDD**FDLD**VVKVSKQDSKIT**P**Q	WKSESVCTPGCVTGVLQTCFLQTITCNCHISK	Stein *et al.*, 2002
Ericin A	*Bacillus subtilis* A1/3	2986	29	+2	MTNMSKFDD**FDLD**VVKVSKQDSKIT**P**Q	VLSKSLCTPGCITGPLQTCYLCFPTFAKC	
Planosporicin (LAB97518)	*Planomonospora* spec.	2194	24	-1	not annotated	ITSVSWCTPGCTSEGGGSGCSHCC	Maffioli *et al.*, 2009; Castiglione *et al.*, 2007
Streptin 1	*Streptococcus pyogenes* M25	2424	23	+3	MNNTIKD**FDLD**LKTNKKDT**A**T**P**Y	VGSRYLCTPGSCWKLVCFTTTVK VGSRYLCTPGSCWKLVCFTTTVK two putative structures predicted	Karaya *et al.*, 2001
Streptin 2		2821	26	+3	MNNTIKD**FDLD**LKTNKKDTA	TPYVGSRYLCTPGSCWKLVCFTTTVK TPYVGSRYLCTPGSCWKLVCFTTTVK two structures predicted (elongated Streptin 1)	Wescombe and Tagg, 2003

Table 1.2 – continued

Salivaricin D	*Streptococcus salivarius 5M6c*	3475	34	+1	MSTKD**FNLD**VEVSKSNTGASA	FTSHSLCTPGCITGVLMGCHIQSIGCNVHIHISK structure predicted	Birri *et al.*, 2012
Epidermin-like lantibiotics							
Epidermin	*Staphylococcus epidermidis*	2164	22	+3	MEAVKEKNDL**FNLD**VKVNAKESNDSG**A**E**P**R	IASKFICTPGCAKTGSFNSYCC AviCys (19 + 22)	Allgaier *et al.*, 1986
[Val1,Leu6] Epidermin	*Staphylococcus epidermidis*	2151	22	+3	MEAVKEKNDL**FNLD**VKVNAKESNDSG**A**E**P**R	VASKFLCTPGCAKTGSFNSYCC AviCys (19 + 22)	Israil *et al.*, 1996
Gallidermin	*Staphylococcus gallinarum* Tü3928	2165	22	+3	MEAVKEKNEL**FDLD**VKVNAKESNDSG**A**E**P**R	IASKFLCTPGCAKTGSFNSYCC AviCys (19 + 22)	Kellner *et al.*, 1988
Staphylo-coccin T	*Staphylococcus cohnii*				MEAVKGKNEL**FDLD**VKVNAKESNDSG**A**E**P**R		Furmanek *et al.*, 1999
BsaA2, Staphylo-coccin Au-26	community-acquired MRSA	2089	22	+2	MEKVLDLDVQVKANNNSNDSAGDER	ITSHSLCTPGCAKTGSFNSFCC AviCys (19 + 22); predicted structure, slightly different *lanA* variants identified, additional non-expressed *lanA*	Daly *et al.*, 2010
Mutacin B-Ny266	*Streptococcus mutans*	2270	22	+3	not annotated	FKSWSFCTPGCAKTGSFNSYCC AviCys (19 + 22); structure predicted (on Edman sequencing results); additional non-expressed *lanA*	Mota-Meira *et al.*, 1997
Mutacin 1140 Mutacin III	*Streptococcus mutans*	2263	22	+3	MSNTQLLEVLGTETFDVQEDLFAFDTTDT TIVASNDDPDTR	FKSWSLCTPGCARTGSFNSYCC AviCys (19 + 22), additional non-expressed *lanA*	Hillman *et al.*, 1998 Qi *et al.*, 1999
Clausin	*Bacillus clausii* OC	?	22	0	not annotated	FTSVSFCTPGCGETGSFNSFCC AviCys (19 + 22)	Bouhss *et al.*, 2009
Mutacin I	*Steptococcus mutans*	2364	24	+2	MSNTQLLEVLGTETFDVQEDLFAFDTTDT TIVASNDDPDTR	FSSLSLCSLGCTGVKNPSFNSYCC AviCys (19 + 22); predicted structure, additional non-expressed *lanA*	Qi *et al.*, 2000
Microbi-sporicin (different variants)	*Microbiospora* spec. (*corallina*)	2246	24	+1	MPADILETRTSETEDLLDLDLSIGVEEIT **A**G**P**A	VTS**W**SLCTPGCTS**P**GGGSNCSFCC AviMeCys (21-24); **Variant 107891 A1**: Chlorotryptophan (4), Dihydroxy-proline (14)	Castiglione *et al.*, 2008
		2230	24	+1		**Variant 107891 A2:** Chlorotryptophan (4), Hydroxyproline (14)	
Pep5-like lantibiotics							
Pep5	*Staphyloccoccus epidermidis*	3488	34	+7	MKNNKNL**FDLE**IKKETSQNTDELE**P**Q	**T**AGPAIRA**S**VKQCQK**T**LKA**T**RLFTVSCKGKNGCK 2-Oxobutyryl (1)	Kaletta *et al.*, 1989
Epilancin K7	*Streptococcus epidermidis* K7	3032	31	+5	MNNSL**FDLN**LNKGVETQKSDLS**P**Q	**S**A**S**VLK**TS**IKVSKKYCKGVTLTCGCNI**T**GGK 2-Hydroxypropionyl (1)	Van de Kamp *et al.*, 1995
Epicidin 280	*Streptococcus epidermidis*	3133	30	+4	MENKKDL**FDLE**IKKDNMENNNELEAQ	**S**LGPAIKA**T**RQVCPKATRFVTVSCKKSDCQ 2-Hydroxypropionyl (1); predicted structure	Heidrich *et al.*, 1998

Table 1.2 – continued

Epilancin 15X	*Streptococcus epidermidis*	3173	30	+7	not annotated	ASIVKTTIKASKKLCRGFTLTCGCHFTGKK Lactate (1)	Ekkelenkamp *et al.*, 2005
Class II lantibiotics (LanM modified, globular peptides)							
Lacticin 481-like lantibiotics							
Lacticin 481	*Lactococcus lactis* CNRZ 481	2901	27	0	MKEQNSFNLLQEVTESELDLILGA	KGGSGVIHTISHECNMNSWQFVFTCCS	Piard *et al.*, 1993
Nukacin ISK-1	*Staphylococcus warneri*	2960	27	+3	MENSKVMKDIEVANLLEEVQEDENEVLGA	KKKSGVIPTVSHDCHMNSFQFVFTCCS predicted structure	Sashihara *et al.*, 2000
Nukacin KQU131	*Staphylococcus hominis KQU-131*	3004	27	+3	MENSKIMKDIEVANLLEEVQEDELNEVLGA	KKKSGVIPTVSHDCHMNSFQFMFTCCS predicted structure	Wilaipun *et al.*, 2008
Mutacin II, JT-8, H-29B	*Streptococcus mutans* T8	3245	27	+1	MNKLNSNAVVSLNEVSDSELDTILGG	NRWWQGVVPTVSYECRMNSWQHVFTCC	Novák *et al.*, 1994
Mutacin K8	*Streptococcus mutans* K8	~ 2734	?	+1	4 *lanA* (MukA1/MukA3 fit to the mass of an antimicrobial peptide produced by this strain; two additional non-expressed *lanA*); unknown/predicted structures (similarity to lacticin 481)		Robson *et al.*, 2007
					MKNTTNEMLELIQEVSLDELDQVIGG	MGKGAVGTISHECRYNSWAFLATCCS	
				+2	MKQSDEMLELIQEVSLDELDQVIGG	AGNGVIRTITQGCRMPNNMQVLFTC	
				+1	MKQSNEMLELIQEVSLDELDQVIGG	MGKGAVGTISHECRYNSWAFLATCCS	
				+1	MKKGTQLYLEALEALQEIKVEELDTFIGG	GGRASNTISSDCRWNSLQAIFSCC	
Streptococcin SA-FF22	*Streptococcus pyogenes SA-FF22*	2795	26	+2	MEKNNEVINSIQEVSLEELDQIIGA	GKNGVFKTISHECHLNTWAFLATCCS structure not fully confirmed, additional non-expressed *lanA*	Jack *et al.*, 1994
Streptococcin AM49	*Streptococcus pyogenes*				MTKEHEIINSIQEVSLEELDQIIGA		Hynes *et al.*, 1994
Macedocin	*Streptococcus macedonicus* ACA-DC198				MEKETTIIESIQEVSLEELDQIIGA		Georgalaki *et al.*, 2002
Macedocin A1		?	23	0	MKNNNKICQDALESLQELKLEEVDELLGG	GHGVNTISAECRWNSLQAIFSCC putative *lanA;* structure predicted (based on similarity to lacticin 481)	Papadelli *et al.*, 2007
Variacin	*Kocuria varians*	2658	25	-1	MTNAFQALDEVTDAELDAILGG	GSGVIPTISHECHMNSFQFVFTCCS structure predicted (similarity to lacticin 481)	Pridmore *et al.*, 1996
Plantaricin C	*Lactobacillus plantarum*	2880	27	+1	not annotated	KKTKKNSSGDICTLTSECDHLATWVCC structure partially elucidated, predicted	González *et al.*, 1994
Ruminococcin A	*Ruminococcus gnavus*	2675	24	0	MRNDVLTLTNPMEEKELEQILGG	GNGVLKTISHECNMNTWQFLFTCC structure predicted, additional non-expressed *lanA*	Dabard *et al.*, 2001

Table 1.2 – continued

Butyrivibrio-cin OR79A	*Butyrivibrio fibrisolvens*	2910	25	0	MNKELNALTNPIDEKELEQILGG	GNGVIKTISHECHMNTWQFIFTCCS structure not fully elucidated/predicted based on similarity to lacticin 481	Kalmokoff *et al* . 1999
Butyrivibrio-cin OR36		?	25	0	MNKDLNALTNPIDEKELEQILGG	GDGVFRTISHECHMNTWMFIFTCCS putative *lanA*, structure predicted based on similarity to lacticin 481	Whitford *et al.,* 2001; Dufour *et al.,* 2007
Butyrivibrio-cin OB247		?	25	0		GDGVFRTISHECAMNTWMFIFTCCS putative *lanA*, structure predicted (similarity to lacticin)	
Salivaricin A	*Streptococcus salivarius* 20P3	2315	22	0	MKNSKDILNNAIEEVSEKELMEVAGG	KRGSGWIATITDDCPNSVFVCC structure not fully elucidated	Ross *et al.,* 1993
Salivaricin B	*Streptococcus salivarius* K12	2740	25	0	MAKQQMNLVEIEAMNSLQELTLEELDNVLGA	GGGVIQTISHECRMNSWQFLFTCCS putative *lanA;* structure predicted based on similarity to lacticin 481	Hyink *et al.,* 2007
Variacin	*Kocuria varians*	2658	25	-1	MTNAFQALDEVTDAELDAILGG	GSGVIPTISHECHMNSFQFVFTCCS structure predicted (similarity to lacticin 481)	Pridmore *et al.,* 1996
Plantaricin C	*Lactobacillus plantarum*	2880	27	+1	not annotated	KKTKKNSSGDICTLITSECDHLATWVCC structure partially elucidated, predicted	González *et al.,* 1994
Bovicin HJ50 Thermophilin 1277	*Streptococcus bovis* HJ50*; thermophilus*	3428	33	+2	MMNATENQIFVETVSDQELEMLIGG	ADRGWIKTLTKDCPNVISSICAGTIITACKNCA ADRGWIKTLTKDCPNVISSICAGTIITACKNCA Disulfide bridge (21+29); structure predicted	Xiao *et al.,* 2004 Kabuki *et al.,* 2007
Salivaricin A1	*Streptococcus agalactiae 120*	2321	22	0	MKNSKDVLNNAIEEVSEKELMEVAGG	KKGSGWFATITDDCPNSVFVCC unknown structure	Nakagawa *et al.,* 2003
	Streptococcus pyogenes MGAS315				MKNSKDILTNAIEEVSEKELMEVAGG		
	S. pyogenes M1				MSFMKNSKDILTNAIEEVSEKELMEVAGG		
	S. pyogenes MGAS10394				MERRMSFMKNSKDILTNVIEEVSEKELMEVAGG		Banks *et al.,* 2004
Salivaricin A2	*Streptococcus salivarius*	?	22	0	MKNSKDILNNAIEEVSEKELMEVAGG	KRGTGWFATITDDCPNSVFVCC unknown structure (*lanA* identified)	Wescombe *et al.,* 2006
Salivaricin A3		?	22	0	MKNSKDVLNNAIEEVSEKELMEVAGG	KKGPGWIATITDDCPNSIFVCC unknown structure (*lanA* identified)	
Salivaricin A4		?	22	0	MKNSKDILNNAIEEVSEKELMEVAGG	KRGPGWIATITDDCPNSIFVCC unknown structure (*lanA* identified)	
Salivaricin A5		?	22	0	MKNSKDILNNAIEEVSEKELMEVAGG	KRGPGWIATITDDCPNSVFVCC unknown structure (*lanA* identified)	
Salivaricin 9	*Streptococcus salivarius strain 9*	2560	24	+2	MKSTNNQSIAEIAAVNSLQEVSMEELDQIIGA	GNGVVLTLTHECNLATWTKKLKCC structure predicted (similarity to lacticin 481)	Wescombe *et al.,* 2011

Table 1.2 – continued

Mersacidin-like lantibiotics							
Mersacidin	*Bacillus amylo-liquefaciens*	1825	20	0	MSQEAIIRSWKDPFSRENSTQNPAGNPFS ELKEAQMDKLV**GA**GDMEAA	CTFTLPGGGGVCTLTSECIC AviMeCys (15+20)	Chatterjee *et al.*, 1992
Actagardine	*Actinoplanes liguriae*	1890	19	0	MSALAIEKSWKDVDLRDGATSHPAGLGFG ELTFEDLREDRTIYAA	SSGWVCTLTIECGTVICAC Lanthionine sulphoxide (14+19)	Zimmermann *et al.*, 1995
Ala(O)-Actagardine	*Actinoplanes liguriae*	1961	20	0	MSALAIEKSWKDVDLRDGATSHPAGLGFG ELTFEDLREDRTIYA	ASSGWVCTLTIECGTVICAC Lanthionine sulphoxide (14+19)	Vertesy *et al.*, 1999
Deoxy-actagardine B (DAB)	*Actinoplanes liguriae* NCIMB 41362		19	0	MSAITVETTWKNTDLREDLTAHPAGLGFG ELSFEDLREDRTIYAA	SSGWVCTLTIECGTLVCAC Lanthionine sulphoxide (14+19)	Boakes *et al.*, 2010
Nai-802	*Actinoplanes, sp* 104802/104771	?	20	+1	?	SSGWVCTLTIECGTVICACR structure predicted based on similarity to Actagardine	Matteo, VAAM Poster, 2011
Michiganin A	*Clavibacter michiganesis*	2145	21	0	MNDILETETPVMVSPRWDMLLDAGEDTSP SVQTQIDAEFRRVVSPYM	SSSGWLCTLTIECGTIICACR predicted structure	Holtsmark *et al.*, 2006
Cinnamycin-like lantibiotics							
Cinnamycin; lantibiotic Ro 09-198	*Streptomyces cinnamoneus/ griseoverticillatus*	2042	19	1	MTASILQQSVVDADFRAALLENPAAFGAS AAALPTPVEAQDQASLDFWTKDIAATEAF A	CRQSC**S**FGPFTFVC**D**GNT**K** Lysinoalanine (19+6); Hydroxyaspartate (15)	Marki *et al.*, 1991 Fredenhagen *et al.*, 1990
Duramycin	*Streptomyces cinnamoneus*	2014	19	0	not annotated	CKQSC**S**FGPFTFVC**D**GNT**K** Lysinoalanine (19+6); Hydroxyaspartate (15)	
Duramycin B	*Strepto-verticillium* spec.	1951	19	0	not annotated	CRQSC**S**FGPLTFVC**D**GNT**K** Lysinoalanine (19+6); Hydroxyaspartate (15)	
Duramycin C	*Streptomyces griseoluteus*	2008	19	-1	not annotated	CANSC**S**YGPLTWSC**D**GNT**K** Lysinoalanine (19+6); Hydroxyaspartate (15)	
Ancovenin	*Streptomyces* spec.	1959	19	0	not annotated	CVQSC**S**FGPLTWSCDGNT**K** Lysinoalanine (19+6)	Kido *et al.*, 1983
Two-peptide lantibiotics (synergistic action)							
Lacticin 3147	*Lactococcus lactis* subsp. *lactis* DPC3147	3322	30	0	MNKNEIETQPVTWLEEVSDQNFDEDVF**GA**	CSTNTF**S**LSDYWGNNGAWCTLTHECMAWCK D-Ala (7)	Ryan *et al.*, 1996
		2847	29	+1	MKEKNMKKNDTIELQLGKYLEDDMIELAE GDESH**GG**	**T**TPA**T**PAI**S**IL**S**AYISTNTCPTTKCTRAC 2-Oxobuturyl (1); D-Ala (9;12)	
Staphylo-coccin C55	*Staphylococcus* aureus C55	3339	30	0	MKSSFLEKDIEEQVTWFEEVSEQEFDDDI F**GA**	CSTNTFSLSDYWGNKGNWCTATHECMSWCK D-Ala (7); structure predicted	Navaratna *et al.*, 1998
		2993	37	-1	MKNELGKFLEENELELGKFSESDMLEITD DEVYAA	GTPLALLGGAA**T**GVIGYISNQTCPTTACTRAC structure predicted	

Table 1.2 – continued

Plantaricin W	*Lactobacillus plantarum*	3223	29	0	MKISKIEAQARKDFFKKIDTNSNLLNVN**GA**	KCKWWNISCDLGNNGHVCTLSHECQVSCN Disulfide brigde (2 + 9); structure predicted	Holo *et al.,* 2001
		3099	32	+6	MTKTSRRKNAIANYLEPVDEKSINESF**GA**↓*GDPEAR*	SGIPCTIGAAVAASIAVCPTTKCSKRCGKRKK structure predicted	
Haloduracin	*Bacillus halodurans* C-125	3043	28	+1	MTNLLKEWKMPLERTHNNSNPAGDIFQELEDQDILAGVN**GA**	CAWYNISCRLGNKGAYCTLTVECMPSCN Disulfide bridge (1-8)	McClerren *et al.,* 2006
	Bacillus halodurans C-125	2330	24	+1	MVNSKDLRNPEFRKAQGLQFVDEVNEKELSSLA**GS**↓*GDVHAQ*	TTWPCATVGVSVALCPTTKCTSQC	McClerren *et al.,* 2006
Smb	*Streptococcus mutans* GS5	?	30	+2	MKSNLLKINNVTEMEKNMVTLIKDEDMLA**GG**	IGTTVVNSTFSIVLGNKGYICTVTVECMRNCSK predicted structure	Yonezawa *et al.,* 2005
		?	32	+2	MKEIQKAGLQEELSILMDDANNLEQLTAG	STPACAIGVVGITVAVTGISTACTSRCINK	
BHT	*Streptococcus ratti* BHT	3375	32	+1	MKEIQKAGLQEELSILMDDANNLEQLTAG	IGTTVVNSTFSIVLGNKGYICTVTVECMRNCQ structure predicted	Hyink *et al.,* 2005
		2802	30	+2	MKSNLLKINNVTEVEKDMVTLIKDEDMELA**GG**	STPACAIGVVGITVAVTGISTACTSRCINK predicted structure	
Lichenicidin	*Bacillus licheni-formis* DSM13/ VK21/ ATCC14580	3250	32	+1	MSKKEMILSWKNPMYRTESSYHPAGNILKELQEEEQHSIA**GG**	TITLSTCAILSKPLGNNGYLCTVTKECMPSCN 2-Oxobuturyl (1)	Begley *et al.,* 2009; Dischinger *et al.,* 2009; Shenkarev *et al.,* 2010, Caetano *et al.,* 2011
		3021	32	+2	MKTMKNSAAREAFKGANHPAGMVSEEELKALV**GG**↓*NDVNPE*	TTPATTSSWTCITAGVTVSASLCPTTKCTSRC 2-Oxobuturyl (1)	
Cytolysin	*Enterococcus faecalis V583*	4164	21	+1	MLNKENQENYYSNKLELVGPSFEELSLEEMEAIQ**GS**↓*GDVQAE*	TTPACFTIGLGVGALFSAKFC structure predicted (2 thioether bridges confirmed)	Booth *et al.,* 1996
		2631	38	+1	MENLSVVPSFEELSVEEMEAIQ**GS**↓*GDVQAE*	TTPVCAVAATAAASSAACGWVGGGIFTGVTVVVSLKHC unknown structure (1 thioether bridge confirmed)	
Pneumo-coccin	*Streptococcus pneumonia* R6	?	38	+1	MTNFNSNEKFCGKSLKSLSADEMSLIY**GA**SDGAEPR	WTPTPIILKSAAASSKVCISAAVSGIGGLVSYNNDCLG structure not fully elucidated (two thioether bridges confirmed of a NisBC modified chimeric peptide)	Majchrzykiewicz *et al.,* 2010
		?	30	+4	MKNDFVIGKSLKELSLEEMQLVY**GG**TDGADPR	STIICSATLSFIASYLGSAQTRCGKDNKKK structure not fully elucidated (two thioether bridges confirmed of a NisBC modified chimeric peptide)	
Enterocin W	*Enterococcus faecalis NKR-4-1*	3256	30	0	MKKEELVGMAKEDFLNVICENDNKLENS**GA**	KCPWWNLSCHLGNDGKICTYSHECTAGCNA structure predicted disulfide bridge (2; 9)	Sawa *et al.*, 2012
		2728	29	+2	MTELNKRLQLKRDVSTENSLKKISNTDETH**GG**	VTTSIPCTVMVSAAVCPTLVCSNKCGGRG structure predicted	
Class III lantibiotics / lantipeptides (lack antibiotic activity, but exhibit different functions such as morphogenetic ones)							
SapB	*Streptomyces coelicolor*	2026	21	0	MNLFDLQSMETPKEEAM**GD**VET	TGSRASLLLCGDSSLSITTCN structure predicted	Kodani *et al.,* 2004

Table 1.2 – continued

SapT	*Streptomyces tendae*	2032	21	0	not annotated	YTQGCSGLCTIVICATVVICG	Kodani *et al.*, 2005
AmfS	*Streptomyces griseus*	?	19	-1	MALLDLQAMDTPAEDSFGELRTGS	QVSLLVCEYSSLSVVLCTP unknown structure	Ueda *et al.*, 2002
Prochlorosin	*Prochlorococcus* MIT9313	-	17 32	-	in total 29 putative LanA genes with homologous leaders; modified by one LanM enzyme (experimentally shown for 6 peptides; different Lan ring structures)		Li *et al.*, 2010
Labyrinthopeptins (labionin containing peptides; LabKC modified)							
Labyrintho-peptin A1	*Actinomadura namibiensis* DSM 6313	?	20	-1	MASILELQDLEVERASSAA	DSNASVWECCSTGSWVPFTCC Labionin (2+5+9; 11+14+19); Disulfide bridge (10+20)	Müller *et al.*, 2010
Labyrintho-peptin A3	*Actinomadura namibiensis* DSM 6313	?	21	-1	MASILELQDLEVERASSAA	SNASVWECCSTGSWVPFTCC Labionin (1+4+8; 10+13+18); Disulfide bridge (9+19)	Müller *et al.*, 2010
Labyrintho-peptin A2		1923	18	-1	MASILELQNLDVEHARGENR	SDWSLWECCSTGSLFACC Labionin (1+4+8; 10+13+17); Disulfide bridge (9+18)	
Lantibiotic peptides without classification/unknown structure and biosynthesis							
Lactocin S	*Lactobacillus sake* L-45	3764	37	+1	MKTEKKVLDELSLHASAKMGARDVESSMN AD	STPVLASVAVSMELLPTASVLYSDVAGCFKYSAKHHC D-Ala (7;11;19)	Mortvedt and Nes, 1990; Skaugen and Nes, 1994
Pediocin PD-1	*Pediococcus damnosus*	2866	27	?	unknown	KKIKKSXSGDIXXLXXEXDHLAXXXXX only partially identified, no structure	Bauer *et al.*, 2005
Carnocin UI 49	*Carnobacterium pisciola*	4635	35 37	?	unknown	GSEIQPR only partially identified (N-terminus), no structure	Stoffels *et al.*, 1992
Paenibacillin	*Paenibacillus* spec.	2983	30	+4	not annotated	ASIIKTTIKVSKAVCKTLTCICTGSCSNCK predicted structure; N-Acetylalanine (1)	He *et al.*, 2007
unnamed	*Bifidobacterium longum* DJO10A	?	?	?	MSINEKSIVGESFEDLSAADMAMLTDRNDDGVAPASLSFAVSVLSVSFSACSVTVVTRLASCGNCK gene cluster identified, predicted: LanD → AviCys or AviMeCys, unknown structure		Lee *et al.*, 2008 / 2011
Venezuelin	*Streptomyces venezuelae*	?	?	?	MENHDIELLAHLHALPETDPVGVDGAPFAA TCECVGLLTLLNTVCIGISCA LanA identified, different variants *in vitro* modified, three putative cleavage sites (GA; AA; PFA) predicted, structure partially elucidated		Goto *et al.*, 2010
Lantibiotic related peptides (formerly falsely classified as lantibiotics)							
Cypemycin	*Streptomyces* spec.	2094	22	+1	MRSEMTLTSTNSAEALAAQDFANTVLSAA APGFHADCETPAM	ATPATPTVAQFVIQGSTICLVC AviCys (19+22); **Linearidin**	Komiyama *et al.*, 1993; Claesen and Bibb, 2010
Sublancin 168	*Bacillus subtilis* 168	3876	37	+3	MEKLFKEVKLEELENQKGS	GLGKAQCAALWLQCASGGTIGCGGGAVACQNYRQFCR Disulfide bridge (7+36; 14+29); Glycosylation (22); **S-linked glycopeptide**	Paik *et al.*, 1998 Oman *et al.*, 2011

Material and Methods

Microbiological methods

Bacterial strains, culture media and growth conditions

All bacterial strains are listed in table 2.1. For long time storage glycerol stocks were prepared. To this end, cells were cultivated overnight, mixed with sterile glycerol to a final concentration of 50% (v/v) and stored at −75°C. Strains were recovered from frozen samples by streaking out on appropriate solid agar plates. If not mentioned otherwise, cultures were maintained at 37°C. Fluid cultures were incubated in a shaking water bath (JULABO Labortechnik, Seelbach, Germany; 200 rpm for *Bacillus* strains, 100-160 rpm for all other strains) or on an orbital shaker (170 rpm, Infors, Bottmingen, Switzerland). For preparation of solid culture media, 14 g agar (Oxoid, Wesel, Germany) were added to 1 l of fluid medium prior autoclaving. Agar plates were cultivated in incubators (Heraeus, Langenselbold, Germany). *Bacillus* strains were grown in tryptic soy broth (TSB, Oxoid) or on tryptic soy agar (TSA, Oxoid). For lantibiotic production, producers and their mutants were cultured in synthetic two-fold *Bacillus* production medium (2xBPM; Bierbaum *et al*., 1995). For aerobic cultivation, Erlenmeyer flasks were maximally filled with 1/100 of the total volume and were closed by a silicon vent containing an ultra-fine glass microfiber filter (BugStopper™, Whatman/GE Healthcare, USA). Streptococci, enterococci and staphylococci were grown on Columbia blood agar plates (Becton and Dickinson, Heidelberg, Germany). For antimicrobial activity testing, indicator strains were maintained in Mueller Hinton (MH) broth (Difco, Detroit, USA) and on MH agar plates (Difco). *Escherichia coli* strains were cultivated in lysogeny broth (LB; Bertani, 1951).

Nostoc punctiforme cells were stored in 1 ml stocks containing 5% DMSO and were recovered by inoculation of 5 ml media with 500 µl totally thawed stock culture (Soule *et al*., 2007). In order to remove DMSO, cells were harvested (8,000g, 10 min, RT) and resuspended in 2 ml fresh medium (ATCC 619). This cell suspension was used for inoculation of 10 ml, 20 ml or 50 ml cultures which were incubated under light (16 h per day) with slow shaking (80 rpm) at 25°C for up to two weeks.

For genetically manipulated strains, antibiotics were added to selection media in the following concentrations: ampicillin [40 µg/ml; AMP]; erythromycin [25 µg/ml; EM]; chloramphenicol [20 µg/ml; CM] and kanamycin [20 µg/ml; KANA].

Table 2.1: Bacterial strains used in this study.

Strain/DNA	Relevant characteristics	Source/references
Microbial strains involved in lantibiotic production		
Bacillus licheniformis DSM 13	lichenicidin producer	DSMZ
B. licheniformis MW3	transformable variant of DSM 13	Waschkau *et al*., 2008
B. licheniformis MW3 LicM1INT	*licM1* insertion mutant	this study
B. licheniformis MW3 LicM2INT	*licM2* insertion mutant	this study
Bacillus sp. HIL Y-85,54728	mersacidin producer	Sanofi-Aventis, Frankfurt, Germany
Bacillus amyloliquefaciens FZB42	harbors the 5' part of the mersacidin gene cluster (*mrsKR2FGE*)	Prof. Borriss, Berlin, Germany
B. amyloliquefaciens mrs1	mutant carrying the mersacidin gene cluster; *mrsA* is replaced by *ermB (mrsKR2FGEermBR1DMT)*	A.M. Herzner, 2008
B. amyloliquefaciens mrs1 + pOPAR1	mersacidin producing mutant: mrs1 strain harboring the *in trans* (pOPAR1) completed mersacidin gene cluster	this study
B. amyloliquefaciens mrs1 + pPAR1/1	mersacidin producing mutant: mrs1 mutant harboring the *in trans* (pPAR1/1) completed mersacidin gene cluster	this study
Nostoc punctiforme ATCC 29133	putative lantibiotic producer: predicted gene cluster of puncticin	ATCC
Caldicellulosiruptor bescii DSM 6725	putative lantibitiotic producer: predicted gene cluster of besciin	genomic DNA (DSMZ)
Intermediate cloning hosts		
E. coli SCS110	intermediate cloning host for plasmids previous to transformation into *Bacillus*; *dcm*$^-$ and *dam*$^-$	Yanisch-Perron *et al*., 1985
E. coli JM109 / *E. coli* JM83	intermediate cloning host for recombinant plasmid construction	Yanisch-Perron *et al*., 1985
Strains involved in heterologous expression of lantibiotic biosynthesis genes		
E. coli Bl21	strain for recombinant protein expression; λDE3-lysogen, T7 RNA polymerase under control of the l*acUV* promotor	Studier and Moffatt, 1986
E. coli C43/C41	Bl21(DE3) derivatives for expression of toxic proteins	Miroux and Walker, 1996

For antimicrobial susceptibility testing the following indicator strains were used: *Bacillus cereus* DSM 31 (DSMZ), *B. halodurans* DSM 18197 (producer of the two-peptide lantibiotic haloduracin; DSMZ), *B. megaterium* ATCC 13632 (KM; ATCC), *B. subtilis* 168 (DSM 402; DSMZ), *Enterococcus faecium* BM 4147–1 (Leclercq *et al*., 1988), *E. faecium* L4001 (clinical isolate), *Lactobacillus sake* 790 E2 (laboratory stock), *Lactococcus lactis* NCTC 497 (NCTC), *Micrococcus luteus* DSM 1790 (lantibiotic susceptible control strain; DSMZ), *M. luteus* ATCC

4698 (lantibiotic susceptible control strain; ATCC), *Staphylococcus aureus* ATCC 33592 (MRSA; ATCC), *S. aureus* ATCC 29213 (MSSA; ATCC), *S. aureus* 1450/94 (northern German epidemic strain; NCS), *S. aureus* Cowan ATCC 12598 (ATCC), *S. aureus* Newman NCTC 8178 (NCTC), *S.* aureus SG511 (lantibiotic susceptible control strain; Sass and Bierbaum, 2008), *S. aureus* Wood 46 (ATCC 10832; ATCC), *Staphylococcus carnosus* TM300 (Rosenstein *et al*., 2009), *Staphylococcus gallinarum* Tü 3928 (producer of the lantibiotic gallidermin; Kellner *et al*., 1988), *Staphylococcus saprophyticus* DSM 20229 (DSMZ), *Staphylococcus simulans* 22 (Bierbaum and Sahl, 1987), *S. aureus* LT440/09 (community acquired MRSA; clinical isolate), *S. aureus* LT420/09 (MRSA; clinical isolate), *S. aureus* LT819/09 (MRSA; Rhine-Hessen epidemic strain; clinical isolate), *Enterococcus faecalis* BM4147_1 (clinical isolate) and *Streptococcus pyogenes* O-19310 (clinical isolate).

Chemicals, antibiotics and solvents

If not mentioned otherwise, all chemicals and solvents used in this work were of analytical grade and were obtained from the following suppliers: AppliChem (Darmstadt, Germany), Sigma-Aldrich (Taufkirchen, Germany) or Merck (Darmstadt, Germany). For Maldi-TOF analysis and HPLC purification MilliQ water (MilliQ Biocel System A10; Millipore GmbH, Schwalbach, Germany) was used for preparation of solutions and sample dilutions.

For commercially used antibiotics, 100 mg/ml stock solutions were prepared and stored at -20°C. Ampicillin and kanamycin were dissolved in sterile MilliQ water whereas chloramphenicol and erythromycin were solved in ethanol (99%). Mersacidin was kindly provided by Sanofi-Aventis and stock solutions (8 mg/ml) were prepared in 80% ethanol. Nisin and Pep5 stocks (AG Sahl, University Bonn) of 8 mg/ml were generated in 0.1 M HCl or MilliQ water, respectively.

Sterilization of media, equipment and bacterial cultures

Plastic labware, media and chemical solutions were sterilized by autoclaving at 121°C for 20 min (Varioklav 75S, H+P Labortechnik AG, Oberschleißheim, Germany). Solutions that contained heat instable substances were sterilized by filtration using sterile syringe filters with 0.2 or 0.4 µm pore size (Supor®Membrane; Pall Life Science Corporation, Ann Arbor, USA). Glass and metallic labware were heat sterilized for 4 h at 200°C (Kelvitron®, Heraeus). Bacterial cultures and contaminated labware were autoclaved at 134°C for 30 min.

Determination of the optical density of a bacterial culture

The optical density of bacterial cultures was determined at a wavelength of 600 nm (OD_{600}) using an UV-160 spectral photometer (Shimadzu, Duisburg, Germany). Cultures with an optical density higher than 0.3 were diluted in appropriate media. OD 1 was assumed to reflect a cell density of $2x10^8$ cells/ml for *Bacillus* strains and a cell density of $2x10^9$ for *S. aureus* and *M. luteus* (Heike Brötz, phD thesis, 1998).

Methods in Molecular Genetics

Purification of nucleic acids

Genomic DNA of *Bacillus* strains was prepared using the PrestoSpinD Bug Kit according to the recommendations of the supplier (Molzym, Bremen, Germany) with following exceptions: 1. for lysis, cells were incubated at 37°C for 30 min in buffer 1 that had been supplemented with 10 µl lysozyme (100 mg/ml); 2. DNA was eluted in 100 µl pre-heated (70°C) MilliQ water and was stored at -20°C.

Chromosomal DNA of *N. punctiforme* was isolated using the GENOMICTip20 Kit (Qiagen, Hilden, Germany). Cells were grown for 2-3 weeks and harvested by centrifugation (8,000 g, RT, 10 min). In order to remove cell surface polysaccharides that might block cell lysis, cells were washed in 1/10 of total culture volume with 5 M NaCl, 1 M sodium iodate and MilliQ water. Each washing step was repeated twice. Cells were aliquoted (250 µl), lyophilized and stored at -20°C until further processing. Cell pellets were thawed on ice and pestled using a metal eppi pistil. For cell lysis, cells were resuspended in 1 ml buffer 1 containing 200 mg/ml lysozyme and were incubated at 37°C for 2 hours. Genomic DNA was further purified following the manufacturer's instructions.

Plasmids were isolated using the Gene-Jet™ Plasmid Miniprep Kit (Fermentas) according to the protocol of the supplier. Cell lysis of *Bacillus* strains was performed by adding 10 µl lysozyme [100 mg/ml] to the resuspension buffer and incubation at 37°C for 30 min. Plasmid DNA was eluted in 40 µl MilliQ water. For elution of plasmids larger than 5 kb, MilliQ water was pre-heated up to 80°C. All plasmids used in this study are listed in table 2.2. Quality and quantity of nucleic acids were analyzed by agarose gel electrophoresis and spectrophotometry.

Overview over plasmids used in this study

All plasmids used in this study are listed in table 2.2. Gene inactivation in *Bacillus* strains was performed by plasmid integration and homologous recombination using recombinant derivatives of the thermo-sensitive shuttle vector pMAD (Arnaud *et al*., 2004; figure 2.1). Recombinant vectors that contain up to 500 bp of the N- and C-terminal sequences of target genes were introduced into the corresponding restrictions sites of the *mcs*. Resulting vectors were introduced in *E. coli* SCS110 as intermediate cloning host and selected on LB agar plates containing ampicillin at 37°C. Finally, these vectors were transformed into *Bacillus* strains and selected on TSA agar plates containing the selection marker erythromycin (5 mg/l) at 30°C. Homologous recombination and insertion of the vectors into target genes was performed by overnight cultivation at the non- permissive temperature (45°C) in TSB (25 mg/l EM) and, subsequent, plating onto TSA agar plates (25 mg/l EM).

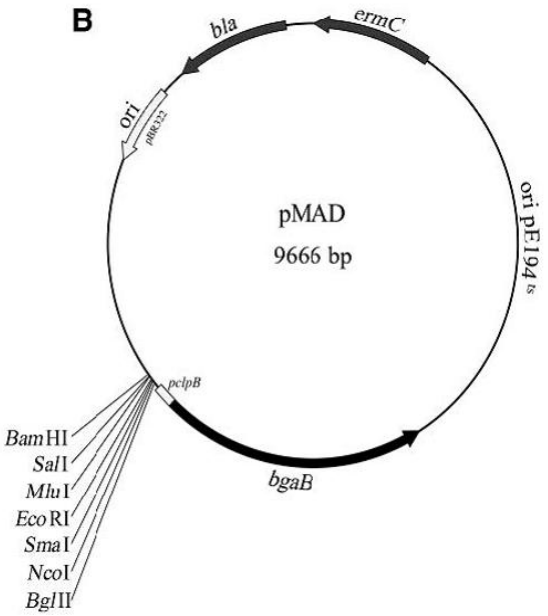

Figure 2.1: Physical map of pMAD (Arnaud *et al*., 2004). The shuttle vector pMAD contains a thermo-sensitive origin of replication and confers resistance to ampicillin (*bla*) and erythromycin (*ermC*). Blue-white screening of recombinants is facilitated by a constitutively expressed β-galactosidase (*bgaB*).

Vectors of the pET expression system (Merck-Novagen, Darmstadt, Germany) were employed for heterologous expression of recombinant, HIS-tagged proteins in *E. coli.* In this system, proteins of interest are expressed under control of a strong bacteriophage T7lac promoter and are repressed by the LacI repressor protein. Expression hosts are lysogenic for

bacteriophage DE3 and, therefore, carry a chromosomal copy of the T7 RNA polymerase gene under control of a *lacUV5* promoter, which is also repressed by LacI. By addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to bacterial cultures, LacI is inactivated which results in the expression of T7 polymerases and, consequently, in expression of target genes. In pET22b, the coding sequence of six histidine residues is located downstream of the multiple cloning site (mcs) and enables a C-terminal fusion of the HIS-TAG to target proteins (figure 2.2). pET28b harbors two putative HIS-TAG sequences for expression of C- or N-terminally HIS-tagged proteins (figure 2.2).

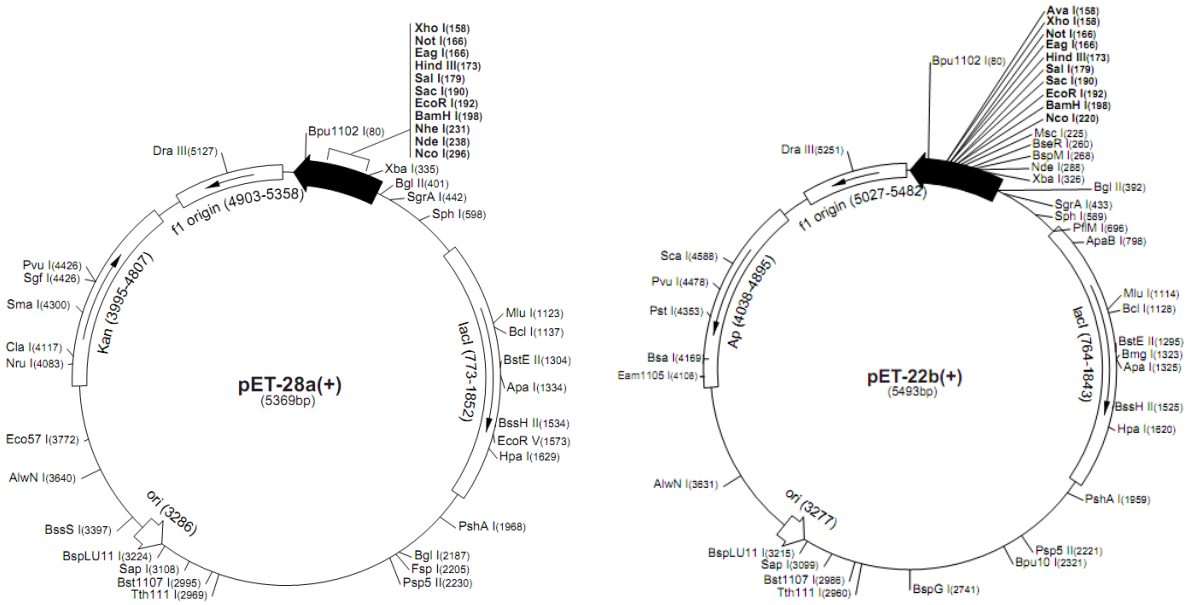

Figure 2.2: Physical map of expression vectors pET22b and pET28a (Novagen/Merck chemicals). Plasmids confer resistance against ampicillin (Ap) or kanamycin (Kan). HIS-TAG coding sequences are located next to the mcs and allow cloning of N- or C-terminal HIS-tagged proteins.

Photometric determination of nucleic acid concentration and purity

UV-light based photometric determination of nucleic acid concentrations was performed using a Nanodrop spectrometer (Nanodrop Technologies, Wilmington, USA) at 260 nm. Purity was determined by the quotient of extinction at 260 nm over 280 nm. A quotient below 1.8 indicated contamination with proteins.

Table 2.2: Plasmids used in this study.

Plasmid	Information	Aim	Source
pMAD	shuttle vector harboring a temperature sensitive ORI, AMP^r, EM^r	homologous recombination in *Bacillus*	Arnaud *et al.*, 2006
pMADLicM1AC	pMAD derivative carrying an approx. 500 bp C- and N-terminal fragment of *bli4126*	knock out of LicM1	this study
pMADLicM1AC	pMAD derivative carrying an approx. 500 bp C- and N-terminal fragment of *bli4128*	knock out of LicM2	this study
pET22b	HIS-TAG expression vector, AMP^r	heterologous expression of C-terminally HIS-tagged proteins in *E. coli*	Novagen
pET22PunM	pET22 derivative harboring the C-terminally HIS-tagged *punM*	heterologous expression of PunM	this study
pET22AnaM	pET22 derivative harboring the C-terminally HIS-tagged *anaM*	heterologous expression of AnaM	this study
pET28b	HIS-TAG expression vector, $KANA^r$	heterologous expression of N-terminally HIS-tagged proteins in *E. coli*	Novagen
pET28PunA1	pET22 derivative harboring the N-terminally HIS-tagged *punA1*	heterologous expression of the PunA1 prepeptide	this study
pET28PunA1_XA	pET22 derivative harboring the N-terminally HIS-tagged *punA1* including a XA-factor cleavage site	heterologous expression of the PunA1 prepeptide	this study
pET28AnaA	pET22 derivative harboring the N-terminally HIS-tagged gene *anaA*	heterologous expression of the AnaA prepeptide	this study
pET28AnaA_XA	pET22 derivative harboring the N-terminally HIS-tagged besciin including XA-factor cleavage site	heterologous expression of the AnaA prepeptide	this study
pUC19	shuttle vector; AMP^r, CM^r	intermediate cloning vector for lantibiotic precursors	Yanisch-Perron *et al.*, 1985
pUC19AnaA	pUC19 derivative harboring the structural gene *anaA*	templates for site directed mutagenesis of protease cleavage sites	this study
pUC19PunA1	pUC19 derivative harboring the structural gene *punA1*		this study
pOPAR1	pCU1 derivative harboring *mrsR1* and *mrsA* including its operator structure	expression of the lantibiotic mersacidin in *B. amyloliquefaciens*	Schmitz *et al.*, 2006
pPAR1/1	pCU1 derivative harboring *mrsR1* and *mrsA*		

DNA sequencing

DNA sequencing was performed by Sequiserve (Vaterstetten, Germany) or Seqlab (Göttingen, Germany) employing the Sanger chain determination method (Sanger *et al*., 1977). For sequencing, recombinant plasmids and PCR products were dissolved in sterile MilliQ water. Integrity of recombinant plasmids was confirmed by sequencing of inserts with vector-specific primers supplied by Sequiserve, if available.

Agarose gel electrophoresis

Standard agarose gel electrophoresis was used to analyze and separate DNA fragments employing 0.8-2% agarose gels (Top Vision™ LE GQ agarose, Fermentas) in a horizontal electrophoresis apparatus (Peqlab, Erlangen, Germany) according to Sambrook *et al.*, 1989. DNA was stained with ethidium-bromide and visualized using ImageMaster VDS (GE Healthcare/Pharmacia).

Polymerase chain reaction (PCR)

Amplification of DNA was performed by the polymerase chain reaction in accordance with Mullis *et al.*, 1986. DreamTaq Polymerase (Fermentas) was used for standard PCR batches. For cloning or subsequent sequencing DNA was amplified using Phusion™HF DNA Polymerase (NEB, Frankfurt/Main, Germany) as detailed in the manual. Desoxynucleotides were ordered as premixed solutions (25 mM each; Fermentas). PCRs were performed in 50 µl batches on the PCR express Thermal Cycler (Hybaid, ThermoLifeScience, Engelsbach, Germany) or the SensoQuest Labcycler (SensoQuest, Göttingen, Germany).

All primers were synthesized by Metabion (Martinsried, Germany) or Microsynth (Balgach, Switzerland) and are listed in table 2.3. Sequencing and standard cloning primers were designed using the Primer3 tool: http://biotools.umassmed.edu/bioapps/primer3_www.cgi.

Enzymatic modification of DNA

Plasmids and PCR products were digested using restriction enzymes (FastDigest™) from Fermentas. Digestion was performed as detailed in the manual. For cloning of large inserts (> 2000 bp), vector backbones were dephosphorylated using alkaline dephosphatase (Fermentas) during digestion. After heat inactivation (10 min, 65°C) digested nucleic acids were stored at -20°C until further use.

Purification of DNA fragments and gel extraction from agarose gels

PCR fragments were purified using the GeneJet™ PCR Purification Kit (Fermentas). For gel extraction, bands of interests were cut from a 0.6% agarose gel with a scalpel and DNA extraction was performed with the GeneJet™ Gel Extraction Kit (Fermentas). Both kits were used according to the manufacturer's instructions with following changes: for cloning DNA was eluted in 35 µl pre-heated (50°C) MilliQ water.

DNA ligation

Recombinant vectors were ligated either for 1 h using T4 DNA Ligase (5u/µl) from Fermentas (20 µl total volume) or overnight using T4 DNA Ligase (10 u/µl) from Roche (10 µl total batch volume). For sticky end ligations a molar ratio of 3:1 (insert to vector) was calculated. For ligation of large inserts, a ratio of 5:1 was chosen and an additional incubation step was performed prior ligation: insert and vector fragments were mixed and subsequently incubated for up to 10 min at 45°C. For blunt end ligation a molar ratio of 1:2 was used and PEG (poly-ethylen-glycol, NEB) was supplemented. Ligations were performed according to the protocol of the manufactures. Ligation batches were used in transformation experiments without prior storage.

Site directed mutagenesis

For introduction of single point mutations, standard cloning PCRs were performed using primers containing the desired base exchange.

In order to introduce up to seven point mutations, inserts of interest were cloned into pUC19. The resulting recombinant vectors were used as templates for a PCR based site directed mutagenesis employing the QuickChange™ Site-Directed-Mutagenesis Kit (Agilent technologies, Waldbronn, Germany) and two primers, comprising the desired mutations.

Both mutagenic primers, each complementary to opposite strands of the vector, were extended employing *Pfu*Ultra HF DNA polymerase (Agilent) without primer displacement. Parental plasmids, that lacked the desired mutations, were digested by *Dpn*I which is specific for (hemi)methylated DNA. Subsequently, batches were transformed into super-competent *E. coli* XL-1 Blue cells (Agilent).

Mutagenesis was performed following the suppliers' instructions. Primers containing the desired exchanges were designed by the following tool: http://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Tool&SubPageType=ToolQCPD&PageID=2296.

Table 2.3: Oligonucleotides used in this study.

Name of the primer	Template	Restriction sites	Sequence (5' - 3')	TM
B. licheniformis				
forΔLicM1FragmentA	*licM1:* bp 4-486	*Bam*H1	GATGGATCCAATGAAAAATCCGCCG	60
revΔLicM1FragmentA		*Mlu*1	TCGACGCGTTCGCCCTTCAATTGATCTTC	60
forΔLicM1FragmentC	*licM1:* bp 2636-3156	*Mlu*1	TTCACGCGTCCTGGCATTTGAAAAGCAGC	62
revΔLicM1FragmentC		*Bgl*II	TTGAGATCTAAACACGTTTTCTCTTTTAAAAGC	62
forDelLicM2A	*licM2:* bp 4-488	*Mlu*1	ATTACGCGTTGTGATAAGTGTTCTCGTCGC	62
revDelLicM2A		*Nco*1	GCACCATGGGTTTTCTTCGCCAAAGGGATG	62
forDelLicM2C	*licM2:* bp 2596-3108	*Bam*H1	ATCGGATCCGCCCGTCGGAATATCGAGA	62
revDelLicM2C		*Mlu*1	CGGACGCGTTGATACACGATGACAGCTGC	62
forhomRecLicM1	*B. licheniformis:* bp 3949702-3949722	-	TGAATCTTCTTATCATCCAGC	58
revhomRecLicM2	*B. licheniformis:* bp 3953148-3953168	-	GCATTTGGATGAAGGTCTTTC	60
pMADmcs1	pMAD: sequencing	-	CTAGCTAATGTTACGTTACAC	58
pMADmcs2neu			GAAGCGAGAAGAATCATAATG	58
Heterologous expression of lantibiotic biosynthesis genes				
forAnaApuc19	*C. bescii:*	*Hind*III	GACAAGCTTGTGCGCACAAAGGGTTATG	62
revAnaApuc19	bp 1199426-1199805	*Eco*R1	CACCTTAAGTGATACGGCGAGAAGACTCG	62
AnaA_XA_Muta_sense	pUC19AnaA	-	AAAGAAATTCAAGAGCAGAATTTGCCAGAAATAGAAGGTA GAGGTACTCCAACTGTAGTTGTTGGTGTG	60
AnaA_XA_Muta_antisense		-	CACACCAACAACTACAGTTGGAGTACCTCTACCTTCTATTTC TGGCAAATTCTGCTCTTGAATTTCTTT	60
forAnaApET28	pUC19AnaA	*Nde*1	ATACATATGAAAGAGTCTACTATCATTAAAAATC	62
revAnaApET28	pUC19AnaA_XA	*Xho*1	AAA CTCGAG TTACCTCATTCTGCAGCTTCTA	62
forAtheMHISpET22	*anaM*	*Nde*1	GAACATATGATGAAACAAGATAATAATTGGCT	62
revAtheMHISpET22		*Xho*1	TTTCTCGAGTTCCAAACATAATATTTCTGGC	62
revAnaM1EcoR1		*Eco*R1	TTGAGAATTCTTAAAACCTCTGT	62
forAnaM1EcoR1		*Eco*R1	TAAGAATTCTCAACAACTCAGTT	62

Table 2.3 - continued

Name of the primer	Template	Restriction sites	Sequence (5' - 3')	TM
SeqpET22AnaMAB1	internal *anaM* primer for sequencing pET22AnaM: bp 1908-1929	-	TTCCCTTTCATTATCGGATTG	59
SeqpET22AnaMAB2	internal *anaM* primer for sequencing pET22AnaM: bp 943-962	-	TCCCAATTTTGCCATAAACA	60
forPunA1pUC19	*N. punctiforme:*	*Eco*R1	AGAGAATTCAACACCGCACAAAACAAGTG	60
revPunA1pUC19	bp 6250388-6250756	*Hind*III	TGGAAGCTTCCTGGCTCGTGCTTCTCTAA	60
PunA1_XA_Muta_sense	pUC19PunA	-	AAAGAAATTCAAGAGCAGAATTTGCCAGAAATAGAAGGT AGAGGTACTCCAACTGTAGTTGTTGGTGTG	60
PunA1_XA_Muta_antisense		-	CACACCAACAACTACAGTTGGAGTACCTCTACCTTCTATTT CTGGCAAATTCTGCTCTTGAATTTCTTT	60
forPunA1pET28	pUC19PunA1	*Nde*1	AGACATATGTTACACCAAATCAAAGAATTA	64
revPunA1pET28	pUC19PunA1_XA	*Xho*1	CTACTCGAGTTATCTCGTTGGACAGTCG	64
forpET22bHISPunM	*punM*	*Nde*1	TCACATATGTCTCAACTATTTGTTCAATC	62
revPunMBamHIMut		*Bam*H1	ATCAGGATCCAGAATCGCTGTC	62
forPunMBamHIMut		*Bam*H1	TTCTGGATCCTGATGGTAGTATC	62
revpET22bHISPunM		*Xho*1	TTGCCTCGAGTTCCCATAACAGCACCGA	62
seqPunM1	internal *punM* primer for sequencing pET22PunM	-	CTGAGGAGCTTGCTGAGG	58
***B. amyloliquefaciens* FZB42**				
GyrA_F	*gyrA*: 41-1066 bp	-	CAGTCAGGAAATGCGTACGTCCTT	58
GyrA_R		-	CAAGGTAATGCTCCAGGCATTGCT	58

Transformation of *Escherichia coli* strains

Preparation of electro-competent *E. coli* strains (Dower *et al*., 1988)

For generation of electro-competent *E. coli* strains, a main culture of 500 ml LB, inoculated with 500 µl of an overnight grown pre-culture, was grown to an optical density of 0.5. After incubation on ice for 30 min, cells were harvested by centrifugation (5,000g, 4°C, 10 min) followed by three washing steps with 500 ml and 250 ml pre-chilled MilliQ water and with 20 ml pre-chilled 10% glycerol solution. Finally, cells were resuspended in 0.8 ml glycerol (10%) and frozen for long time storage at -75°C in 50 µl aliquots.

Transformation of electro-competent *E. coli* strains

Frozen cells were thawed on ice, immediately mixed with 2 µl of ligation batches or 100 ng plasmid DNA, followed by electroporation employing a pre-chilled electro cuvette (1 mm) in a Biorad gene pulser (program EC2, Biorad, Munich, Germany). Cells were resuspended in 1 ml LB and, for regeneration, incubated under shaking at 37°C for up to 1 h. Finally, transformants were plated onto appropriate selection media (undiluted, 1:10, 1:100 diluted and concentrated samples).

Preparation of chemo-competent ($CaCl_2$) *E. coli* strains

For preparation of super-competent cells (Morrison, 1977), *E. coli* strains were freshly streaked out onto LB agar plates. Cultures (50 ml TYM media) were inoculated with 100 µl of an overnight grown LB pre-culture and cells were aerobically grown to a cell density of 0.6. Cells were harvested (5,000 g, 4°C, 5 min, Acc. 9, Br. 7) and the resulting cell pellets were resuspended in 5 ml of pre-chilled and filter-sterilized TFBI. After centrifugation cells were resuspended in 2.5 ml pre-chilled TFBII medium and aliquoted into 100 µl samples. Cells were shock-frozen in liquid nitrogen and stored at -75°C until further use.

Table 2.4. Media for preparation of chemo-competent *E. coli* strains.

Medium	Volume	Chemical ingredients
TYM broth	50 ml	1 g Bacto-Trypton (Difco); 0.25 g yeast extract (Difco); 292 mg NaCl; 123 mg $MgSO_4$
TFBI	50 ml	5 M potassium acetat (0.3 ml); $MnCl_2$ (495 mg); 2 M KCl (2.5 ml); 1 M $CaCl_2$ (0.5 ml) and glycerol (7.5 ml)
TFBII	50 ml	2 M MOPS pH7 adjusted with 5 M KOH (2.5 ml); 2 M KCl (0.25 ml); 1 M $CaCl_2$ (3.7 ml) and glycerol (7.5 ml)

Transformation of chemo-competent ($CaCl_2$) *E. coli* strains (Morrison, 1977)

Frozen chemo-competent *E. coli* cells were thawed on ice immediately before use. 50 µl cell suspension were mixed with DNA (10-20 µl of a ligation batch or 100-200 ng plasmid DNA) and incubated on ice for 30 min. For transformation, cells were exposed to a heat shock treatment at 42°C for 30 seconds followed incubation on ice for 3 min. Transformed cells were resuspended in 500 µl pre-warmed LB. After regeneration for 45-60 min at 37°C with shaking, 100 µl of cell suspension were plated onto appropriate agar plates in 10- and 100-fold dilutions, and as undiluted and concentrated samples.

Transformation of *Bacillus* strains

Natural competence transformation of *B. amyloliquefaciens* FZB42 and its mutants

B. amyloliquefaciens FZB42 is a naturally competent *Bacillus* strain. It harbors a competence mediating signal cascade system consisting of kinases, transporters and transcription factors that allow uptake of heterogeneous DNA fragments resulting in integration of these fragments into its chromosomal DNA by homologous recombination.

In order to generate genetically manipulated *B. amyloliquefaciens* strains, a modified protocol according to Anagnostopoulos and Spizizen (1961) was employed. Cells were aerobically grown at 37°C in 20 ml HS medium to an OD_{600} of 1.5 to 2. Cells were harvested (5,000 g, 5 min, RT) and resuspended in 20 ml LS medium. After 45 min incubation, EDTA was added followed by an additional incubation for 45 min. In successful transformation batches, 1 ml culture was mixed with 8 µg of linearized plasmid DNA followed by incubation for additional 2 h. 300 µl transformants were plated onto appropriate selective agar plates.

Table 2.5: Solutions and media for competence transformation of *Bacillus* strains.

Solution	Volume	Chemical ingredients
10fold-S-Base	100 ml	2.0 g $(NH_4)_2SO_4$; 14.0 g K_2HPO_4; 6.0 g KH_2PO_4; 1.0 g NaCitrate x 2 H_20; ad 100 ml *aqua dest*.; after autoclaving 1 M $MgSO_4$ (0.1 ml) was added.
LS medium (low sulfate)	20 ml	sterile *aqua dest*. (16.5 ml); 10fold-S-Base (2.0 ml); 20% glucose (0.5 ml); 1 mg/ml L-Trp (0.1 ml); 1% caseine hydrolysate (0.1 ml); 0.5 M spermine (0.2 ml); 0.1 M $MgCl_2$ (0.5 ml); 0.1 M $CaCl_2$ (0.1 ml)
HS medium (high sulfate)	20 ml	sterile *aqua dest*. (13.4 ml); 10fold S-Base (2 ml); 20% glucose (0.5 ml); 1 mg/ml L-Trp (1 ml); 1% caseine hydrolysate (0.2 ml); 2% yeast extract (1 ml); 8% L-Arg 0.4% L-His (2 ml)

Protoplast transformation of natural competence deficient *B. licheniformis* DSM 13

B. licheniformis DSM 13 harbors an insertion of an IS element in the competence gene *comP* and therefore this organism cannot be transformed by natural competence transformation. Transformation was performed according to the protoplast transformation protocol published by Waschkau *et al.* (2008) and use of the transformable mutant *Bacillus licheniformis* MW3.

Moleculargenetic bioinformatic tools and accession numbers

For bioinformatic analysis of DNA sequences, the tools listed below were used. Accession numbers of genomes and genes (NBCI data base) that were employed in sequence alignments are shown in table 2.6.

Blasts were performed at the NCBI nucleotide website:

- http://blast.ncbi.nlm.nih.gov/Blast.cgi

Multi sequence alignments were done by Clustal W2; LALIGN or the PC tool DNAman:

- http://www.ebi.ac.uk/Tools/msa/clustalw2/
- http://www.ch.embnet.org/software/LALIGN_form.html

Phylogenetic trees were calculated employing:

- http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::clustalw-multialign

Bacillus transcription factor binding motifs were predicted by the DBTBS database:

- http://dbtbs.hgc.jp/

Restriction analyses were performed using the NEBcutter:

- http://tools.neb.com/NEBcutter2/index.php

GC content of DNA sequences was determined with the GEMS launcher (Genomatix):

- http://www.genomatix.de/cgi-bin/tools/tools.pl

A subcellular localization of proteins was predicted using the online tool CELLO v.2.5:

- http://cello.life.nctu.edu.tw/

Vector cards were created by the use of the PlasMapper 2.0:

- http://wishart.biology.ualberta.ca/PlasMapper/index.html

Table 2.6: Accession numbers of genomes and genes.

Strain/DNA	Accession No. (NCBI data base; GeneBank)
B. amyloliquefaciens FZB42	CP000560
B. licheniformis DSM 13	AE017333
B. licheniformis ATCC 14580	CP000002.3
N. punctiforme ATCC 29133	CP001037.1
C. bescii DSM 6725	CP001393
gyrA	JF519633
mersacidin gene cluster	AJ250862

Protein- and biochemical methods

Preparation of lantibiotic peptides

(a) Production of lichenicidin

For production of lantibiotics *B. licheniformis* DSM 13 and MW3 were cultured in 50 ml 2×BPM at 37°C for up to 48 h. Production experiments with the *B. licheniformis* MW3 insertion mutants LicM1INT and LicM2INT were performed at 45°C. After 48 h of incubation, the cells were pelleted by centrifugation at 10,000 g and 4°C for 30 min. For further analysis culture supernatant was sterilized by filtration and stored at −20°C. Cell pellets were immediately washed with 35 ml 70% isopropanol (adjusted to pH2 with HCl) and incubated at 4°C for 4 h. Cells were removed by centrifugation and the supernatant was sterilized by filtration. For the following analysis of the cell wash extract, isopropanol was removed by rotary evaporation (Rotavapor RE11, Essen, Germany) and the resulting protein solution was stored at −20°C. Antimicrobial activity was determined in agar well diffusion assays against different indicator strains, mainly against *M. luteus*.

(b) Production of mersacidin by *B. amyloliquefaciens*

Production of mersacidin by *B. amyloliquefaciens* mrs1 harboring pPAR1/1 or pOPAR1 was assayed in 50 ml synthetic medium (2x BPM). Cells were grown for 24 h at 37°C with agitation (180 rpm). For further analyses, culture supernatants were sterilized by filtration and stored at -20°C. Detection of an antimicrobial activity was performed by agar well diffusion assays against *M. luteus*, *S. aureus* SG511 und *B. megaterium* using 50 µl culture supernatant.

Table 2.7: Preparation of 2xBPM (Bierbaum *et al*., 1995).

Medium	Chemical ingredients
Wolfe's mineral salts	Titriplex I (500 mg/l); ZnSO4·7 H_2O (180 mg/l); $CuSO_4$·5 H_2O (50 mg/l); $CoCl_2$·6 H_2O (170 mg/l); $CaCl_2$·2 H_2O (130 mg/l); $NaMoO_4$·1 H_20 (11 mg/l); $Al_2(SO_4)_3$ (26 mg/l); H_3BO_3 (10 mg/l); 1 M NaOH (5 ml/l)
Mineral salts	10 mM $CaCl_2$; 2 mM $FeSo_4$; 1 mM $MnSO_4$
2xBPM	0.25 M $(NH_4)_2SO_4$ (20 ml); 0.1 M $MgSO_4$ (2 ml); 2 M K-P-buffer pH 7.0 (2 ml); 0.5 M Tris-maleate pH 7.0 (20 ml); Wolfe's mineral salts (2 ml); *aqua dest*. (27.5 ml); after autoclaving 2.5 M glucose (16 ml); mineral salts (10 ml) were added to a total volume of 100 ml.

HPLC purification of lantibiotics

(a) Purification of lichenicidin

2 ml cell wash extracts containing 0.1% TFA were applied to a POROS II RP-HPLC-column (10R2, 100×4.6 mm, Perseptive Biosystems, Freiburg, Germany) and eluted in a gradient of 20-55% acetonitrile or isopropanol (containing 0.1% TFA). Peaks were detected by measuring absorbance at 210, 220 and 266 nm. Fractions were collected, assayed for their antimicrobial activity against the indicator strain*M. luteus* in agar well diffusion assays and analyzed by MALDI-TOF spectrometry.

(b) Purification of mersacidin

For purification of mersacidin produced by *B. amyloliquefaciens* FZB42 mutant strains, 5 ml culture supernatants containing 0.1% TFA were applied to a POROS II RP-HPLC-column and eluted in a gradient of 30-42% acetonitrile (containing 0.1% TFA). Peaks were detected measuring absorbance at 210 or 220 and 266 nm. Fractions were collected and assayed for an antimicrobial activity against *M. luteus* in agar well diffusion assays. All active fractions were combined and lyophilized. Resulting lyophilisates were resuspended in 15 ml 5% acetonitrile supplemented with 0.1% TFA and further purified using a RP C-18 column (Nucleosil-100-C18, 250 x 4.5 mm; Schambeck SFD GmbH, Bad Honnef, Germany). Active fractions were eluted in a gradient of 50-65% acetonitrile (containing 0.1% TFA).

Antimicrobial susceptibility testing

(a) Determination of the minimal inhibitory concentration (MIC)

Minimal inhibitory concentrations (MIC) of antimicrobial substances were determined by the geometric broth microdilution method. Serial two-fold dilutions of antimicrobials were prepared in 96 well polystyrene round bottom microtiter plates (Greiner, Frickenhausen, Germany) using half-concentrated MH broth. For *M. luteus,* MIC determinations were performed in fully concentrated MH broth. Cells were grown to an OD_{600} of 1. An inoculum of 5×10^5 CFU/ml was employed in a final volume of 200 µl. For MIC determination of antimicrobials that were further assayed in cell lysis experiments, an inoculum of 10^7 CFU/ml was used. Prior to incubation, the inoculated plates were shaken at RT for 10 min (4,000 rpm; Titertek Flow Laboratories,). MICs were calculated from the lowest concentration resulting in the complete inhibition of visible bacterial growth after 16 h and 24 h of incubation. MIC determinations were performed three times. For antimicrobial activity testing of lantibiotics, $CaCl_2$ was added to a final concentration of 1 mM.

(b) Agar well diffusion assay

Antimicrobial activity of culture supernatants, cell wash extracts and HPLC fractions was determined by the agar well diffusion method. One colony of the indicator strain was resuspended in 5 ml MH medium. 1 ml of this diluted cell suspension was used for inoculation of a second 5 ml MH culture and this dilution was used for seeding MH agar plates with indicator strains. Therefore, the surface of agar plates was overlaid with cell suspensions that immediately were removed by pipetting followed by incubation for 15 min at RT in order to air-dry the plates. Wells (diameter of 7 mm) were introduced into the surface using a sterile cork borer. 50 µl of each sample were added to a well, unless indicated otherwise, and dried for 15 min at RT. After incubation at 37°C overnight, activity was determined by the diameter (in cm) of inhibition zones around the wells.

For determination of active HPLC fractions, each fraction was either directly pipetted onto the surface of indicator seeded agar plates (5 - 15 µl) or 50 µl were tested in prepared wells. Since *M. luteus* is known to be one of the most sensitive indicator strains for lantibiotics, all fraction activity testings were performed with *M. luteus* as a standard indicator organisms.

(c) Determination of arbitrary units of antimicrobially active samples

In order to compare the antibacterial efficacy of lichenicidin to other lantibiotics the arbitrary units of the cell wash extract containing lichenicidin in comparison to other lantibiotics e.g. Pep5, nisin and mersacidin were determined. Serial dilutions (each representing a 1:2 dilution) of the lantibiotics and the cell wash extract were prepared in

half-concentrated MH broth. Fresh colonies of indicator organisms were used to prepare cell suspensions of about 5×10^6 CFU/ml which were employed to inoculate MH agar plates. 10 µl of lantibiotic solutions were spotted onto plates that had been seeded with the indicator. The highest dilution yielding a clear inhibition zone on the lawn was detected by visual inspection after incubation overnight.

(d) Stability assays

Thermal stability of antimicrobial compounds was analyzed by incubating 2 ml culture supernatants and cell wash extract at 37°C, 45°C, 65°C, 80°C and 100°C for up to 240 min. Stability of antimicrobial activities against treatment with proteases was determined by adding 10 mg/ml of proteinase K, pronase E, α-chymotrypsin and trypsin (Serva, Feinbiochemica, Heidelberg) to cell-free supernatant and cell wash extract and incubating at 37°C for up to 240 min. Influence of different pH on the activity was examined by adjusting the pH to 1.5, 3, 9 and 13 and incubating for 30 min at RT. Residual antimicrobial activities were then determined in agar well diffusion assays against *M. luteus*.

(e) Growth assays in presence of antimicrobial agents

Growth assays in presence of antimicrobial agents were performed against *M. luteus* in 96 well polystyrene round bottom microtiter plates in a total volume of 200 µl. 25 ml MH broth were inoculated with 500 µl pre-culture and were grown to an optical density of 0.2. 100 µl of this cell suspension was used for inoculation. Peptides were added in concentrations corresponding to 5 times the MIC and in 1:2 dilutions. Growth curves were recorded by measuring the optical density over the time (12 h) in a microtiter plate absorbance reader (Sunrise™, Tecan Group, Switzerland).

(f) Whole-cell biosensor assay

By use of a β-galactosidase-based promotor-induction assay (Burkhard and Stein, 2005; Starón *et al*., 2011) antibiotic solutions were analyzed for their capacity to cause cell envelope stress. *B. subtilis* reporter strains express a chromosomally encoded β-galactosidase under control of promotors of the antibiotic sensing and detoxification modules BceRC-AB (TMB279), YxdJK-LM (TMB299), PsdRS-AB (TMB588) and LiaRS (TMB016). Cell envelope stress caused by antibiotic samples was assayed by the agar well diffusion method. To this end, reporter strains were seeded onto MH agar plates containing X-Gal (5-bromo-4-chloro-indolyl-galactopyranoside; Fermentas) in a final concentration of 400 µg/ml. 50 µl of peptide solution were applied to each well (7 mm). In a qualitative β-galactosidase assay, plates were scored for the appearance of blue rings at or near to the edges of inhibition zones after incubation at 37°C for 24 h.

Detection of protease activity

Protease activity in cell-free culture supernatants was detected on skim milk agar plates (3 g/l yeast extract, 5 g/l peptone, 2.5 g/l skim milk powder, 15 g/l agar, pH 7.2 (M. Nagel, diploma thesis 2007)). 50 µl of fresh and filter-sterilized supernatant were added into wells that had been introduced into agar surfaces with a sterile cork borer. Protease activity was determined by the appearance of clear zones around the wells after incubation at 37°C overnight.

Heterologous protein expression of HIS-tagged proteins by *E. coli* Bl21 and *E. coli* C43

For heterologous expression of HIS-tagged lantibiotic modification enzymes and precursors, expression strains were grown in 0,5-1 l LB main cultures containing ampicillin (pET22 derivatives) or kanamycin (pET28 derivatives). 10 ml overnight pre-cultures were used for inoculation of main cultures that were grown to an OD_{600} of 0.5-0.6 at 30°C. Heterologous expression was induced with a final concentration of 1mM IPTG. Cells were pelleted by centrifugation (10 min, 6,000 rpm, 4°C, Sorvall, Heraeus). Cell pellets were resuspended in 20 ml lysis buffer and stored at -20°C.

Cells were lysed by addition of 15 µl of lysozyme (10 mg/ml; 30 min incubation on ice) followed by sonification (8x15 sec intervals at 60% including 15 sec of cooling on ice; Sonifier TM W250; Branson). Lysates were incubated for 15 min on ice after addition of 10 µl RNAse and DNAse [10 mg/ml]. Cell debris and intact cells were removed by centrifugation (6,500 g, 10 min, 4°C). Purification of HIS-tagged proteins and peptides was performed using the Nickel-NTA purification system following the manual of the supplier (Qiagen) and was attempted accordingly to protein purification protocols published by McClerren *et al*., (2006) and Türck & Bierbaum (2011) with several variations and combinations thereof. Proteins were eluted in 500 µl aliquots, mixed with 100% glycerol to a final concentration of 50% and stored at -20°C.

Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the NuPAGE system (Invitrogen)

Purified proteins and peptides were analyzed via standard SDS-PAGE (Laemmli, 1970) and in a Mini Protean III vertical electrophoresis apparatus (BioRad). For small peptides, 20-24% SDS-gels were prepared or 4-12% gradient Novex Bis-Tris gels were used in the NuPAGE electrophoresis system (MES-buffer, XCell SureLock® Mini-Cell, Invitrogen) according to the supplier's instruction. LanM proteins were analyzed on 10% SDS-gels. 10xLDS sample buffer

(Invitrogen) was added to each sample. Samples were heated (70°C) for 10 min prior to electrophoresis. Gels were stained with PageBlue™ Protein Stain (Fermentas) or silver stain according to Heukeshoven and Dernick (1988).

Table 2.8: **Buffers for SDS-gel-electrophoresis.**

Buffer	Chemical ingredients
Solution A	3 M Tris-HCl; pH 8.5
Solution D	0.96 M Tris; 0.8% (w/v) SDS
Running Buffer	25 mM Tris; 192 mM glycine; 0.1 (w/v) SDS; pH 8.5
10% separating gel	40% (19:1) acrylamide/bisacrylamide (2.25 ml); solution A (2.25 ml); 0.21% APS (1.2 ml); *aqua dest*. (3.126 ml); 20% SDS (0.06 ml); TEMED (6 µl)
20% separating gel	40% (19:1) acrylamide/bisacrylamide (4.5 ml); solution A (2.25 ml); 0.21% APS (1.2 ml); *aqua dest*. (0.876 ml); 20% SDS (0.06 ml); TEMED (6 µl)
4% stocking gel	40% (19:1) acrylamide/bisacrylamide (0.238 ml); solution D (0.3 ml); 0.21% APS (0.8 ml); *aqua dest*. (1.03 ml); TEMED (5 µl)

Mass spectrometry by MALDI-TOF

Mass spectrometry of peptide preparations was performed using a MALDI-TOF mass spectrometer (Bruker Biflex, Bruker Daltonics, Bremen, Germany). 1 µl of cell wash extracts or culture supernatants were mixed with 2 µl matrix (α-cyano-4-hydroxycinnamic acid in acetonitrile: 0.1% TFA in MilliQ water, 1:3). For MALDI-TOF analysis of active HPLC fractions, 20 µl of each fraction were concentrated 1:10 using a rotational vacuum concentrator (RVC 2–18, Christ, Osterode, Germany). Samples were spotted onto the MALDI target and air-dried. Mass spectra were measured in the positive ion mode and analyzed by FlexAnalysis 2.0 (Bruker Daltonics).

Protein biochemical bioinformatic tools

Potential protease cleavage sites were observed using the ExPasy PeptideCutter Tool:

- http://www.expasy.ch/tools/peptidecutter/

Transmembrane sequences were identified using the TMHMM web server v. 2.0.:

- http://www.cbs.dtu.dk/services/TMHMM/

Blasts were performed at the NCBI nucleotide website:

- http://blast.ncbi.nlm.nih.gov/Blast.cgi

Molecular weight and charges were predicted by the PC tool DNAman or by PROTEIN CALCULATOR v3.3:

- http://www.scripps.edu/~cdputnam/protcalc.htm

Multi sequence alignments were done by Clustal W2 or the PC tool DNAman:

- http://www.ebi.ac.uk/Tools/msa/clustalw2/
- Protein sequences that were chosen for blast and alignment analysis of putative lantibiotic and TOMM modification enzymes are listed in table 2.9.

Table 2.9: Overview of biosynthetic enzymes that were used for alignments and blasts of putative lantibiotic and TOMM biosynthesis enzymes (*according to Haft 2009 and Haft *et al*., 2010).

Query for	Protein	Substance	Protein ID (NCBI)
Standard LanM			
One-peptide lantibiotics	MrsM	Mersacidin	CAB60261
	LctM	Lacticin 481	AAC72258
Two-peptide lantibiotics	HalM1 HalM2	Haloduracin	BH0452 BH0455
Lantipeptides	ProcM	Prochlorosin	PMT0250
Standard TOMM dehydrogenase (TIGR03605)*			
Microcin B17	MccC	Microcin B17	P23185
Streptolysin S	SagB	Streptolysin S	AAC25178
Thiopeptides	TpaE TpaF	Thiopeptide TP-1161	ZP_06427466 ZP_06427458.1
Heterocycloanthracins	predicted in *B. cereus* (Haft, 2008)		BA_1266
Plantazolicin	PznB	Plantazolicin	RBAM007480
Standard TOMM cyclodehydratase (TIGR03603)*			
Microcin B17	MccB	Microcin B17	P23184
Streptolysin S	SagC	Streptolysin S	AAC25179
Thiopeptides	TpaC TpaD	Thiopeptide TP-1161	ACR48336 YP_055576.1
Standard TOMM cyclodehydratase (TIGR03603)*			
Heterocycloanthracins	predicted in *B. cereus* (Haft, 2008)		BA_1268
Plantazolicin	PznC	Plantazolicin	RBAM007460
Standard TOMM scaffold protein (TIGR03604)*			
Microcin B17	MccD	Microcin B17	P23186
Streptolysin S	SagD	Streptolysin S	YP_002285592
Heterocycloanthracins	predicted in *B. cereus* (Haft, 2008)		BA_1267
Plantazolicin	PznD	Plantazolicin	RBAM007470
TOMM scaffold-cyclodehydratase fusion proteins (TIGR03882)*			
Cyanobactins	TruD	Trunkamide	ACA04490

Results

PART 1: Novel lantibiotic gene clusters and producers

Aim of this project

This project aimed at the identification of novel lantibiotic gene clusters that are encoded in publicly available sequenced bacterial genomes, but have not been characterized yet. Identification of novel lantibiotics and producers, especially in bacterial taxa not considered to comprise potential lantibiotic producers might enlarge the potential of therapeutical use of lantibiotics by discovery of new features and actions.

Biosynthetic enzymes, which are involved in post-translational modifications of lantibiotic precursors share conserved structures and sequences. To this end, they are useful blueprints for the bioinformatical identification of homologous proteins and, thus, for predicting novel lantibiotic gene clusters.

The lantibiotic mersacidin shows promising antimicrobial activities even against MRSA strains. Therefore, in this study, the aa sequence of its modification enzyme (MrsM) was chosen for a BlastP search in the NCBI genome database. As a result, various new putative lantibiotic-like gene clusters were identified. In this thesis, three putative lantibiotic producers, *Bacillus licheniformis* DSM 13, *Nostoc punctiforme* ATCC 29133 and *Caldicellulosiruptor bescii* DSM 6725, were chosen for a closer analysis. To this end, the predicted producers were either 1. analyzed for production of an antimicrobial activity that was subsequently demonstrated to be based on the predicted cluster by plasmid integration mutagenesis targeting the putative lantibiotic modification enzymes; or 2. heterologous expression of the biosynthetic enzymes and lantibiotic precursors of predicted gene clusters for subsequent *in vitro* modification experiments was aimed in this thesis.

A: The novel lantibiotic gene cluster of *Bacillus licheniformis* DSM 13

Genomic data mining based searches identified a putative two-peptide lantibiotic gene cluster in two isogenic *Bacillus* strains, *B. licheniformis* DSM 13 and ATCC 14580. As *B. licheniformis* strains are known for production of proteases, their lantibiotics were predicted to exhibit enhanced stability against bacterial proteases, an interesting feature that might also confer peptide stability in therapeutic enteral applications.

In this thesis, homologous expression of the new lantibiotic was achieved, followed by its partial purification and characterization. *B. licheniformis* DSM 13 was assayed for production of an antimicrobial activity that could be attributed, by MALDI-TOF analysis and plasmid integration mutagenesis, to production of a lantibiotic encoded by the predicted gene cluster. Additionally, a detailed bioinformatic analysis of the gene cluster was performed.

Description and bioinformatic analysis of the novel two-peptide lantibiotic gene cluster of *B. licheniformis* DSM 13 / ATCC 14580

Annotation of the sequenced chromosomes of *B. licheniformis* DSM 13 and ATCC 14580 revealed two putative lantibiotic modification enzymes of the LanM-type (BLi04126/BL00928 and BLi04128/BL00928; Veith *et al*., 2004; Rey *et al*., 2004). For following descriptions of the predicted lantibiotic gene cluster, locus tags of the DSM 13 strain were used.

Both LanM-coding ORFs were recognized in data base searches, since both display significant aa identity to MrsM (33%) and to LanM enzymes of known two-peptide lantibiotics, like haloduracin (38%). Interestingly, these *lanM*-like genes are encoded on both DNA strands and are separated by a putative lantibiotic structural gene (BLi04127, *licA1*). This predicted lantibiotic precursor is located upstream of the putative modification enzyme (LicM1) encoded by *bli04126* and was previously annotated as lichenicidin (Veith *et al*., 2004). It exhibits a high sequence similarity to other LanA peptides, like mersacidin (36%) and to the LanA1 peptide of haloduracin (40%).

Presence of two LanM-coding genes, their arrangement within the cluster and their aa similarity to two-peptide lantibiotic modification enzymes, suggested a second precursor that might have been overlooked during annotation. This has also been postulated in a recent publication dealing with the two-peptide lantibiotic haloduracin (McClerren *et al*., 2006). Lantibiotic structural genes can be identified by cumulative occurrence of Cys, Ser, Thr residues in the C-terminus of prepeptides and their conserved cleavage sites. Indeed, a closer analysis of the intergenic region upstream of *licA1* revealed a second precursor (*licA2*; bp 3949269-3949514) in front of the second LanM homolog (*licM2; bli04128*). Eight bp downstream of a conserved AGGAGG Shine-Dalgarno sequence, this ORF encodes a peptide with 72 aa, consisting of a leader (34 aa) and a propeptide (38 aa) that are separated by a GG cleavage site. Like the above mentioned genes, this peptide also displays the highest similarity to corresponding gene of the *hal* gene cluster, e.g. to the β-peptide HalA2 (52%).

Further downstream of *licA1*, additional ORFs are present that show sequence similarity to proteins involved in lantibiotic production. BLi04125 constitutes a transporter with a peptidase domain and 49% similarity to HalT(P). BLi4124 encodes a protein with an N-

terminal signal domain, which is similar to the peptidase involved in processing of the lantibiotic cytolysin (CylA, 37%) and to a peptidase encoded in the genome of *B. halodurans* C-125 (BH1491, 49%) that, so far, has not been described to be part of the *hal* gene cluster. Based on these results, a role in transport and processing of the lichenicidin peptides was postulated and they were named LicT(P) and LicP, respectively.

BLi04122 is a helix-turn-helix regulator protein, which showed 60% similarity to a protein that is encoded near the haloduracin gene cluster (BH0460) and might be involved in gene regulation by acting as transcriptional regulator. BLi04121 is a small protein with similarity (61%) to a protein encoded downstream of the haloduracin gene cluster (BH0459) and it harbors three transmembrane helices. BLi04123 is a small hypothetical protein, which showed no significant sequence similarity to known proteins.

Following ORFs might be involved in producer self-protection: BLi04120 is the ATP binding domain of an ABC transporter; BLi04119 did not show any sequence similarity to known proteins, however, six transmembrane domains were predicted for this protein; BLi04118 showed low similarity to MrsE, an ABC transporter subunit of the mersacidin gene cluster (21%) and likewise, a computer analysis showed six membrane-spanning regions. The N-terminal two thirds of BLi04117 appeared to be homologous to 1. the bacitracin transporter of *B. licheniformis*, a protein that is able to prevent binding of bacitracin to the undecaprenyl-pyrophosphat of its target lipid II, 2. to the ATPase component of the CcmA multidrug resistance system and 3. to the ATPase subunit of the haloduracin gene cluster (HalF1, 35.8%). BLi04116 again is a transmembrane protein with six membrane-spanning helices and had similarity to the BcrB subunit of the bacitracin transporter (52%). The gene downstream to BLi04116 encodes a protein with similarity to the ferrochelatase HemH, indicating that the gene cluster ends here. Upstream, the cluster is bordered by BLi04129, encoding a pectate lyase.

In conclusion, the novel gene cluster, spanning bp 3938843 to 3953096, comprises fourteen ORFs including two lantibiotic structural genes, two modification enzymes, a peptidase, an exporter, one regulator and, probably, two different immunity systems (an ABC transporter BLi04120-18 and a transporter with homology to the bacitracin transporter BLi4117/16) (figure 3.1.1)

The GC content (45.1%) of the cluster is quite similar to the average GC content of the whole genome (46.2%) and the gene cluster does not seem to be associated with any mobile element. After searching both annotated sequences for “free” gene designations, according to established lantibiotic gene nomenclature and predicted functions, following gene

designations for the ORFs involved in biosynthesis of lichenicidin were proposed: *licA1* (BLi04127, structural gene of the mature α-peptide Licα), *licA2* (structural gene of the mature β-peptide Licβ); *licM1* (BLi04128 encoding the LanM that modifies LicA1), *licM2* (BLi04126 encoding the LanM modifying LicA2), *licT(P)* (BLi04125 encoding the processing transporter) and *licP* (BLi04124 encoding the peptidase).

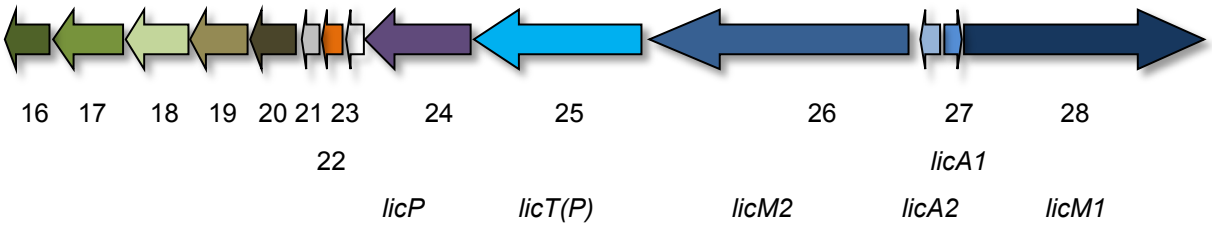

Figure 3.1.1: Gene cluster of lichenicidin. The gene cluster of the novel lantibiotic lichenicidin is located at the far end of the chromosome of *B. licheniformis* DSM 13 and covers bp 3938843-3953096 (locus tags BLi04116 to BLi04128 and an additional, not yet annotated ORF). Genes of prepeptides are **light blue**; genes of modification enzymes are **dark blue**. Other genes are marked as follows: **processing transporter**, **peptidase**, **regulator**, genes that might be involved in **immunity** and encode proteins that have similarity to transporters. Numbers give the last two digits of DSM 13 annotations. For small **grey** ORFs, no functions could be assigned up to now, however, similar ORFs are encoded in vicinity of the haloduracin gene cluster.

Detailed bioinformatical analysis of the lantibiotic prepeptides LicA1 and LicA2

AA sequences of the predicted lantibiotic precursors LicA1 and LicA2 were compared to sequences of previously described two-peptide lantibiotics (figure 3.1.2) e.g. haloduracin, lacticin 3741, SMB, BHT, plantaricin W, staphylococcin C55 and to mersacidin.

Lantibiotic prepeptides of the two-peptide lantibiotic subgroup are characterized by typical LanA1 and LanA2 motifs. Such motifs were identified in both predicted lichenicidin peptides as shown in figure 3.1.2. Since these motifs include conserved Ser,Thr and Cys, the lantibiotic peptides harbor at least partial conserved lanthionine ring patterns. Based on this sequence similarity, bridging patterns were predicted for both lichenicidin peptides, that correspond to those of the closely related haloduracin peptides. Since the LicA1 propeptide contains a further Cys as well as four hydroxy-aa in its N-terminal part, an additional, fourth ring was predicted for this peptide. Moreover, similarity to the Lanα-peptides of haloduracin and plantaricin W suggested, that the serine (30) within the C-terminal ring most probably is not

(A)

```
LicA1:
MSKKEMILSWKNPMYRTESSYHPAGNILKELQEEEQHSIAGGTITLSTCAILSKPLGNNGYLCTVTKECMPSCN

MrsA:       GDMEAACTFTLPGGGGVCTLTSECIC
HalA1:       CAWYNISCRLGNKGAYCTLTVECMPSCN                    28
LicA1:  TITLSTCAILSKPLGNNGYLCTVTKECMPSCN                     32
PlwA1:      KCKWWNISCDLGNNGHVCTLSHECQVSCN                    29
LtnA1:     CSTNTFSLSDYWGNNGAWCTLTHECMAWCK                    30
SacA1:     CSTNTFSLSDYWGNKGNWCTATHECMSWCK                    30
SmbA1:  IGTTVVNSTFSIVLGNKGYICTVTVECMRNCSK                    32
BhtA1:  IGTTVVNSTFSIVLGNKGYICTVTVECMRNCQ                     32
Motif:             SxxxGNxGxxCTxTxECmxxC
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(B)

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LicA2:
MKTMKNSAAREAFKGANHPAGMVSEEELKALVGGNDVNPETTPATTSSWTCITAGVTVSASLCPTTKCTSRC

HalA2:   GDVHAQ......TTWPC.ATVGV..S.VALCPTTKCTSQC          30
LicA2:   NDVNPETTPATTSSWTC.ITAGVTVS.ASLCPTTKCTSRC           38
PlwA2:   GDPEAR......SGIPCTIGAAVAAS.IAVCPTTKCSKRCGKRKK     37
SmbA2:               STPACAIGGVVGITVAVTGISTACTSRCINK         31
BhtA2:               STPACAIG.VVGITVAVTGISTACTSRCINK         30
LtnA2:         TTPAT....PAISILSAYIS.TNTCPTTKCTRAC            29
C55A2:   GTPLAL...LG...GAATG.VIGYIS.NQTCPTTACTRAC           32
Motif:                           sxxxxcpsTxCsxxC
                                 t      t   t
```

Figure 3.1.2: AA alignment of two-peptide lantibiotics and lichenicidin. AA sequence alignment of LicA1 and LicA2 propeptides with Lanα (A) and LanA2 propeptides (B) of the two-peptide lantibiotics plantaricin (PlwA1, AAG02567; PLWA2, AAG02566), staphylococcin C55 (SacA1, BAB78438; SACA2, BAB78439), lacticin 3147 (LtnA1, O87236; LtnA2, O87237), haloduracin (HalA1, BAB04173; HALA2, BAB04172), BHT (BhtA1, AAZ76603; BhtA2, AAZ76602) and SMB (SmbA1, BAD72777; SmbA2, BAD72776) and mersacidin (MrsA, Z47559). AA identities are highlighted in blue (100%), red (75%) and green (35%). AA that are removed by additional proteolysis are denoted in **violet**. AA, which are thought to be involved in additional modifications are marked in **red** (proteolysis) and yellow ((Me)Lan ring). The thioether bridging pattern represent that of the Hal and Plw peptides.

dehydrated as it had been previously described for the corresponding Ser in these peptides (McClerren *et al.*, 2006 and Holo *et al*., 2004).

For LicA2, an additional processing step resulting in removal of six N-terminal residues was postulated. As this includes loss of two negatively charged aa, the active β-peptide gains a positive net charge. Such a second processing step has been demonstrated for other β-peptides as well. This prediction was based on the presence of a protease within the Lic gene cluster, that has aa sequence similarity to proteases involved in second processing of plantaricin W and haloduracin β-peptides.

Antimicrobial activity testing of the culture supernatant and an isopropanol cell wash extract of *B. licheniformis* DSM 13

In order to test, if *B. licheniformis* DSM 13 produces an antimicrobial substance, cells were cultivated in synthetic medium (2xBPM), TSB, LB and MH broth at 37°C for up to 48 h. In agar well diffusion assays of the culture supernatant against different Gram-positive indicator strains (*Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Staphylococcus simulans* and enterococci), the highest production was observed for incubation in 2xBPM. Neither hemolysis nor growth-inhibition of Gram-negative bacteria was observed. For detailed analysis, cells were grown in 50 ml 2xBPM for 24 h. Cells were harvested by centrifugation, culture supernatant was removed and resulting cell pellets were resuspended in 15 ml 70% isopropanol (pH2).

These cell wash extracts and crude culture supernatants were assayed for antimicrobial activity in agar well diffusion assays against different indicator strains. Both samples exhibited an antibacterial activity against Gram-positive bacteria, including several MRSA strains. However, they had a slightly different spectrum of activity, indicating that culture supernatant and isopropanol cell wash extract might contain different substances (figure 3.1.3). Gram-negative bacteria and eukaryotic cells were not affected (data not shown). As previously determined for culture supernatant, isopropanol extracts also showed no hemolytic activity on Columbia blood agar plates.

Additionally, cross resistance of *Bacillus halodurans* C-125, which produces the closely related two-peptide lantibiotic haloduracin, was also tested; *B. halodurans* C-125 was sensitive to both fractions. Similar results were obtained with the mersacidin producer *Bacillus spec*. HIL Y-85.54728.

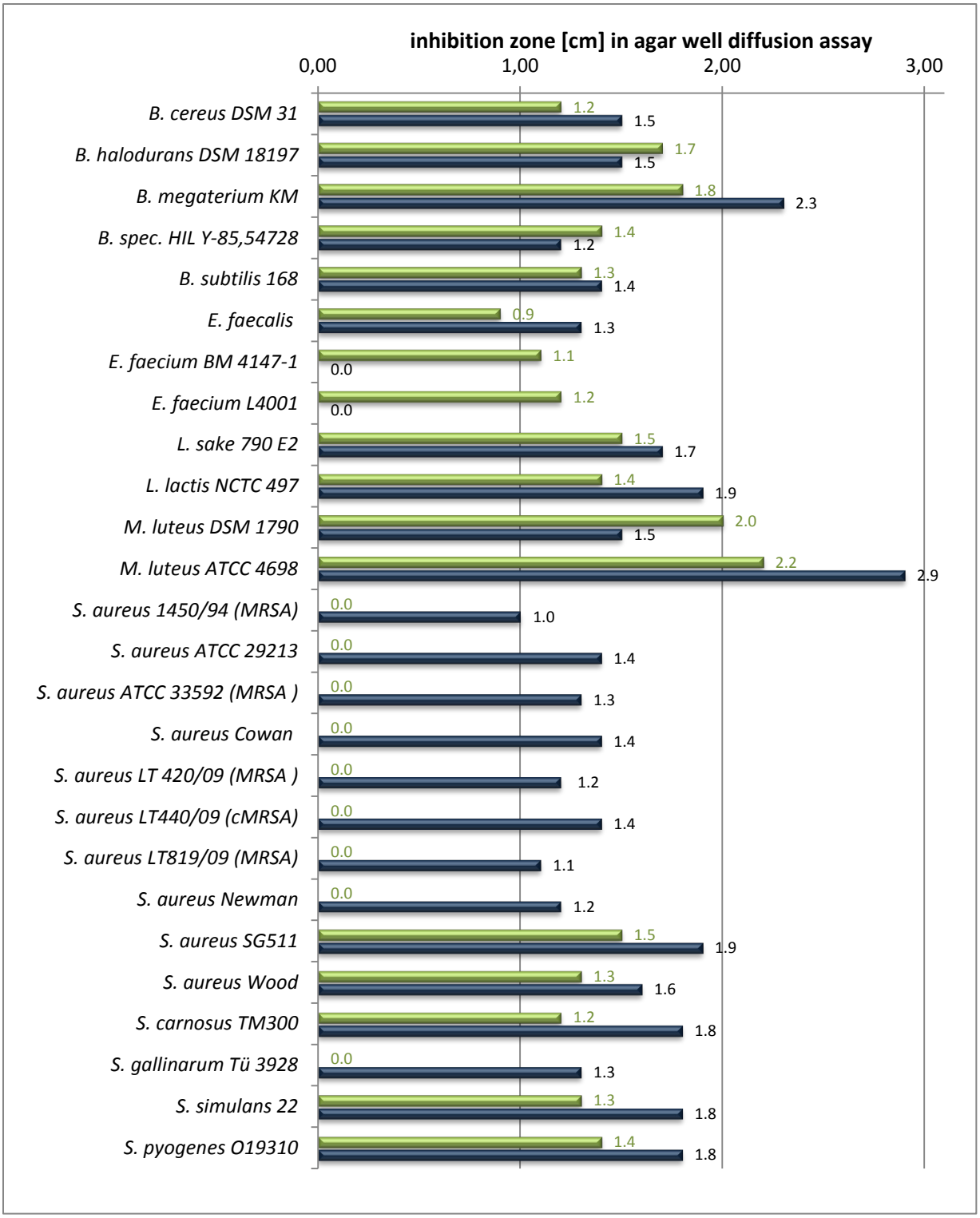

Figure 3.1.3: In agar well diffusion assays. *B. licheniformis* DSM 13 culture supernatant and isopropanol cell extract were active against Gram-positive bacteria, but showed slightly different spectra of activity, indicating that they contained different antibacterial compounds.

Stability testing of the antimicrobial activities produced by *B. licheniformis* DSM 13

For further characterization of both active fractions, heat stability, resistance against protease treatment and pH stability were determined. In all stability testings, the cell wash extract was tested after evaporation of isopropanol. After incubation the samples were tested in agar well diffusion assays in order to analyze and compare inhibiting effects on the antimicrobial activity.

The supernatant contained a substance that displayed only limited heat stability and was inactivated within 2 h at 80°C and within 30 min by boiling, whereas the activity of the cell wash extract was heat stable and even withstood 4 h at 100°C without any loss (figure 3.1.4).

Both active fractions inhibited growth of the indicator strain *M. luteus* in a deferred antagonism test at pH 1.5. 3 and 9; however, the supernatant did not show any antibacterial activity at pH 13. In contrast, the cell extract was initially active, but inactivated after incubation for 4 h at pH 13 at RT (figure 3.1.5).

Additionally, resistance of both substances against treatment with different proteases was tested. Incubation in presence of proteolytic enzymes (pronase E, proteinase K, trypsin, chymotrypsin) did not affect the antibacterial activity present in the supernatant. The activity of the isopropanol-extract was stable in the presence of trypsin and chymotrypsin, but, addition of proteinase K and pronase E led to inactivation within 4 h of incubation, indicating the proteinaceous nature of this substance (figure 3.1.6).

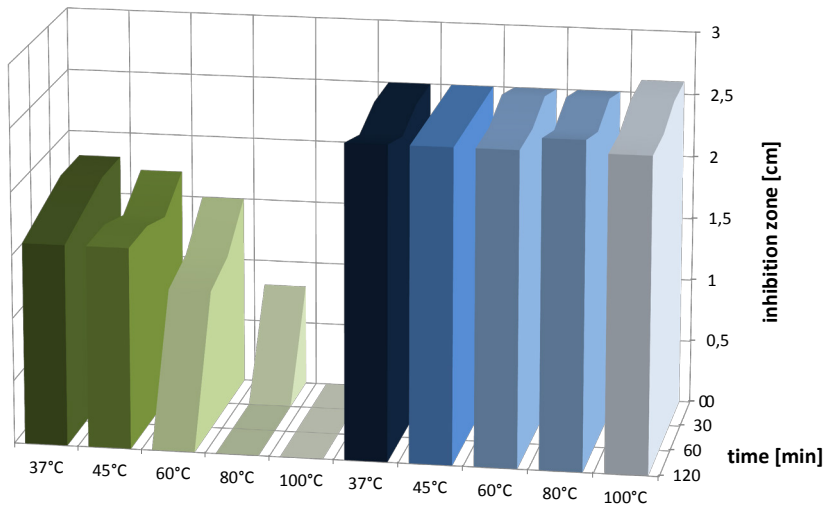

Figure 3.1.4: Stability assay: heat treatment of culture supernatant and cell wash extract. The substance present in the cell wash extract was not affected, whereas the substance in culture supernatant was inactivated by heat.

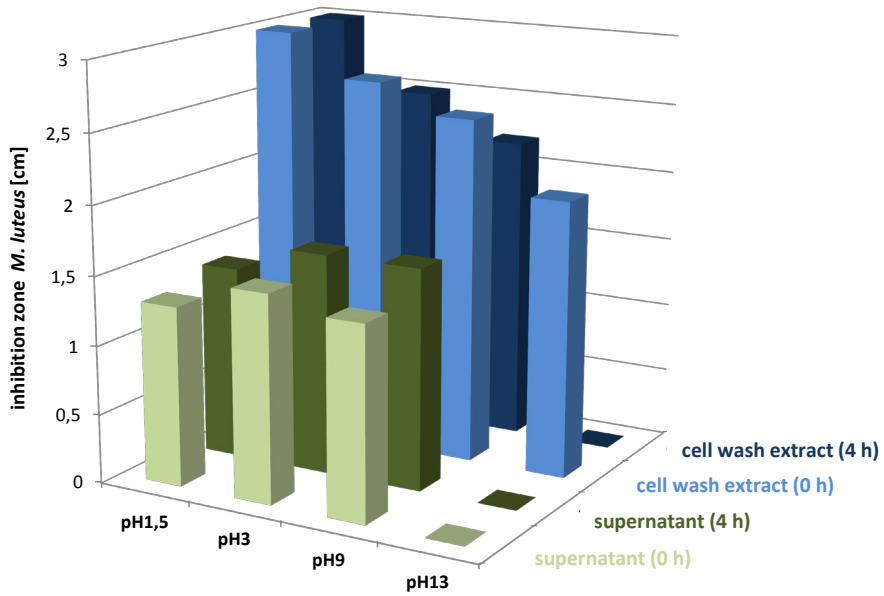

Figure 3.1.5: Stability assay: In antagonism tests at different pH supernatant and **cell wash extract** were only slightly affected by pH 1.5 to 9, but inactivated at pH 13.

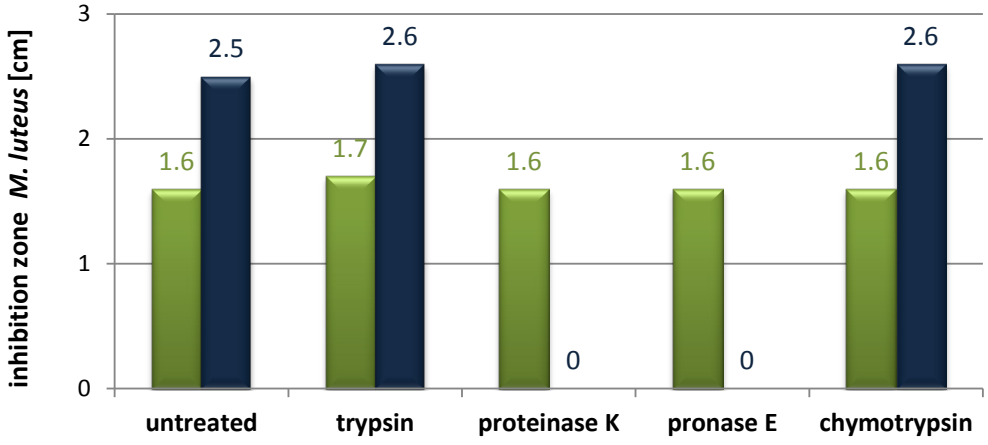

Figure 3.1.6: Stability assay: Treatment with proteases. No loss of activity was detected after treatment with trypsin and chymotrypsin. The substance in the **cell wash extract** was inactivated by proteinase K and pronase E, while the antimicrobial activity in the **supernatant** was unaffected.

Preliminary characterization of the antimicrobial effects of both antimicrobial substances produced by *B. licheniformis*

First indications that the action of the antibiotic substances might target the cell envelope were obtained from a test of four different *B. subtilis* reporter strains for a cell envelope stress antibiotic screening in a qualitative β-galactosidase based agar well diffusion assay (Burkhardt and Stein, 2008; Starón *et al*,. 2011). Here, the cell wash extract and supernatant were screened for their capacity to induce expression of LacZ-promotor fusions of the detoxification systems BceAB (TMB294) PsdA (TMB299), YxdL (TMB588) and LiaI (TMB016) that are involved in peptide sensing and detoxification of cell wall active antibiotics in *Bacillus* strains.

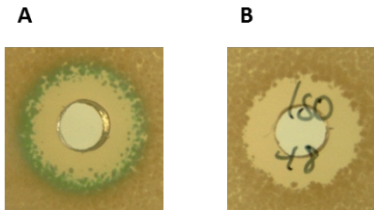

Figure 3.1.7: Qualitative β-galactosidase based agar well diffusion assay against the reporter strain *B. subtilis* TMB016 indicated presence of a cell envelope stressing antibiotic in the culture supernatant (A), whereas the antibacterial substance within the cell extract is not recognized by the pLIAI reporter system (B).

All four reporter strains were affected by culture supernatant and cell extract of *B. licheniformis* in agar well diffusion assays (1.3 cm), but a positive lipid II interaction was only detected in the pLiaI based whole cell biosensor as indicated by occurrence of a blue ring around the inhibition zone against the reporter strain *B. subtilis* TMB016 on MH agar plates containing X-Gal (figure 3.1.7 A). In contrast, the cell wash extract showed no lipid II interaction (figure 3.1.7 B).

In a growth assay, the growth of the indicator *M. luteus* was inhibited in presence of 4xMIC culture supernatant (10 µl in 200 µl) and cell wash extract (7.5 µl in 200 µl) of *B. licheniformis* (figure 3.1.8). The antimicrobial compound of the cell wash extract completely inhibited growth of the indicator strain. In contrast, growth of *M. luteus* was observed in presence of antimicrobially active culture supernatant for 200 min and only then lysis started. These results also indicated production of two distinct antimicrobial substances with different modes of action by *B. licheniformis* DSM 13.

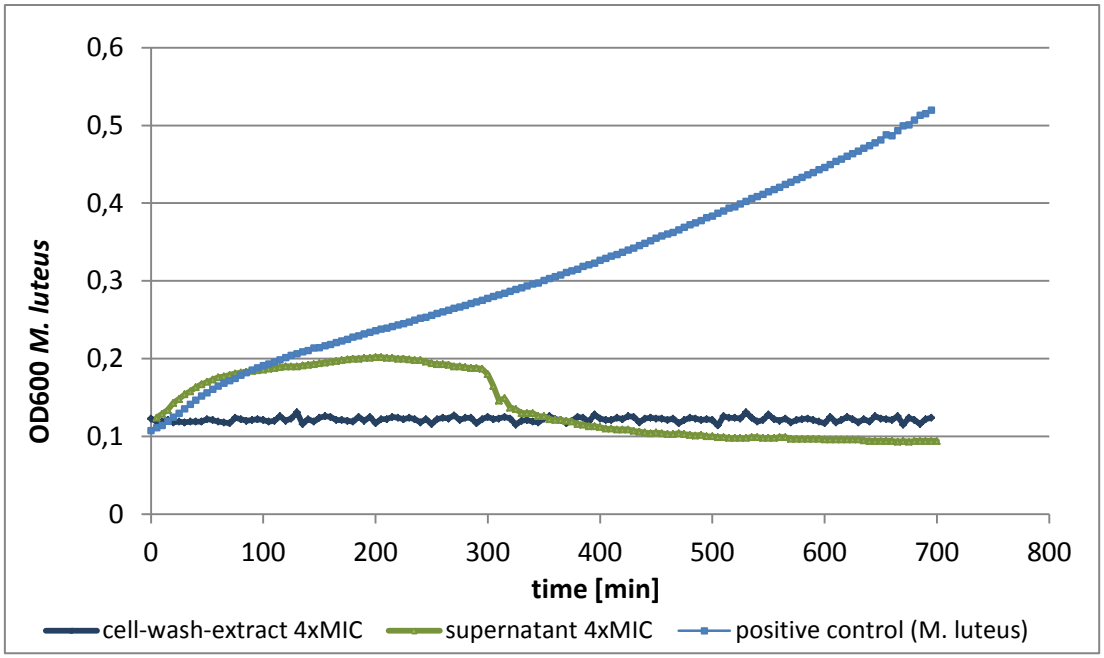

Figure 3.1.8: Cell growth assay of culture supernatant and isopropanol cell wash extract.
The indicator strain *M. luteus* was grown in presence of 4xMIC of the culture supernatant and the cell wash extract. Both inhibited growth of the indicator, but apparently differed in their MoA.

MALDI-TOF analyses of culture supernatant, whole cells and isopropanol cell extract of *B. licheniformis* DSM 13

For the two-peptide lantibiotics haloduracin and lacticin 3147 an accumulation of lantibiotic peptides on the surface of producer cells has been demonstrated and MALDI-TOF analysis of whole cells (colony mass spectrometry; CMS) identified these peptides. For the putative lichenicidin producer, *B. licheniformis* DSM 13, mass signals (3021 Da, 3038 Da, 3054 Da, 3071 Da, 3268 Da, 3284 Da, 3305 Da) within the relevant range of 1600 to 5000 Da were detected in mass spectra of whole cells and corresponding masses were additionally found in the analyses of the cell wash extract (figure 3.1.8 A and C). These signals clearly matched the calculated masses of both peptides encoded by LicA1 and LicA2, whereas the masses 3268 Da, 3284 Da and 3305 Da might probably denote the LicA1 propeptide containing 4-6 of 8 putative dehydrations (table 3.1.1). Masses of 3021 Da, 3038 Da, 3054 Da and 3071 Da were predicted to represent different Licβ-peptides that have lost the 6 N-terminal aa by proteolytic processing and harbor 9-12 dehydrated residues. These masses were not detected in mass spectra of the culture supernatant (figure 3.1.9 C), thus indicating a cell surface accumulation of the lichenicidin peptides, too. Five signals (2622 Da, 3199 Da, 3520 Da, 3710 Da, 4762 Da) were identified within the spectra of the culture supernatant.

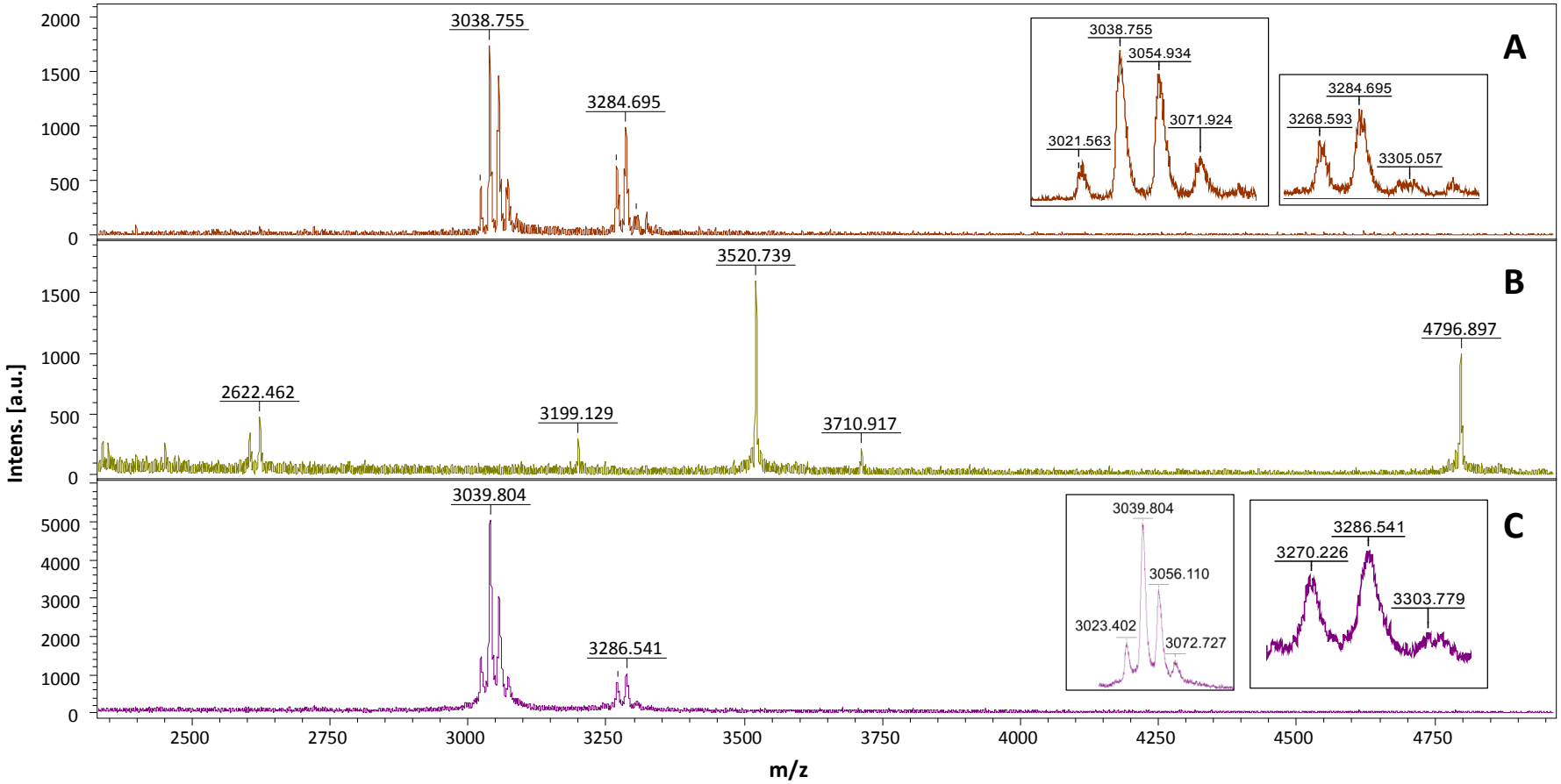

Figure 3.1.9: MALDI-TOF analyses of whole cells (CMS, A), culture supernatant (B) and cell wash extract (C) of *B. licheniformis* DSM 13. Two predominant mass signals (3038 Da; 3284 Da) were detected in mass spectra of whole cells and isopropanol extract, but were not observed in mass spectra of the culture supernatant.

Table 3.1.1: Calculation of putative MWs of Licα and Licβ peptides depending on the dehydration state of active peptides. Masses. that were predicted to represent the MW of active peptides. as suggested by MALDI TOF analysis, are highlighted in blue (present in CMS; isopropanol extract) or green (present in supernatant). AA, that might be dehydrated are shown in **blue** and Cys residues are marked in **violet**.

putative aa sequence of Licα	calculated MW [Da] of the Licα-peptide depending on the dehydration state					
	8	7	6	5	4	3
TITLSTCAILSKPLGNNGYLCTVTKECMPSCN	3232.9	**3250.9**	**3268.9**	**3286.9**	**3304.9**	3322.9
ITLSTCAILSKPLGNNGYLCTVTKECMPSCN	-	3149.8	3167.8	3185.8	3203.8	3221.8
TLSTCAILSKPLGNNGYLCTVTKECMPSCN	-	3036.7	3054.7	3072.7	3090.7	3108.7
LSTCAILSKPLGNNGYLCTVTKECMPSCN	-	-	2953.6	2971.6	2989.6	3007.6
STCAILSKPLGNNGYLCTVTKECMPSCN	-	-	2840.4	2858.4	2876.4	2894.4
TCAILSKPLGNNGYLCTVTKECMPSCN	-	-	-	2771.3	2789.3	2807.3
CAILSKPLGNNGYLCTVTKECMPSCN	-	-	-	-	2688.2	2706.2
AILSKPLGNNGYLCTVTKECMPSCN	-	-	-	-	2585.1	2603.1
ILSKPLGNNGYLCTVTKECMPSCN	-	-	-	-	2514	2532
LSKPLGNNGYLCTVTKECMPSCN	-	-	-	-	2400.8	2418.8
SKPLGNNGYLCTVTKECMPSCN	-	-	-	-	2287.7	2305.7

putative aa sequence of Licβ	calculated MW [Da] of the Licβ-peptide depending on the dehydration state											
	15	14	13	12	11	10	9	8	7	6	5	4
NDVNPETTPATTSSWTCITAGVTVSASLCPTTKCTSRC	3635.3	3653.3	3671.3	3689.3	3707.3	3725.3	3743.3	3761.3	3779.3	3797.3	3815.3	3833.3
DVNPETTPATTSSWTCITAGVTVSASLCPTTKCTSRC	3521.2	3539.2	3557.2	3575.2	3593.2	3611.2	3629.2	3647.2	3665.2	3683.2	3701.2	3719.2
VNPETTPATTSSWTCITAGVTVSASLCPTTKCTSRC	3406.1	3424.1	3442.1	3460.1	3478.1	3496.1	3514.1	3532.1	3550.1	3568.1	3586.1	3604.1
NPETTPATTSSWTCITAGVTVSASLCPTTKCTSRC	3307	3325	3343	3361	3379	3397	3415	3433	3451	3469	3487	3505
PETTPATTSSWTCITAGVTVSASLCPTTKCTSRC	3192.9	3210.9	3228.9	3246.9	3264.9	3282.9	3300.9	3318.9	3336.9	3354.9	3372.9	3390.9
ETTPATTSSWTCITAGVTVSASLCPTTKCTSRC	3095.8	3113.8	3131.8	3149.8	3167.8	3185.8	3203.8	3221.8	3239.8	3257.8	3275.8	3293.8
TTPATTSSWTCITAGVTVSASLCPTTKCTSRC	2966.6	2984.6	3002.6	**3020.6**	**3038.6**	**3056.6**	**3074.6**	3092.6	3110.6	3128.6	3146.6	3164.6
TPATTSSWTCITAGVTVSASLCPTTKCTSRC	-	2883.5	2901.5	2919.5	2937.5	2955.5	2973.5	2991.5	3009.5	3027.5	3045.5	3063.5
PATTSSWTCITAGVTVSASLCPTTKCTSRC	-	-	2800.4	2818.4	2836.4	2854.4	2872.4	2890.4	2908.4	2926.4	2944.4	2962.4
ATTSSWTCITAGVTVSASLCPTTKCTSRC	-	-	-	2721.3	2739.3	2757.3	2775.3	2793.3	2811.3	2829.3	2847.3	2865.3
TTSSWTCITAGVTVSASLCPTTKCTSRC	-	-	-	2650.2	2668.2	2686.2	2704.2	2722.2	2740.2	2758.2	2776.2	2794.2
TSSWTCITAGVTVSASLCPTTKCTSRC	-	-	-	-	2567.1	2585.1	2603.1	2621.1	2639.1	2657.1	2675.1	2693.1

None of these signals clearly fit to calculated masses that might represent the active Licα-peptide (figure 3.1.9 C; table 3.1.1). In contrast, two masses (2622 Da and 3520 Da) would matched calculated masses of the Licβ-peptide (figure 3.1.9 and table 3.1.1) representing 1. the LicA2 propeptide that has lost the first N-terminal aa and that harbors fifteen dehydrations and 2. a propeptide sequence with eight dehydrations that misses eleven aa at its N-terminus.

Taken together, these MALDI-TOF results along with the results of agar well diffusion assays and stability testings, suggested the production of two different antimicrobials by *B. licheniformis* DSM 13. The antimicrobial substance of the cell wash extract most likely represented the lantibiotic lichenicidin, whereas the antimicrobial activity of the culture supernatant was predicted to reflect an additional, so far uncharacterized substance.

Since *B. licheniformis* is known for its impressive production of seven different proteases, it was hypothesized that lantibiotic peptides might be inactivated within the culture supernatant by proteolytic degradation performed by at least one of these proteases.

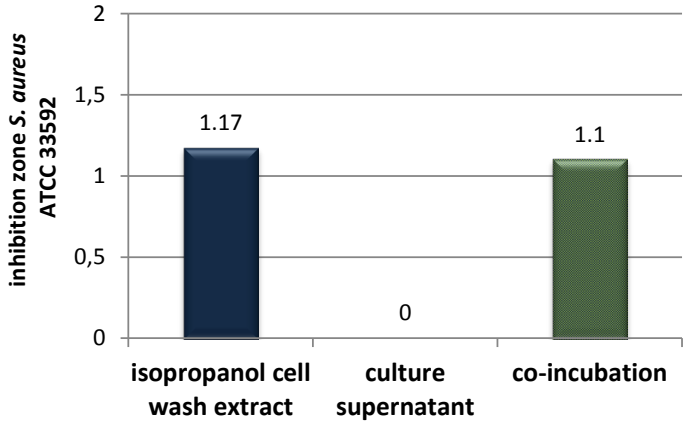

Figure 3.1.10: Co-incubation of cell wash extract and culture supernatant. In agar well diffusion assays, the cell wash extract showed an antimicrobial activity against *S. aureus* ATCC 33592 (**blue bar**) while the culture supernatant was inactive. Co-incubation of both extracts (**blue-green bar**), had no effect on activity of the extract, indicating that the antimicrobial substance was stable against proteases excreted by the producer strain.

Production of proteases under lantibiotic production condition was verified on skim milk agar plates by application of 50 µl per well of protease containing culture supernatant Subsequently, the cell wash extract (25 µl), after evaporation of isopropanol, was incubated with the culture supernatant (25 µl) in order to test protease stability of lichenicidin. After 2 h of incubation, activity against *S. aureus* ATCC 33592 or *S. gallinarum* (data not shown) was not diminished, indicating that the lichenicidin peptides were indeed resistant against proteases excreted by the producer strain (figure 3.1.10).

SDS-PAGE analysis of the pre-purified peptides of *B. licheniformis* cell wash extracts

As indicated by MALDI-TOF analysis of cell wash extracts, *B. licheniformis* DSM 13 produces two most likely cell-surface associated peptides, that might represent the both lichenicidin peptides. For a first characterization of the substance associated with the inhibitory effect of the cell wash extract and in order to correlate the antimicrobial activity to these peptides, bacterial SDS-PAGE overlay assays were performed.

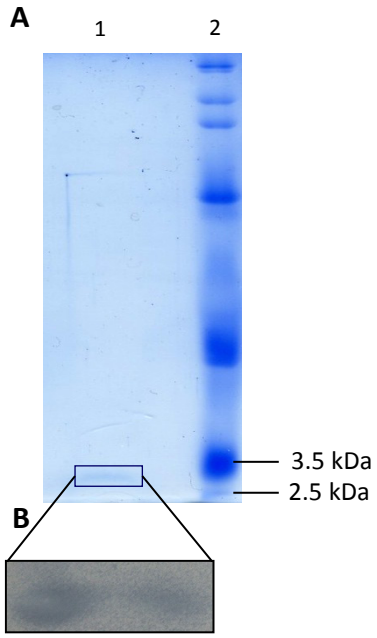

Figure 3.1.11: SDS-PAGE analysis and in situ overlay assay of concentrated and pre-purified peptides of the cell wash extract. After evaporation of isopropanol, the cell wash extract was pre-purified by RP-HPLC. Fractions that were characterized by an antimicrobial activity against *M. luteus* were pooled and concentrated by evaporation. A peptide band (A) of < 3.5 kDa was detected, that caused an activity against *M. luteus in situ* (B).

First peptide purifications by reversed phase HPLC (POROS II column, isopropanol-water gradient with 0.1% TFA) resulted in pre-purification of antimicrobial peptides (data not shown). Fractions, which had been eluted with 36-42% isopropanol were analyzed for their antimicrobial activity against the indicator strain *M. luteus*. To determine the size of the substance responsible for antimicrobial activity, active fractions were pooled, concentrated by evaporation and lyophilization and, finally, were assessed for their protein composition by SDS-PAGE (20%) analysis. Figure 3.1.11A showed presence of a band with a MW < 3.5 kDa, that was correlated to the antimicrobial activity *in situ* by overlaying a corresponding unstained SDS-gel with the indicator strain *M. luteus* (figure 3.1.11 B).

Inactivation of the putative lantibiotic modification enzymes LicM1 and LicM2

In order to clearly link the observed antimicrobial activities and peptide masses of the cell wash extract to the putative lantibiotic gene cluster in *B. licheniformis* DSM 13, the biosynthetic enzymes LicM1 and LicM2 were genetically inactivated. Inactivation was performed by plasmid integration using derivatives of the thermo-sensitive shuttle vector pMAD (Arnaud *et al*,. 2004). The recombinant vector pMADDelLicM1AC carries 5' and 3' parts (each appr. 500 bp) of the gene *bli04128* (*licM1*) and lacks start and stop codon of the gene. Insert fragments were generated *via* PCR using the oligonucleotide pair forΔLicM1fragmentA and revΔLicM1fragmentA for amplification of the 5' part of *licM1* and the primer pair forΔLicM1fragmentC and revΔLicM1fragmentC for the 3' part. PCR products were digested by *Mlu*I, *Bgl*II and *BamH*I following the manufacturer's instructions. Both fragments were ligated (T4 ligase, Fermentas) in tandem into corresponding restriction sites of pMAD. The resulting vector pMADDelLicM1AC was introduced into *E. coli* SCS110 as intermediate cloning host by electroporation.

According to this protocol, a second derivative of pMAD, called pMADLicM2AC, was generated for inactivation of LicM2. 3' and 5' parts of the gene *bli04126* were amplified using the primer pair forDelLicM2A and revDelLicM2A and forDelLicM2C and revDelLicM2C, respectively.

Both recombinant vectors were introduced into *B. licheniformis* MW3 *via* protoplast transformation and transformants were selected at 30°C. Recombinants, that had inserted the plasmids into their genomes, were selected after overnight cultivation at a non-permissive temperature (45°C) in TSB (25mg/l EM) and, subsequent, plating onto TSA agar plates (25 mg/l EM). Insertions of vectors into target genes were confirmed by PCR using primers that anneal within genomic sequences, which flank target genes (forhomRecLicM1,

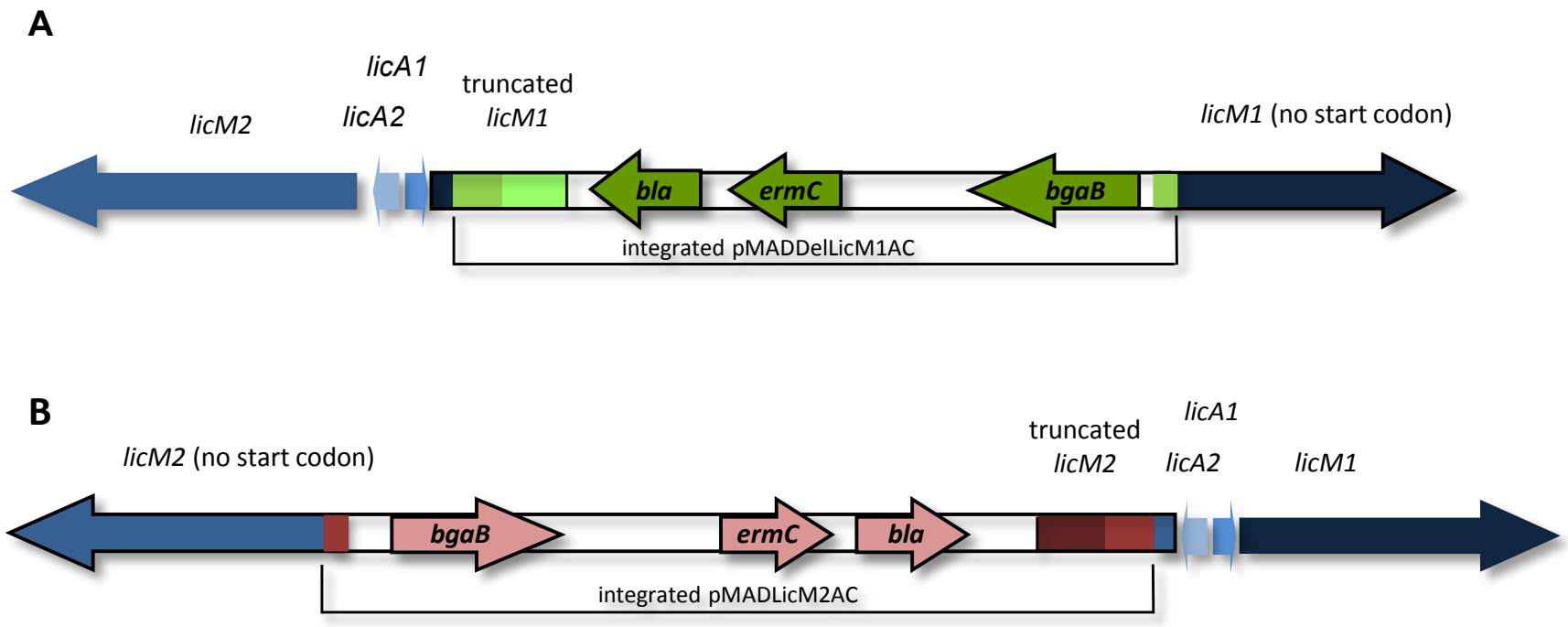

Figure 3.1.12: Gene inactivation of *licM1* and *licM2* was performed by homologous recombination and plasmid integration using the recombinant plasmids pMADDelLicM1AC and pMADLicM2AC. Both integration mutants *B. licheniformis* LicM1INT (A) and LicM2INT (B) harbor two copies of the targeted *lanM* genes: a copy that is transcribed, but is truncated and a second copy that does not dispose of promoter, Shine Dalgarno sequence and start codon. Genes that derive from the plasmid pMADDelLicM1AC are marked in **green** and those deriving from pMADLicM2AC are marked in **red**.

revhomRecLicM1, forhomRecLicM2, revhomRecLicM2) and a primer that anneals within the vector sequence (pMADmcs2neu). Resulting strains were named *B. licheniformis* MW3 LicM1INT and LicM2INT.
Both integration mutants, harbor two copies of the LanM enzyme in their genome (figure 3.1.12). The transcribed copy of *licM1* which is located downstream of *licA1* harbors an internal deletion that leads to loss of 718 aa. The second copy is located downstream of the *bga* gene on the opposite strand and does not dispose of promoter, Shine-Dalgarno sequence and start codon. The first copy of *licM2* leads to transcription of a protein with a C-terminal deletion of 873 aa and the second copy again cannot be transcribed.

Furthermore, integration of the plasmid into BLi04126 was expected to exert a polar effect and to inhibit transcription of the downstream genes of the gene cluster i.e. of the transporter and peptidase, which might be involved in export and processing of both peptides (figure 3.1.12). After inactivating of the LicA1 modifying enzyme, the LicM1 insertion mutant was characterized by loss of activity of the cell wash extract against *M. luteus* in agar well diffusion assays. In contrast, activity of the culture supernatant was not affected. MALDI-TOF mass spectrometry showed loss of a mass signal with 3251.7 Da $(m+1H)^{1+}$ in the cell wash extract of this mutant (figure 3.1.13). After removal of the leader at the conserved GG cleavage site, the unmodified propeptide LicA1 possessed a calculated molecular mass of 3376.9 Da. The missing mass signal might correspond to the mature Licα where post-translational modification resulted in dehydration of seven (-126 Da) of eight possible sites. Furthermore, antibacterial activity of the cell wash extract was also lost in the LicM2 integration mutant. The antibacterial activity in the culture supernatant remained unaffected, indicating that this activity definitely is not related to the lantibiotic gene cluster. MALDI-TOF spectra of the cell wash extract lacked the mass signals $[(m+1H)^{1+}]$ at 3251.7 Da, at 3021.7 and a minor signal at 3039.6 Da that was present in some preparations indicating a correlation of all three masses to the lichenicidin gene cluster (figure 3.1.13). The calculated mass of the unmodified propeptide LicA2 amounts to 3905.3 Da. A mass of 3020.6 Da was predicted for a Licβ-peptide that has lost its six N-terminal aa by proteolytic processing and harbors twelve dehydrated residues and three hydroxy-aa.

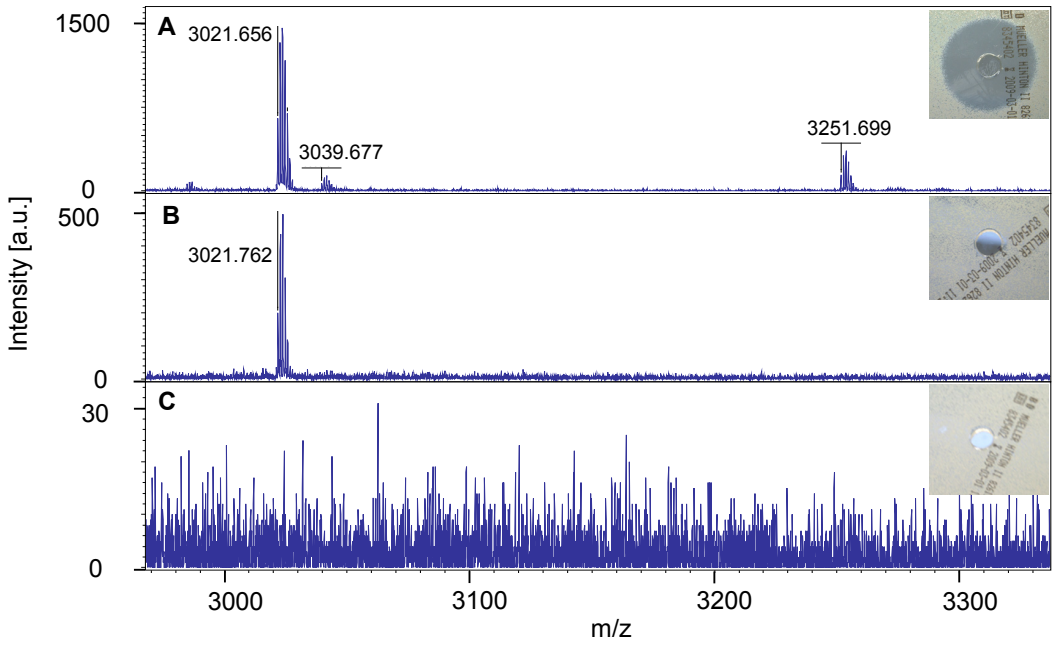

Figure 3.1.13: MALDI-TOF mass spectra of *B. licheniformis* MW3 wild-type (A) and its insertion mutants LicM1INT (B) and LicM2INT (C). Isopropanol extracts of both insertion mutants were characterized by loss of activity against *M. luteus*. MALDI-TOF spectra of these cell wash extracts in comparison to the wild-type (A) showed loss of a mass signal at 3251 Da in the case of the LicM1 insertion mutant (B) indicating that this mass represents the protonated form of the active Licα-peptide. Plasmid insertion into *licM2* inactivated this enzyme and most probably exerted a polar effect on the downstream genes, thus affecting production of both peptides. This mutant did not produce the Licα-peptide and is further characterized by the absence of a 3021 Da peak, which might represent the protonated form of the mature Licβ-peptide, harboring twelve dehydrated residues (C). In some cultivations a further mass signal of 3039 Da was observed, which might denote a Licβ-peptide with only eleven dehydrations.

HPLC purification of lichenicidin

In order to show that the lost masses corresponded to the antibacterial activity, the cell wash extract was fractionated by HPLC and masses of active fractions were determined. 150 ml isopropanol cell wash extract (10 cultivations) were evaporated resulting in approx. 15 ml yellow peptide solution that was applied to a POROS II RP-HPLC column. Active fractions eluted in 36-42% of an isopropanol-water gradient (0.1% TFA), were pooled (22 ml), and lyophilized after evaporation of isopropanol. The resulting pellet was resuspended in 5 ml 5% acetonitrile (0.1% TFA) and applied to a Nucleosil C18 RP-HPLC column.

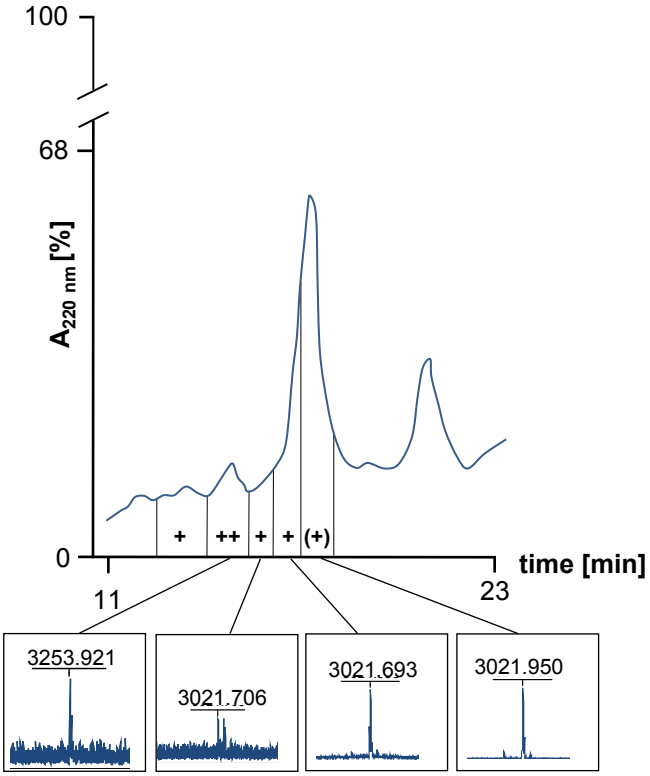

Figure 3.1.14: HPLC chromatogram of the *B. licheniformis* cell extract. Pre-purified cell wash extract was applied to a RP-HPLC column (Nucleosil, C18) and eluted in a gradient of 20-55% acetronitrile (containing 0.1% TFA). MALDI-TOF analysis of active fractions [+ medium activity, ++ strong activity and (+) poor activity] showed presence of masses representing the Licα or the Licβ peptide.

Active fractions eluted in a gradient of 20-55% acetronitrile (containing 0.1% TFA). As shown in figure 3.1.14 the masses for Licα (fraction 9) and Licβ (fraction 10-13) were associated with these active fractions confirming that these peptides represent the two-peptide lantibiotic lichenicidin.

For other two-peptide lantibiotics a synergistic effect of α- and β-peptides has been described. This could also be shown for both lichenicidin peptides. 25 µl of above characterized fractions showed no antimicrobial activity in diffusion assays when tested separately. In contrast, a combination of these (each 25 µl) showed enhanced activity, indicating a synergistic action of both peptides (figure 3.1.15).

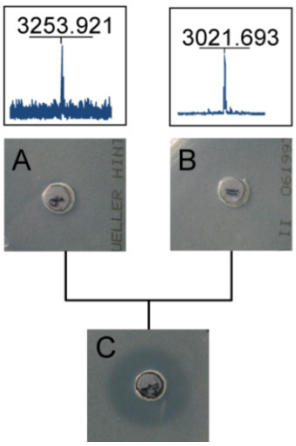

Figure 3.1.15: Synergistic effect of fractions containing Licα- or Licβ-peptides. 25 µl HPLC fractions that contained Licα or Licβ, as determined by mass spectrometry, were tested separately and in combination against *M. luteus*. Only combination of both peptides showed an antimicrobial activity indicating that both, Licα and Licβ, are required for an optimal effect.

Antibiotic efficacy of lichenicidin against MRSA in comparison to other lantibiotics

In order to compare the antibiotic efficacy of lichenicidin against MRSA strains to that of other lantibiotics, solutions containing lichenicidin, mersacidin, Pep5 and nisin were tested against *S. aureus* SG511 (methicillin sensitive strain, MSSA). *S. aureus* ATCC 33592 (methicillin resistant strain; MRSA) and *S. aureus* LT440/09 (community acquired MRSA). All lantibiotics had to be used at higher concentrations to inhibit growth of MRSA strains, compared to that resulting in growth inhibition of MSSA strains; the minimal inhibitory concentrations on agar plates were 2-fold (ATCC 33592) or 4-fold (LT440/09) higher for nisin. 6-fold (both strains) higher for mersacidin, 8-fold higher for lichenicidin (both strains) and 16- (LT440/09) or 32-fold (ATCC 33592) higher for Pep5 than the MIC against *S. aureus* SG511. These results indicated, that the novel lantibiotic lichenicidin exhibits an antibiotic efficacy against MRSA strains that is comparable to that of other lantibiotics, like mersacidin.

Analysis of lichenicidin production by other *Bacilli* strains

An additional *B. licheniformis* strain (I5), which was isolated from the Lake Bogoria (Kenya), was tested under the above mentioned production conditions. Isopropanol wash extracts and culture supernatants were tested against *M. luteus* and analyzed by MALDI-TOF. This strain showed neither production of an antimicrobial substance nor presence of lichenicidin-related masses in the MALDI-TOF spectra. In order to demonstrate presence of a lichenicidin gene cluster, PCRs employing primers that anneal within both biosynthetic enzymes were

performed. No PCR products were detected in agarose gel electrophoresis. Additionally, PCRs using LanM-specific degenerated primers, that were designed to screen for genes that code for lantibiotic modification enzymes of the LanM-type, failed (data not shown), indicating that this *B. licheniformis* strain does not harbor the lichenicidin cluster.

Bacillus strains that were isolated from infested soil samples in this study showed the production of antimicrobially active substances in the cell wash extracts. MALDI-TOF analysis of one of these strains indicated production of two cell-envelope associated peptides with MWs of 3021 Da and 3251 Da (figure 3.1.16), that most probably represent the lantibiotic lichenicidin. In line with this, partial amplifications (500 bp) of the genes coding for the modification enzymes of lichenicidin were obtained by PCR and use of the primer pairs forΔLicM1fragmentA and revΔLicM1fragmentA as well as forΔLicM2fragmentA and revΔLicM2fragmentA (data not shown) thus indicating presence of a lichenicidin cluster in this strain.

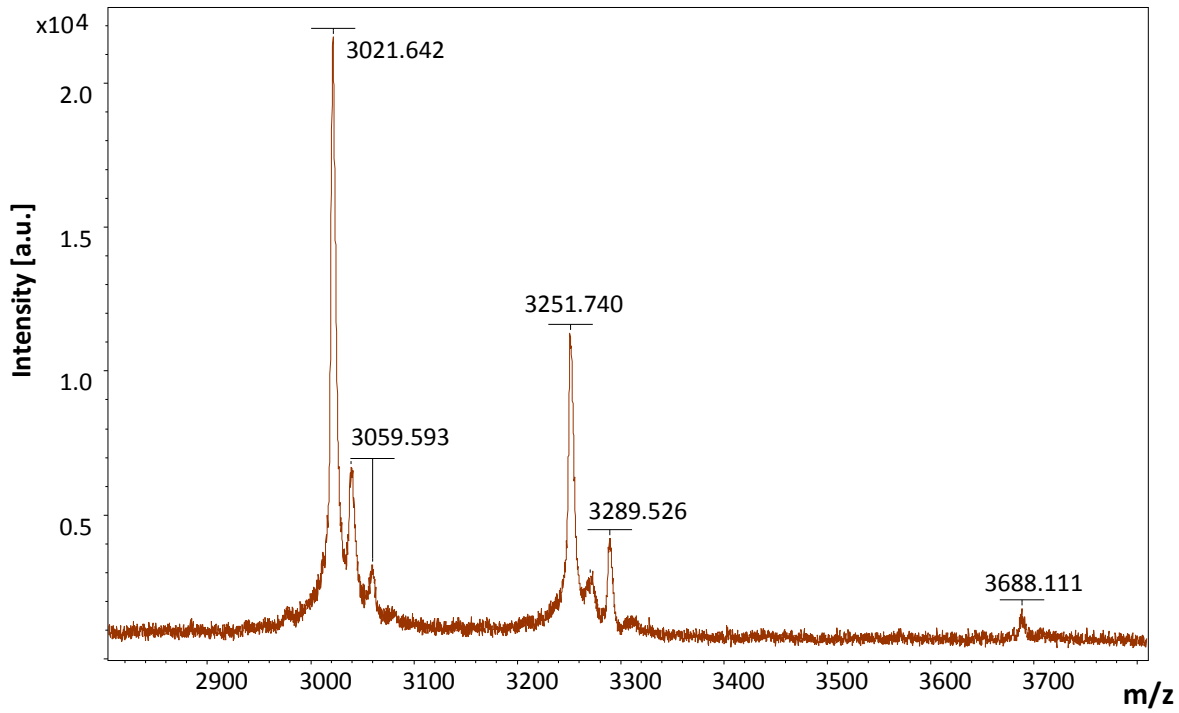

Figure 3.1.16: MALDI-TOF analysis of the isopropanol cell wash extract of a *Bacillus* strain isolated from an infested soil sample. The antimicrobially active cell wash extract was characterized by the presence of two mass signals, 3021 and 3251 Da, indicating the production of lichenicidin.

B: Novel lantibiotic-like gene clusters of *Nostoc punctiforme* ATCC 29133 and *Caldicellulosiruptor bescii* DSM 6725

In addition to the gene cluster coding for lichenicidin, other lantibiotic-like clusters were identified by genomic data mining for novel lantibiotic modification enzymes. Two of those clusters, which were predicted to contain all genes necessary for lantibiotic production and were supposed to be complete clusters, were chosen for a detailed characterization in this thesis. The clusters were found within the genomes of the nitrogen-fixing cyanobacterial strain *Nostoc punctiforme* ATCC 29133 and the Gram-positive clostridial strain *Anaerocellum thermophilum* DSM 6725, that has recently been renamed *Caldicellulosiruptor bescii* sp. nov. DSM 6725 (Yang *et al*., 2010).

Of note, cyanobacteria like *Nostoc* are referred to as Gram-negative bacteria, which had not been considered potential lantibiotic producers until identification of the cyanobacterial lantipeptide prochlorosin in 2010 (Li *et al*., 2010). All other known producers of lantibiotics and lantipeptides are Gram-positive bacteria.

The aim of the present study was the heterologous expression of the HIS-tagged biosynthetic enzymes and their corresponding prepeptides by *Escherichia coli* for subsequent *in vitro* modification assays.

In silico analysis of lantibiotic-like biosynthesis genes found in *Nostoc punctiforme*

Genome sequence annotation identified four LanM-like proteins (Npun_F5047, NpunR3312, Npun_AF076, Npun_R3205) in the genome of *N. punctiforme* ATCC 29133 (Meeks *et al*., 2001). A more detailed analysis of neighboring genes revealed a lantibiotic-like gene cluster (14892 bp; Npun_F5043-Npun_F5052) in vicinity of the LanM-coding gene Npun_F5047 (figure 3.2.2). In agreement with the classical lantibiotic designation scheme, the name puncticin (Pun) was proposed for this gene cluster.

The LanM homolog Npun_F5047 (PunM) showed the highest sequence similarity (app. 70%) to three other LanM proteins identified in the genome of *N. punctiforme* ATCC 29133, and had an aa sequence similarity of 27% to MrsM. Contrary to other LanM, the conserved third zinc binding motif (CHG; Li *et al.*, 2006; Zhu *et al.*, 2003) of PunM appeared to be altered to CCG (figure 3.2.1). A similar aa exchange is present in the sequence of the LanM protein, that modifies the cyanobacterial lantipeptide prochlorosin (ProcM; Li *et al*., 2010). Interestingly, this sequence alteration was found in all LanM-like proteins present in *N. punctiforme.*

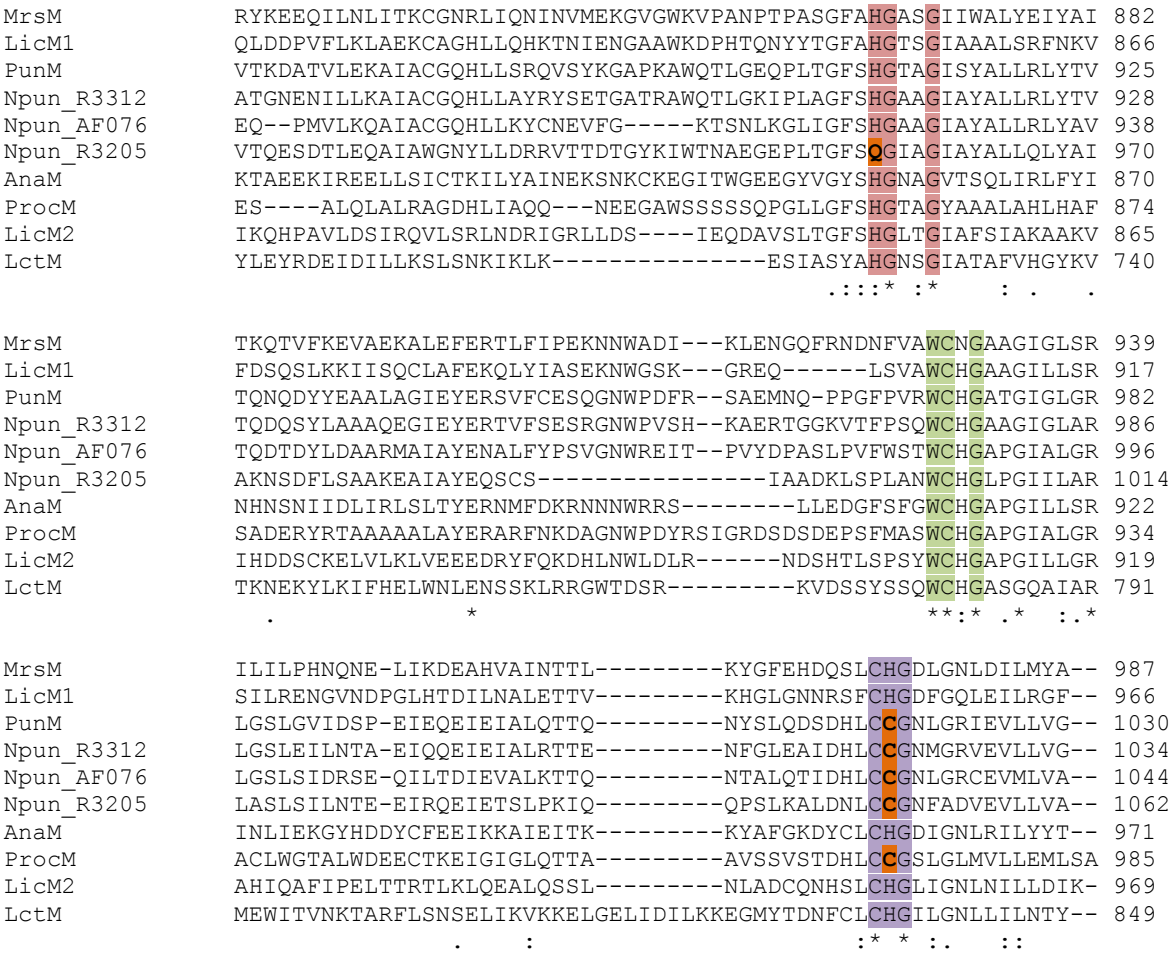

Figure 3.2.1: Partial alignment of aa sequences of (putative) LanM proteins.

AA sequences of lantibiotic modification enzymes of mersacidin (MrsM), lichenicidin (LicM1; LicM2), lacticin 481 (LctM) and of the lantipeptide prochlorosin (ProcM) were compared to those of the LanM-like proteins that were identified within the genomes of *C. bescii* (AnaM) and *N. punctiforme* (PunM, Npun_R3312, Npun_R3205, Npun_AF076). Conserved zinc binding motifs (Paul *et al.*, 2007; Li *et al.*, 2006; Zhu *et al.*, 2003) are marked in violet, red and green. In all putative LanM found in *N. punctiforme,* aa alterations **(orange)** within the third motif were observed. A similar aa alteration was also found in the sequence of ProcM.

Npun_F5045 and Npun_F5046, both coding for hypothetical peptides with a length of 91 aa, appeared to be the corresponding lantibiotic structural genes, *punA1* and *punA2*. They are characterized by a high amount of Cys, Thr and Ser residues, that are predominantly located in their C-terminal sequences. With exception of a putative conserved GG cleavage site, which apparently separates the precursors into extremely long leaders (73 aa) and short propeptide sequences (18 aa), their aa sequence was not similar to that of other lantibiotics. Both peptides have almost similar sequences and only differ in three aa; two of which are within the predicted leaders and one is found within the propeptide sequences. The latter is

located at the far end of the propeptide and results in replacement of the C-terminal Lys (PunA2) by an Arg (PunA1) which is also positively charged. As these aa exchanges are caused by single point mutations in almost all cases, structural genes might have evolved by gene duplication. This hypothesis is supported by the highly similar intergenic regions, that directly flank *punA1* and *punA2* (upstream flanking region: 44 bp 80.4% sequence similarity; downstream flanking region: 120 bp 77%). Three Cys and four hydroxy-aa were found within both predicted peptides and, therefore, formation of up to three thioether-bridges is likely.

Npun_F5050 and Npun_F5052 resemble typical lantibiotic exporters (29-38% sequence similarity). As only Npun_F5050 possesses an N-terminal protease domain, it was believed to be responsible for export and processing of the lantibiotic peptides.

A regulatory function was predicted for proteins encoded by Npun_F5044 and Npun_F5043, due to aa similarity to histidine kinases and transcriptional regulators, respectively.

Npun_F5048, Npun_F5049 and Npun_F5051 had no significant sequence similarity to proteins associated with lantibiotic production. Blast analysis suggested a relation of Npun_F5048 to membrane fusion proteins of the superfamily of HylD-like secretion proteins. Npun_F5049 codes for a hypothetical protein of 116 aa that contains a conserved motif with unknown function, that is discussed to be associated with membrane proteins. An additional membrane fusion protein is encoded by Npun_F5051 that appeared to be homologous to a periplasmatic subunit (ErmA) of an ABC transporter involved in antibiotic efflux. Therefore, these proteins might have a function in immunity or in the export of puncticin.

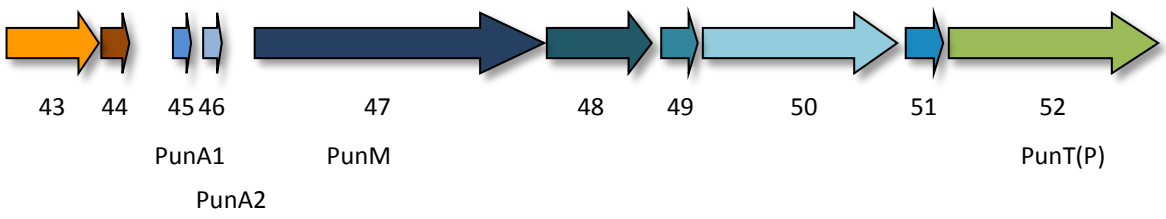

Figure 3.2.2: Predicted lantibiotic-like gene cluster of *N. punctiforme* ATCC 29133 (puncticin)

The putative lantibiotic-like gene cluster covers bp 6247825-6262716 (locus tags Npun_F5043 to Npun_F5052). Genes of the prepeptides (*punA1*; *punA2*) are **light blue**; the gene of the modification enzyme (PunM) is **dark blue**. Other genes are marked as follows: **transporter with protease domain**, **kinase**, **transcriptional regulator**, subunits of ABC transporters and membrane proteins are highlighted in **green-blue** and **turquoise** colors. Numbers give the last two digits of the gene annotations.

The cluster is flanked by Npun_R5042 and Npun_R5053, both of which are on the opposite DNA strand. Npun_R5042 encodes a protein of the cupin 2 barrel superfamily that includes proteins with diverse actions and functions, such as proteins associated with secondary metabolism or non-ribosomal antibiotic biosynthesis machineries. NpunR_5053 is a protein with unknown function. Neither these two, nor other loci in vicinity to the proposed puncticin cluster resemble loci known to be associated with lantibiotic production and, thus, they were not believed to be part of the biosynthesis cluster.

A GC content of 42% was calculated for the cluster, that slightly differs from the GC content of the *N. punctiforme* genome (45.7%). The gene cluster did not appear to be associated with any genetic mobile element, like transposons or IS-elements.

In addition to the PunM of the puncticin gene cluster, three further LanM-like proteins (NpunR3312, Npun_AF076 (plasmid associated), Npun_R3205) were identified in the genome of *N. punctiforme* (table 3.2.1). These *lanM* genes are often flanked by short hypothetical genes that are annotated as Nif11-like peptides. Interestingly, the N-termini of some of these short genes and the leader sequences of both puncticin precursors are similar (up to 65% sequence similarity on aa level; table 3.2.1). Although all peptides contain a putative GG cleavage site, Cys residues are not present in any of the peptides (figure 3.2.3). Therefore, Npun_F3224-26 and Npun_F6115 cannot represent lantibiotic prepeptides and the status of Npun_R3313 and Npun_AF077 is unclear.

Table 3.2.1: Lantibiotic-like biosynthesis and structural genes found in the genome of *Nostoc punctiforme* ATCC 29133.

Protein ID	Similarity to PunA1 leader	*lanM* in vicinity	*Nif11* annotation	Length (aa)
Npun_F5045 (PunA1)	100%	Npun_F5047	-	91
Npun_F5046 (PunA2)	97%	Npun_F5047	-	91
Npun_R3313	65%	Npun_R3312	+	93
Npun_AF077	33%	Npun_AF076	+	103
Npun_F3224	47%	? (Npun_R3205)	+	95
Npun_F3225	44%	? (Npun_R3205)	+	95
Npun_F3226	42%	? (Npun_R3205)	+	97
Npun_F6115	43%	?	+	77

Figure 3.2.3: AA sequence alignment of peptides that share aa sequence similarity to leader sequences of the putative PunA prepeptides. In blast analyses employing leader sequences of PunA1/PunA2, six short, hypothetical peptides were identified in *N. punctiforme*. Some of these peptides appeared to be located next to putative *lanM* genes. Putative leader cleavage sites are marked in **red**, Cys residues are highlighted in **pink** and Ser/ Thr in **blue**.

In silico analysis of lantibiotic-like gene cluster *of C. bescii* DSM 6725

A putative lantibiotic modification enzyme of the LanM-type was identified in the genome of the completely sequenced strain *C. bescii* DSM 6725 (Athe_1107; Kataeva *et al*., 2009). The corresponding aa sequence harbored LanM-typical active sites and zinc binding motifs (figure 3.2.1) and showed up to 33% aa identity to the lantibiotic modification enzymes HalM1 (haloduracin), LicM1 (lichenicidin) and MrsM (mersacidin).

In vicinity to this predicted LanM, additional ORFs were located, that shared significant aa sequence similarity with proteins or functions involved in production of lantibiotics. Together, these genes (Athe_1106-1112; bp 1199478-1208568) may form a gene cluster coding for a novel lantibiotic named besciin. In the following, the designation Ana is used for genes associated with this cluster.

A putative *anaA* structural gene (265 bp) is encoded by the ORF Athe_1106. The prepeptide consists of 95 aa and contains three Gly residues (41-43 bp) that might include the typical GG cleavage site. The predicted AnaA sequence exhibited an aa similarity of about 40% to the LanA1 precursor of lichenicidin (LicA1) and to the predicted precursor of ruminococcin B

(figure 3.2.4 B). This similarity was mainly restricted to the leader sequences. Besides, the precursor and both lantibiotics had only low aa similarity. Interestingly, conserved residues included three Cys, three hydroxy-aa and a conserved Glu residue, that resembles the typical lipid II binding motif of class II lantibiotics. This similarity raised the question, whether the C-terminus of the active besciin peptide might have a thioether bridging pattern similar to the conserved bridging patterns of LanA1 and mersacidin-like peptides. Since AnaA contains additional hydroxy-aa in this region, the bridging pattern could not be predicted clearly.

The putative propeptide (53/52 aa) is extremely long compared to other lantibiotics. The presence of three Cys residues and a total of twelve hydroxy-aa (six Ser and six Thr) allows introduction of up to three thioether-aa and twelve dehydrations (figure 3.2.4 A). A net positive charge of 1.88 was calculated at pH7.

A

MKESTIIKNPVLRNKVNAKIYNPAGDIVKEIQEQNLPEQAGGGTPTVVVGVISAVTAVTNLAFSIDQA
ITKYYACSLVYTYSAECRSDGRSCRMR

B

```
LicA1           MSKKEMILSWKNPMYR---TESSYHPAGNILKELQEEEQ-HSIAGGTITLSTCAILSKP- 55
RumB            MKKN-----YRNPMTR---PENFMNPAGNVMKEIKEADL-NNFSAG---------AGEPR 42
AnaA            MKES---TIIKNPVLRNKVNAKIYNPAGDIVKEIQEQNLPEQAGGGTPTVVVGVISAVTA 57
                *.:.      :**: *     .  :***:::**::* :  .. ..*          . .

LicA1           LGNNGY----------LC----TVTKEC-----MPSCN-- 74
RumB            VSDGSQ----------FC----TSTKECNWGTIMFVCC-- 66
AnaA            VTNLAFSIDQAITKYYACSLVYTYSAECR--SDGRSCRMR 95
                : : .            *    * : **        *
```

Figure 3.2.4: AA sequence of the predicted AnaA precursor (A) and alignment (B) with the lantibiotic prepeptides of lichenicidin (LicA1) and ruminococcin B (RumB). (Putative) leader cleavage sites are marked in **red**. For the AnaA precursor, Cys residues are highlighted in **pink** and Ser/ Thr in **blue**. For LicA1, residues involved in (Me)Lan bridge formation are highlighted in colored boxes, whereas subsidiary residues of one thioether aa are marked in the same color. Hydroxy-aa that are dehydrated to Dha and Dhb in the active peptide Licα are marked in **orange**.

Athe_1109-11 code for ATPase (Athe_1109) and permease (Athe_1110 + ATHE_1111) subunits of ABC-transporters and, thus, might possibly play a role in assembly of a LanEFG immunity transporter. The gene product of *athe_1112* (737 aa) resembles a typical class II

lantibiotic exporter containing an internal protease domain. A blast analysis, demonstrated up to 41% sequence identity to exporters of the lantibiotics haloduracin and lichenicidin.
Athe_1108 is annotated as FMN-reductase and was predicted to be an NADPH-dependent dehydrogenase. It had no sequence similarity to proteins involved in biosynthesis of lantibiotics. Enzymes of the FMN-reductases superfamily are associated with production and modification of another class of ribosomally synthesized peptides, the TOMMs. Some TOMM gene clusters had been shown to hold similarity to lantibiotics regarding 1. the presence of LanM-like modifications enzymes and 2. the leader structures of the precursors (Haft *et al*., 2010). Therefore, the aa sequence of Athe_1108 was compared to FMN dehydrogenases present in gene clusters of typical TOMM peptides (e.g. microcin B17, streptolysin S, thiopeptides TP-1161, heterocycloanthracin, plantazolicin, trunkamide), but no significant aa similarity was found. In addition, no other modification enzymes usually found in TOMM-like gene clusters, scaffold proteins or cyclodehydratases involved in class specific peptide modifications, were detected in the genome of *C. bescii*.
Mersacidin is modified by an FMN-dependent decarboxylase (MrsD), which catalyzes the introduction of the C-terminal AviMeCys residue. The sequence of Athe_1108 is dissimilar to that of MrsD and the corresponding propeptide sequence of AnaA does not contain a C-terminal Cys residue, thus raising the question, whether this enzyme might represent a new type of lantibiotic biosynthesis enzyme. In line with these findings, a blast analysis with the ORF Athe_1108 identified as yet uncharacterized LanM- and LanA-like proteins in vicinity of homologous FMN-reductases in *Bacillus cereus* AH621 (38%), *Clostridium botulinum* H04402 065 (35%), *B. cereus* SJ1 (34%) and *Clostridium beijerinckii* NCIMB 8052 (34%). The same blast analysis did not find a homologous FMN-reductase in any known lantibiotic producer.

At both ends, the predicted besciin gene cluster is flanked by two almost identical GCN5-related N-acetyltransferase coding genes (91% DNA, 98% aa sequence identity) that both are located on the opposite DNA strand. Proteins of this N-acetyltransferase family perform diverse functions such as promotion of antibiotic resistance, but no correlation of these enzymes and lantibiotic production has been reported up to now. Therefore, these genes probably do not belong to the besciin gene cluster. This notion is supported by presence of quite similar genes in other *Caldicellulosiruptor* strains with up to 94% aa sequence similarity - a feature that is uncommon for genes involved in production of lantibiotics. Additionally, in these strains GCN5-related N-acetyltransferase coding genes are not co-localized with LanM-like enzymes or other genes associated with lantibiotic production.

Taken together, the besciin gene cluster (figure 3.2.5) comprises the structural gene *anaA*, along with one modification enzyme of the LanM-type (AnaM;), an additional gene with an unknown function, ABC transporter subunits with similarity to lantibiotic immunity-transporters and an exporter containing a protease domain (AnaT(P)).

The GC content of the putative lantibiotic cluster was calculated to be 28.7% and differed significantly from the average GC content (35.2 %) of the whole genome suggesting an uptake by horizontal gene transfer. Presence of two nearly identical flanking regions and absence of associated mobile elements might indicate integration by homologous recombination.

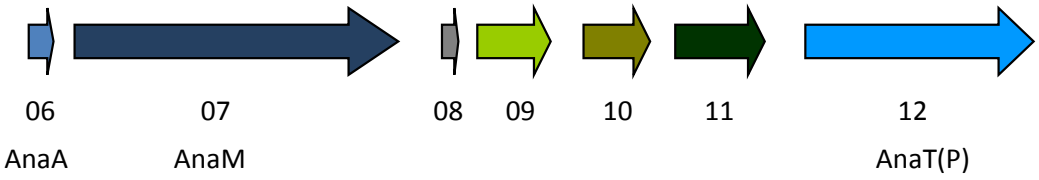

Figure 3.2.5: The lantibiotic-like gene cluster of *C. bescii* DSM 6725 (besciin)

The lantibiotic-like gene cluster covers bp 1199478-1208568 (locus Athe_1106 to Athe_1112). The gene of the predicted prepeptide AnaA is **light blue**; the gene of the modification enzyme (AnaM) is dark blue. Other genes are marked as follows: **transporter** with protease domain, subunits of ABC transporters and membrane proteins are highlighted in **green** colors. A gene with an unknown function is marked in **grey**. Numbers give the last two digits of the locus tags.

Introduction of a XA-factor cleavage site into lantibiotic-like precursors

In order to facilitate removal of the leader sequence subsequently to *in vitro* modification and processing assays, an engineered XA-factor protease cleavage site was introduced into the structural genes found in *N. punctiforme* and *C. bescii*.

The putative structural genes coding for puncticin (*punA1*) and besciin (*anaA*) were introduced into pUC19 (2686 bp). PCR employing the primer combination forAnaApUC19 and revAnaApUC19 resulted in amplification of a fragment including *anaA* (285 bp) and downstream (53 bp) and upstream (40 bp) flanking sequences. Using primers forPunA1pUC19 and revPunA1pUC19, *punA1* itself (276 bp) and flanking regions (68 bp at the 5'end; 25 bp at the 3'end) were amplified. The PCR products were digested by *Eco*R1 and *Hind*III. Vector and insert were ligated using T4 ligase (Fermentas) and were directly

transferred in *E. coli* JM109. Plasmid integrity of pUC19AnaA and pUC19PunA1 was confirmed by sequencing of both inserts.

Finally, these recombinant plasmids were used as templates in a site directed mutagenesis approach using the QuickChange™ Site-Directed-Mutagenesis Kit (Agilent). For introduction of cleavage sites specific for XA-factor proteases, the natural GG protease cleavage sites and two aa upstream were replaced by the site specific recognition sequence (IEGR) of the XA-factor protease according to the protocol for *in vitro* modification and processing of the heterologous expressed haloduracin peptides (McClerren *et al*., 2007; table 2.5).

Corresponding mutations were introduced by mutagenic primers, AnaA_XA_Muta_sense and AnaA_XA_Muta_antisense for the *anaA* gene, or PunA1_XA_Muta_sense and PunA1_XA_Muta_antisense for mutagenesis of PunA1. The resulting plasmids harboring the desired mutations were confirmed by sequencing of the inserts and were named pUC19 AnaA_XA and pUC19PunA1_XA.

Table 3.2.2: Introduction of XA-factor cleavages sites into PunA1 and AnaA by primer mutagenesis.

AnaA												
aa sequence within AnaA		**Q**			**A**			**G**			**G**	
DNA sequence	C	A	A	G	C	T	G	G	T	G	G	T
position in *anaA*	115	116	117	118	119	120	121	122	123	124	125	126
position in pUC19AnaA	405	406	407	408	409	410	411	412	413	414	415	416
desired aa sequence		**I**			**E**			**G**			**R**	
mutated DNA sequence	**A**	**T**	A	G	**A**	**A**	G	G	T	**A**	G	**A**
PunA1												
aa sequence within PunA		**V**			**S**			**G**			**G**	
DNA sequence	G	T	T	A	G	T	G	G	A	G	G	A
position in *punA1*	208	209	210	211	212	213	214	215	216	217	218	219
position in pUC19PunA1	677	678	679	680	681	682	683	684	685	686	687	688
desired aa sequence		**I**			**E**			**G**			**R**	
mutated DNA sequence	**A**	T	T	**G**	**A**	**G**	G	G	**A**	A	G	A

Construction of recombinant pET vectors for heterologous expression of lantibiotic-like biosynthesis genes of *C. bescii* and *N. punctiforme* in *E. coli*

For heterologous expression of the putative *lanM* and *lanA* genes of *N. punctiforme* and *C. bescii*, the putative structural genes (*anaA*; *punA1)* and both LanM-coding sequences (*anaM*; *punM*) were cloned into the expression vectors pET28b and pET22b, respectively.

(A) Cloning of the structural genes *anaA* and *punA1* and their mutated variants into pET28

In order to obtain N-terminally HIS-tagged prepeptides by heterologous expression in *E. coli, punA1* and *anaA* and their mutated variants, were cloned into pET28b. PunA1 and PunA1_XA were amplified by the primer pair forPunApET28 und revPunA1pET28. For amplification of the AnaA coding ORF, the primers forAnaApET28 and revAnaApET28 were employed. PCR fragments lacking the original start codon were subsequently digested by *Nde*I and *Xho*I and ligated into corresponding restriction site of pET28. The resulting expression vectors were named pET28AnaA, pET28AnaA_XA, pET28PunA1 and pET28PunA1_XA and were confirmed by sequencing. Finally, recombinant vectors were introduced into the expression strains *E. coli* Bl21 and *E. coli* C43.

(B) Cloning of PunM and AnaM into pET22

Genes coding for modification enzymes of the lantibiotic-like gene clusters of *N. punctiforme* (PunM) and *C. bescii* (AnaM) were introduced into pET22b. Complete amplification of the PunM coding ORF was achieved using primers forpET22HisPunM and revpET22HisPunM. The resulting PCR fragment was 3318 bp long and lacked a terminal stop codon. In a complimentary approach, the target gene was cloned stepwise, by insertion of two fragments (4-2150 bp; 2150-3466 bp), into pET22b. To this end, the primer pair forpET22HisPunM and revPunMBamHIMUT was used to amplify the N-terminal part of the gene (4-2150 bp). The C-terminal *punM* sequence (2150-3466 bp) was amplified by PCR employing the primers forPunMBamHIMUT and revpET22HisPunM. An additional restriction site for *BamH*I (2148-2153 bp) was introduced into *punM* by primer mutagenesis that generated a silent mutation at position bp 2148 (C→G; table 3.2.3).

Table 3.2.3: Introduction of an additional *Bam*H1 restriction site into *punM*.

aa sequence within PunM	**L**			**D**			**P**		
DNA sequence within *punM*	C	T	C	G	A	T	C	C	T
position (bp)	2146	2147	2148	2149	2150	2151	2152	2153	2154
mutated sequence	C	T	**G**	G	A	T	C	C	T
***Bam*H1 site**			**G**	**G**	**A**	**T**	**C**	**C**	

Cloning of *anaM* was performed likewise. The complete AnaM coding sequence without the stop codon was amplified with the primers forAtheMHispET22 and revAtheMHispET22. In a complimentary approach, the target gene was cloned stepwise, by insertion of two fragments (4-1157 bp; 1157-3306 bp) into pET22b. An N-terminal fragment of *anaM* was generated with the primer combination forAtheMHispET22 and revAnaMEcoR1. PCR using primers forAnaMEcoR1 and revAtheMHispET22 generated a C-terminal fragment. A "G to A" exchange at position 1155 bp by primer mediated mutagenesis, introduced an additional *EcoR*1 restriction site into *anaM* (bp 1153-1158; table 3.2.4).

Table 3.2.4: Introduction of an additional *Eco*R1 restriction site into *anaM*.

aa sequence within AnaM	**R**			**I**			**L**		
DNA sequence within *anaM*	A	G	G	A	T	T	C	T	C
position (bp)	1153	1154	1155	1156	1157	1158	1159	1160	1161
mut. sequence	A	G	**A**	A	T	T	C	T	C
***Eco*R1 site**		**G**	**A**	**A**	**T**	**T**	**C**		

Successful ligations were performed using T4 Ligase (Roche) overnight. Dephosphorylated vector backbones and inserts were incubated in an 1:5 ratio. Subsequently, *E. coli* JM109 or JM83 were transformed with these ligation samples.

Recombinant vectors carrying the coding sequences for PunM or AnaM could be generated by two-step cloning of two *lanM* fragments only and the resulting vectors were named pET22PunMAB and pET22AnaMAB. After confirmation of plasmid integrity by sequencing, recombinant plasmids were finally transferred into the expression hosts *E. coli* Bl21 or *E. coli* C43 for overexpression.

First attempts to purify of heterologous expressed, HIS-tagged lantibiotic precursors and biosynthetic enzymes by NI-NTA affinity chromatography

Heterologous expression of LanM-like proteins and LanA-like peptides was attempted according to the expression and Ni-NTA purification protocols published by McClerren *et al.* (2006). As positive control, the N-terminally HIS-tagged mersacidin modification enzyme was expressed by the expression host *E. coli* Bl21 pET28MrsM.

Successful purification of HIS-tagged MrsM was achieved with the LanM expression and purification protocol of McClerren *et al*. (2006). Eluates were analyzed by SDS-PAGE gel electrophoresis (figure 3.2.6) and showed expression and purification of a protein with app. 120 kDa that matched with the calculated mass for HIS-tagged MrsM protein (123 kDa; appendix page 167).

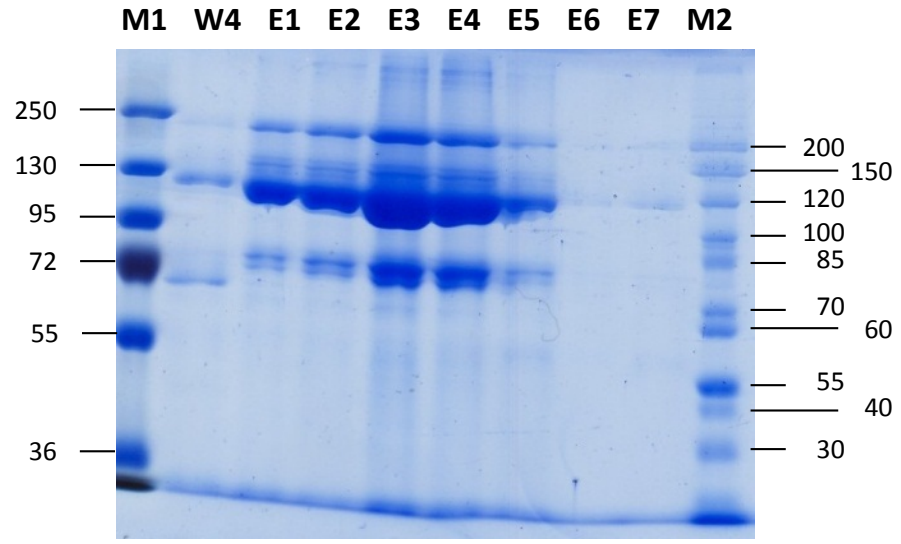

Figure 3.2.6: SDS-PAGE analysis of HIS-tagged MrsM purified by Ni-NTA affinity chromatography.
HIS-tagged MrsM protein was expressed in *E. coli Bl21* pET28MrsM (1 l LB, 1 mM IPTG, 20 h, 18°C) and purified according to the LanM purification protocol of McClerren *et al*., 2006. Purified proteins were analyzed by SDS-PAGE gel (10%) electrophoresis and stained with PageBlue™ protein staining solution (Fermentas). Lane M1 = prestained protein marker (kDa), lane W4 = wash fraction 4, lanes E1-E7 = eluates, lane M2 = unstained protein marker (kDa).

HIS-tagged PunM and AnaM could not be purified under the previously established expression and purification conditions employing *E. coli* Bl21 as expression host. Growth curves of PunM or AnaM expressing *E. coli* Bl21 strains were compared to that of *E. coli* Bl21 pET28MrsM (figure 3.2.7). For the latter strain, a final optical density of 2.5 was obtained in expression cultures, whereas significantly lower OD_{600} values of 1.9 and 1.4, respectively, were observed for PunM and AnaM expression strains 20 h after induction. Additionally, a slower growth rate indicated a cell lytic effect, as did a decrease in cell density of the *E. coli* Bl21 pET22AnaMAB expression culture 4 h after induction of protein expression.

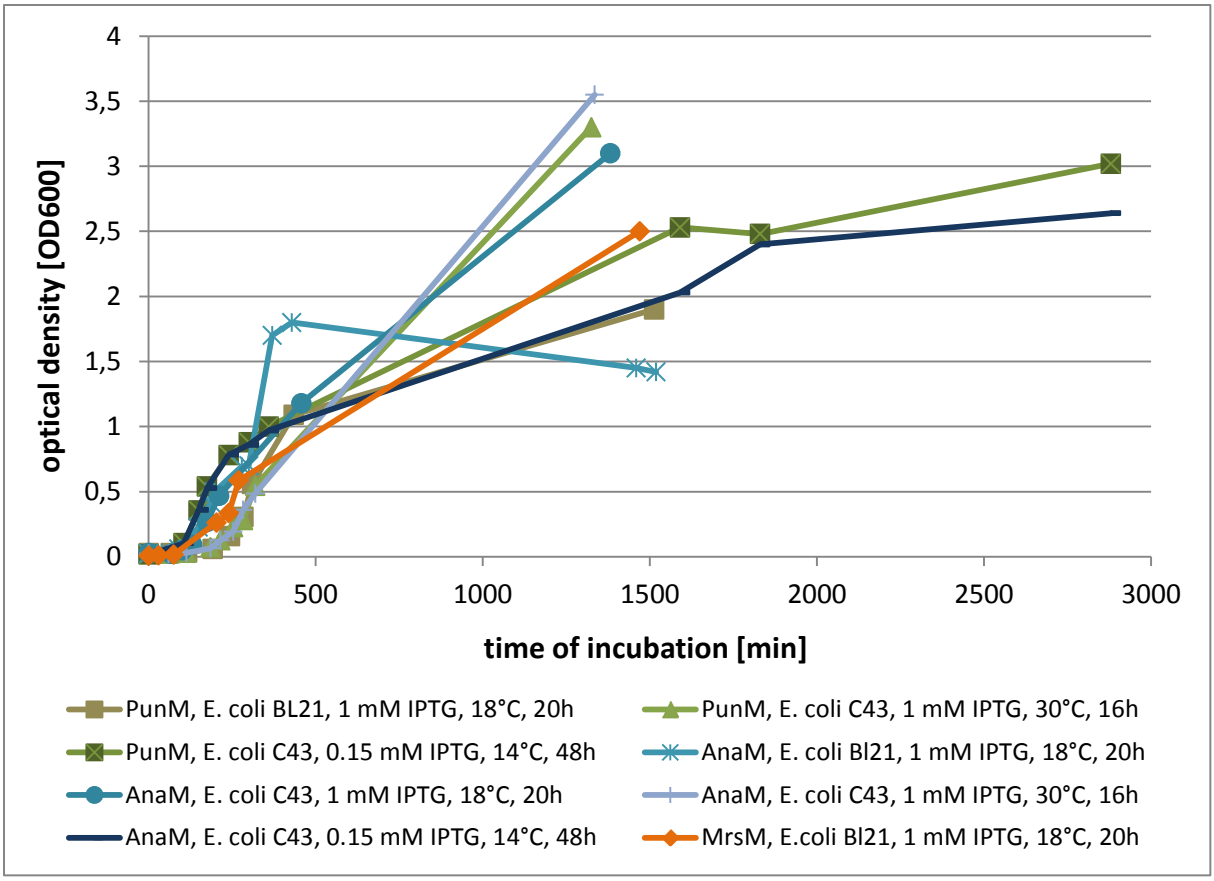

Figure 3.2.7: Growth curves of LanM expressing cultures. Growth of *E. coli* Bl21 and *E. coli* C43 cultures harboring the recombinant vectors pET22AnaMAB and pET22PunM were monitored in comparison to the MrsM expressing strain *E. coli* Bl21 pET28MrsM after induction of protein expression. In contrast to *E. coli* Bl21 expressing MrsM and all tested *E. coli* C43 strains, expression of the recombinant PunM and AnaM proteins apparently caused cell lysis of *E. coli* Bl21 due to protein toxicity of overexpressed proteins as indicated by slower growth rates and lower cell densities.

Based on these results, it was suggested that the recombinant AnaM and PunM proteins might be toxic to *E. coli* Bl21 when overexpressed. Therefore, pET22AnaMAB und pET22PunMAB were transferred into *E. coli* C43, which is optimized for overexpression of toxic proteins.

HIS-tagged AnaM and PunM proteins were expressed in these hosts and the bacterial growth of corresponding expression cultures was compared under different induction and expression conditions (figure 3.2.7). *E. coli* C43 clones were grown in 1 l LB in presence of the selection marker ampicillin (40 mg/l) and under following conditions: 1. overexpression was induced at OD_{600} 0.5 with a final concentration of 1 mM IPTG. Cells were cultivated for additional 20 h at 18°C (according to McClerren *et al.*, 2006); 2. cultures were induced at

OD_{600} 0.5 and subsequently grown for 16 h at 30°C (according to Türck and Bierbaum, 2012); 3. protein expression was induced with 0.15 mM IPTG at OD_{600} 0.6-0.8 and cells were grown for 48 h at 12°C (Müller *et al*., 2011). Under these tested conditions, all *E. coli* C43 mutant strains reached a final OD of 3-3.5 (figure 3.2.7) indicating that toxicity of overexpressed AnaM and PunM might be overcome in these expression strains proteins.

The resulting cell pellets were purified by Ni-NTA affinity chromatography under native conditions (LanM purification protocol of McClerren *et al*., 2006), denaturative conditions (LanA purification protocol of McClerren *et al*., 2006) or using a purification protocol for membrane proteins according to Türck and Bierbaum (2012). Unfortunately, purification of HIS-tagged AnaM and PunM was not successful.

Following the same expression and purification strategies, overexpression of both lantibiotic-like precursors, AnaA and PunA1, and their mutated variants, AnaA_XA and PunA1_XA, was aimed in *E. coli* Bl21. No toxic effect could be observed after induction of protein overexpression in *E. coli* Bl21 mutants. Finally, successful heterologous production of HIS-tagged PunA1_XA and AnaA_XA was achieved in 500 ml cultivation of *E. coli* Bl21 in presence of the selection marker kanamycin (25 mg/l). Overexpression was induced by 1 mM IPTG and followed by incubation at 37°C for 3 h. Ni-NTA purification was performed according to McClerren *et al*., 2006. Eluates were analyzed by Nu-PAGE gel electrophoresis (4-12% Bis-Tris gradient gel, MES buffer, Invitrogen; figure 3.2.7 (A) and (B)) and showed for both batches expression and purification of peptides with a mass <15 kDa that matched with the calculated masses of the HIS-tagged PunA1_XA (12.077 kDa; appendix page 165) and AnaA_XA (12.633 kDa; appendix page 166) peptides. Additionally, in both gels bands with app. 24 kDa were observed that might represent dimers of the HIS-tagged precursors.

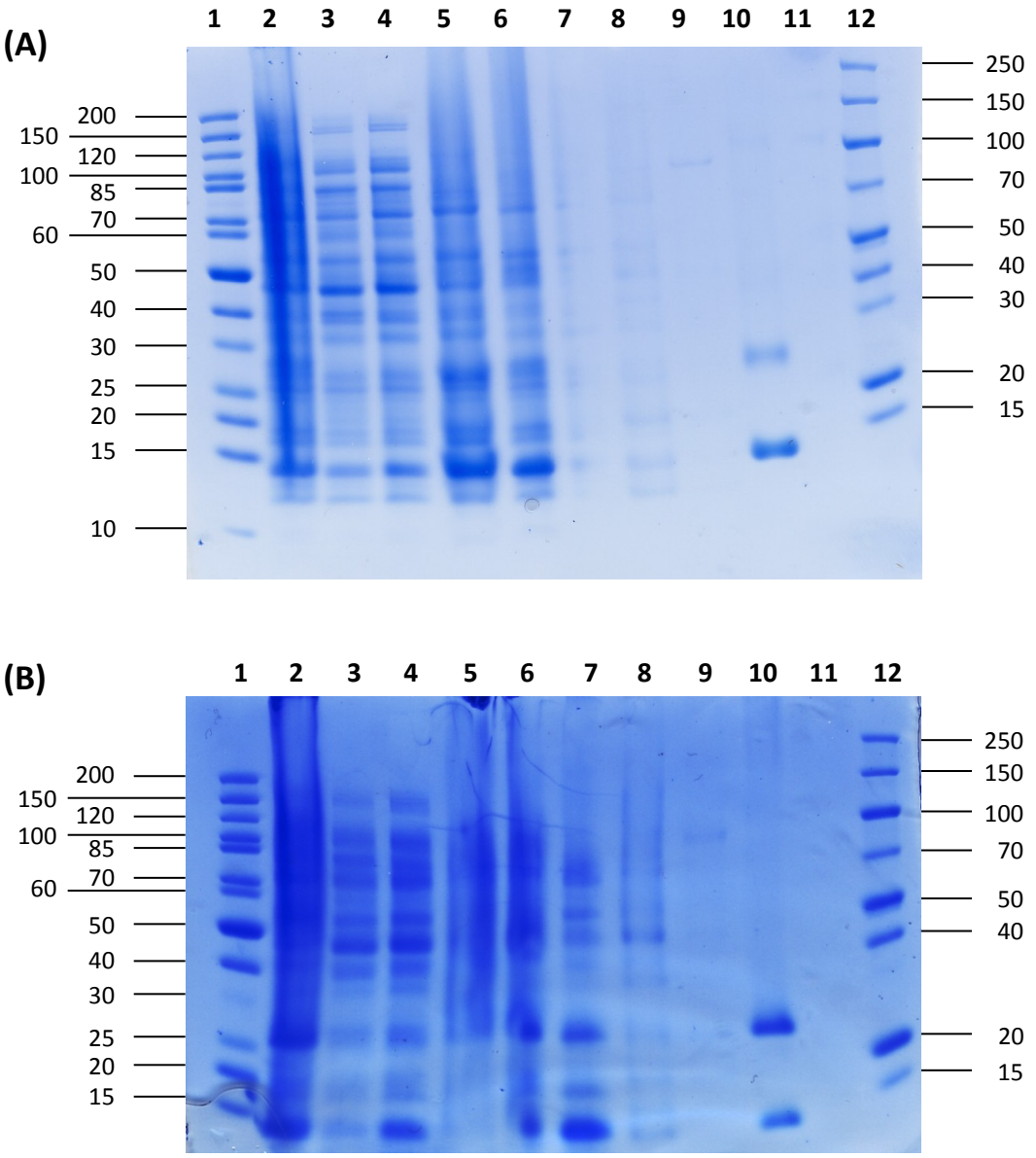

Figure 3.2.7: Nu-PAGE analysis of heterologous expressed and N-terminally HIS-tagged PunA1_XA (A) and AnaA_XA peptides (B) purified by Ni-NTA affinity chromatography.

HIS-tagged precursors were expressed in *E. coli* Bl21 pET28PunA1_XA and pET28AnaA_XA (0.5 l LB, 1 mM IPTG, 3 h, 37°C) and purified according to the LanA purification protocol of McClerren *et al.*, 2006. Purified proteins were analyzed by Nu-PAGE gel (Bis-Tris 4-12% gradient gel) electrophoresis and stained with PageBlue™ protein staining solution (Fermentas). Lane 1 = protein marker SM0661 (Fermentas; kDa), lane 2 = cell lysat; lane 3 = supernatant after first sonification step; lane 4 = supernatant after second sonification; lane 5 = pellet after third sonification; lane 6 = pellet after centrifugation of combined supernatants; lane 7 = flow through; lane 8 = wash step 1; lane 9 = wash step 2; lane 10 = combined eluates 1-4; lane 11 = combined eluates 5-8; lane 12 = protein marker SM1881 (Fermentas; kDa).

PART 2: Mersacidin production by *Bacillus amyloliquefaciens* FZB42

Introduction and aim of the project

Mersacidin, a class II lantibiotic, is one of the best characterized lantibiotic peptides. It consists of 20 aa, of which nine are subjected to post-translational modifications that result in four intramolecular rings and a globular structure (figure 3.3.1; Chatterjee *et al.*, 1992). The active lantibiotic peptide has a MW of 1825 Da.

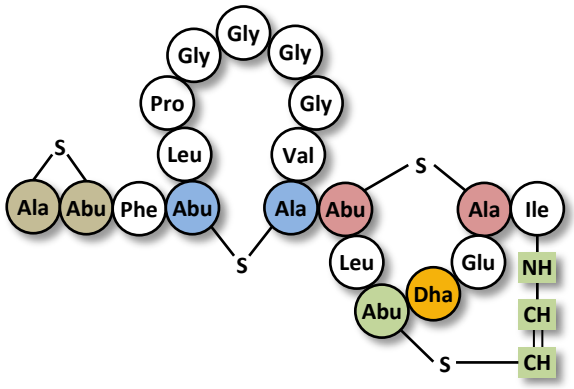

Figure 3.3.1: Structure of mersacidin

Mersacidin consists of 20 aa and has four intramolecular rings including three methyllanthionines and a C-terminal aminovinyl-methyl-D-cysteine.

Mersacidin is produced by a not yet closer characterized *Bacillus* strain called *B.* sp. HIL Y-85,54728 which is not naturally competent (Chatterjee *et al*., 1992). In order to enable genetic manipulation of the mersacidin gene cluster, this project mainly focused on the transfer of the mersacidin biosynthetic gene cluster (figure 3.3.2 A) to an easily transformable *Bacillus* strain.

Total genome sequencing identified a partial mersacidin cluster in the genome of the naturally competent *B. amyloliquefaciens* FZB42 (Chen *et al*., 2009). This partial cluster contains the genes *mrsE;F;G;R2;K2* that are involved in producer self-protection (figure 3.3.2 B). *MrsFGE* encode an ABC transporter that inhibits binding of mersacidin to producer cells (Guder *et al*., 2002), and *mrsK2R2* is a two-component regulatory system that induces expression of *mrsFGE* in presence of mersacidin (Schmitz *et al*., 2006). The biosynthesis part of the mersacidin gene cluster (*mrsA;M;D;R1;T*) was transferred into *B. amyloliquefaciens*, resulting in strain *B. amyloliquefaciens* mrs1 (figure 3.3.2 C; A.M. Herzner, diploma thesis, 2008). This mutant harbors a complete mersacidin gene cluster with exception of the structural gene *mrsA,* which is replaced by an erythromycin resistance cassette (*ermB)* that served as selection marker.

Comparison of DNA sequences, including 16S rRNA genes, and biochemical testings of *B. amyloliquefaciens* FZB42 and *B. sp*. HIL Y-85,54728 indicated that the latter might be a member of the species *B. amyloliquefaciens* (A.M. Herzner, diploma thesis, 2008).

The study presented hereafter aimed a complete reconstitution of the mersacidin gene cluster in *B. amyloliquefaciens* mrs1 to allow production of mersacidin by this mutant. Furthermore, the relatedness of the mersacidin wild-type producer and the *amyloliquefaciens* group were evaluated more in detail.

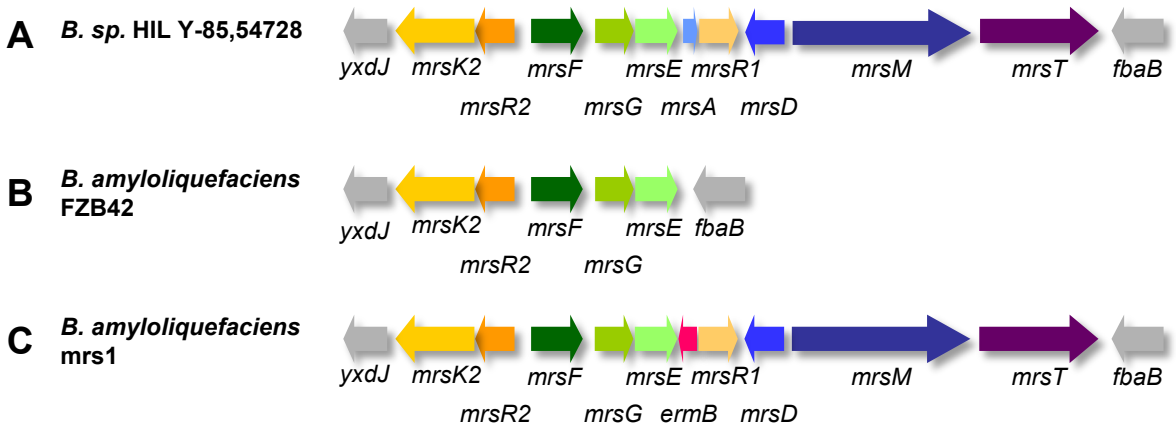

Figure 3.3.2: (Partial) mersacidin gene clusters of *B.* sp. HIL Y-85,54728 (A; wild-type producer), *B. amyloliquefaciens* FZB42 (B) and the mutant *B. amyloliquefaciens* mrs1 (C).

(A) The mersacidin gene cluster (12.3 kb; Altena *et al*., 2000) consists of immunity genes *mrsFGE* (green), the structural gene *mrsA* (light blue), modification enzymes *mrsD;M* (dark blue), an exporter containing a protease domain *mrsT* (purple) and regulatory genes *mrsR1;R2;K2* (yellow and orange).

(B) *B. amyloliquefaciens* FZB42 harbors a partial mersacidin gene cluster consisting of immunity genes *mrsFGE* and regulatory genes *mrsK2;R2* that are found at the same site as in the original producer strain.

(C) In *B. amyloliquefaciens* mrs1, competence transformation using genomic DNA of a mersacidin deletion mutant (*B*. sp. HIL Y-85,54728 Rec1; Schmitz *et al*., 2006) resulted in a partial completion of the mersacidin gene cluster. The gene *mrsA* is replaced by *ermB*. *MrsR1,* most probably, is not transcribed due to a polar effect.

Bioinformatic analysis of the partial mersacidin gene cluster present in *Bacillus amyloliquefaciens* FZB42

In a bioinformatic analysis the partial mersacidin gene cluster, identified in *B. amyloliquefaciens* FZB42, was compared to corresponding sequences of the wild-type producer. Coding sequences showed up to 99.6% aa identity. MrsE and MrsF proteins of both strains differ in two aa, whereas regulators representing MrsR2 differ at one position only. Four exchanges were found in both MrsK2 coding sequences. None of these exchanges were found within active sites or domains. For MrsG, a lower level of identity was obvious and both annotations mainly differ at their N-termini. A detailed analysis of both *mrsG* DNA sequences indicated, that a thymidine residue was missing in position 980 of the original sequence, resulting in definition of a false stop codon and, thus, in annotation of a shortened MrsG (R. Reinartz, diploma thesis, 2005). After sequence correction, both *mrsG* sequences were identical.

Additionally, corresponding non-coding sequences were compared and were found to have a high degree of sequence similarity, with exception of an intergenic region that is located between *mrsE* and *fbaB* (figure 3.3.3). Here, a short sequence of 147 bp is inserted in the genome of *B. amyloliquefaciens* FZB42 (bp 3774591-3774738) that is not present in the wild-type producer strain and did not yield any hits in databases. This sequence is flanked by two distinct regions that are similar to intergenic regions found within the mersacidin gene cluster. The upstream 38 bp region is similar to the sequence found downstream of *mrsE*, i.e. the sequence upstream of the putative *mrsA* operator. The 89 bp region downstream of the 147 bp insertion is homologous to the sequence downstream of *mrsT* and upstream of *fbaB* and contains the inverted repeat that is thought to delimit the mersacidin gene cluster (G. Bierbaum, personal communication). *FbaB*, which encodes a fructose-bi-phosphate aldolase, reflects the 3' flanking ORF of the mersacidin gene cluster in the wild-type producer.

In conclusion, a partial mersacidin gene cluster was identified in *B. amyloliquefaciens* FZB42 that is highly similar to the corresponding genes of the mersacidin wild-type producer and reflects the complete mersacidin immunity operon. Even DNA sequences, including intergenic and non-coding sequences, have a high degree of similarity. These results might indicate that the biosynthetic part of the mersacidin cluster was lost from *B. amyloliquefaciens* FZB42 during evolution. Therefore, this organism was chosen as amenable to mersacidin production and reconstitution of the cluster was performed by transferring the biosynthetic part into *B. amyloliquefaciens* FZB42.

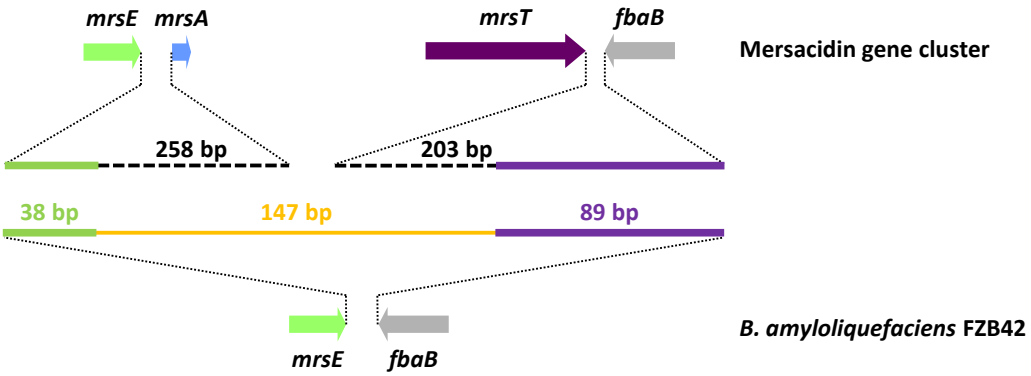

Figure 3.3.3: Intergenic region between *mrsE* and *fbaB* in *B. amyloliquefaciens* FZB42. The intergenic region contains 5' and 3' sequences that are homologous to corresponding sites in the mersacidin gene cluster. In between, there is an insertion of 147 bp that was found neither in other *B. amyloliquefaciens* strains nor in the mersacidin wild-type producer.

Determination of the minimum inhibitory concentration of mersacidin against *Bacillus amyloliquefaciens*

Producer self-protection of strains producing class II lantibiotics is mediated by an ABC-transporter whose subunits are encoded by *lanEFG* genes. Comparison of MIC values of the wild-type mersacidin producer and its *mrsK2R2* knockout clone, which does not express the mersacidin immunity genes *mrsFGE*, had demonstrated that expression of *mrsK2R2FGE* increased resistance to mersacidin about threefold (Guder *et al*., 2002). Identification of the complete mersacidin immunity operon in *B. amyloliquefaciens* raised the question, whether this strain would be immune to mersacidin. Therefore, MIC values of *B. amyloliquefaciens* FZB42 and its mrs1 mutant, which only harbors a partially reconstituted gene cluster, were determined in comparison to the mersacidin wild-type producer. All three strains exhibited MICs in the same range (25 µg/ml) indicating that *B. amyloliquefaciens* FZB42 and its mutants were at least as resistant to mersacidin as the producer strain and, therefore, amenable to heterologous mersacidin production.

Generation of mersacidin producing *B. amyloliquefaciens* mutants

In order to facilitate generation of a mersacidin producing *B. amyloliquefaciens* mutant, the pCU1 based, recombinant plasmids pPAR1/1 and pOPAR1 (Schmitz *et al*. 2006), both harboring the structural gene of mersacidin (*mrsA*) were introduced into *B. amyloliquefaciens* mrs1. This mutant harbors a partially reconstituted mersacidin gene cluster in which the structural gene and its promoter are replaced by an erythromycin

resistance cassette (A.M. Herzner, diploma thesis, 2008). The regulator MrsR1 is essential for mersacidin biosynthesis (Guder *et al*., 2002) and is transcribed from the *mrsA* promoter (Bierbaum, unpublished results). Due to insertion of *ermB*, transcription of this regulator is likely to be affected by a polar effect in this mutant strain.

Successful generation of *B. amyloliquefaciens* mersacidin-producing mutants was achieved by *in trans* completion of *mrsA* and *mrsR1* using the recombinant plasmids pOPAR1 and pPAR1. Both plasmids harbor a fragment of the gene cluster that encodes *mrsA* and *mrsR1*, and those are arranged in the same orientation as in the chromosomal context (Schmitz *et al*., 2006). The vectors pOPAR1 and pPAR1, that differ in the presence of an operator sequence upstream of *mrsA* (figure 3.3.4), were prepared from *E.coli* strains, that served as intermediate hosts, and were directly transferred by competence transformation into *B. amyloliquefaciens* mrs1. Transformants were selected on TSA (containing 5 µg/ml chloramphenicol) and were subsequently cultured on TSA agar plates (25 µg/ml CM). The *in trans* completed gene cluster harboring strains were named *B. amyloliquefaciens* mrs1 pPAR1/1 or *B. amyloliquefaciens* pOPAR1 (figure 3.3.5). For control purposes a mutant strain that carries the empty pCU1 vector also was transformed and this strain was named *B. amyloliquefaciens* mrs1 pCU1. Successful transformation was confirmed by plasmid preparation and gel electrophoresis.

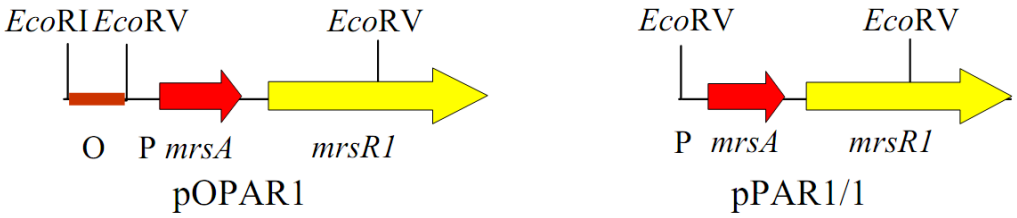

Figure 3.3.4: Inserts of the pCU1-based, recombinant vectors pOPAR1 and pPAR1/1. O = putative operator structure, P = promotor; *mrsA* = structural gene coding for the lantibiotic prepeptide of mersacidin; *mrsR1*= gene coding for a transcription factor of the mersacidin gene cluster (A.M. Herzner, diploma thesis, 2008 and Schmitz *et al*., 2006).

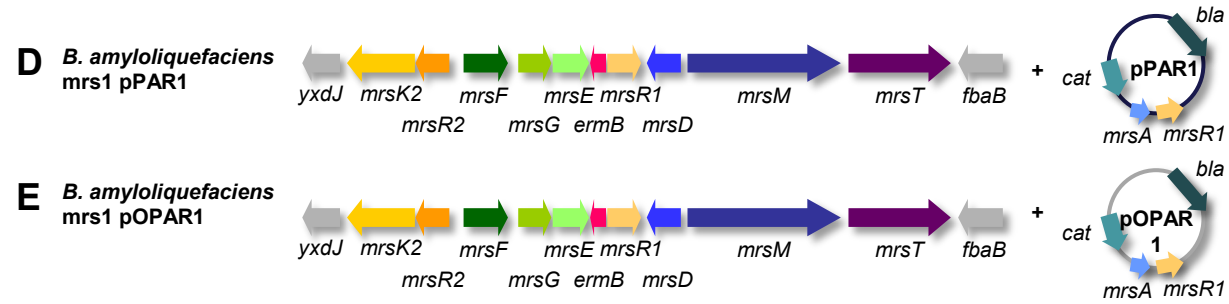

Figure 3.3.5: *In trans* completed mersacidin gene clusters of *B. amyloliquefaciens* mrs1 pPAR1 (D) and pOPAR1 (E). Successful generation of mersacidin-producing mutants was achieved by *in trans* completion of *mrsA* and *mrsR1* using pOPAR1 and pPAR1.

Production of mersacidin by *B. amyloliquefaciens* mrs1 pPAR1 and pOPAR1

In order to prove heterologous expression of mersacidin*, B. amyloliquefaciens* FZB42 and its mutant strains were grown for 24 h in 2xBPM. Sterile culture supernatants were analyzed for production of mersacidin by MALDI-TOF and assayed for antimicrobial activity after sterile filtration.

MALDI-TOF spectra of culture supernatants of *B. amyloliquefaciens* mrs1 pPAR1 and pOPAR1 were compared to those of *B. amyloliquefaciens* FZB42 and the mrs1 strain (figure 3.3.6) and to purified mersacidin. The latter showed three characteristic signals, with 1826 Da being the protonated lantibiotic peptide itself, 1848 Da and 1864 Da respectively representing sodium and potassium adducts of mersacidin. These characteristic mersacidin masses were also detected in culture supernatants of the mutant strains harbouring pPAR1 or pOPAR1, indicating expression of fully modified mersacidin. In contrast, spectra of *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* mrs1 did not show any mersacidin-related mass signals.

Besides, the spectra of mersacidin-producing cultures showed additional mass signals (e.g. 1965 Da and 1981 Da) that were not detected in supernatants of the FZB42 wild-type and the mrs1 mutant. Therefore, these peptides were thought to correlate with mersacidin production and their MWs (3 Da differences in mass) are in line with calculated masses of different incompletely modified or partially processed mersacidin variants and the corresponding potassium and sodium adducts thereof (table 3.1)

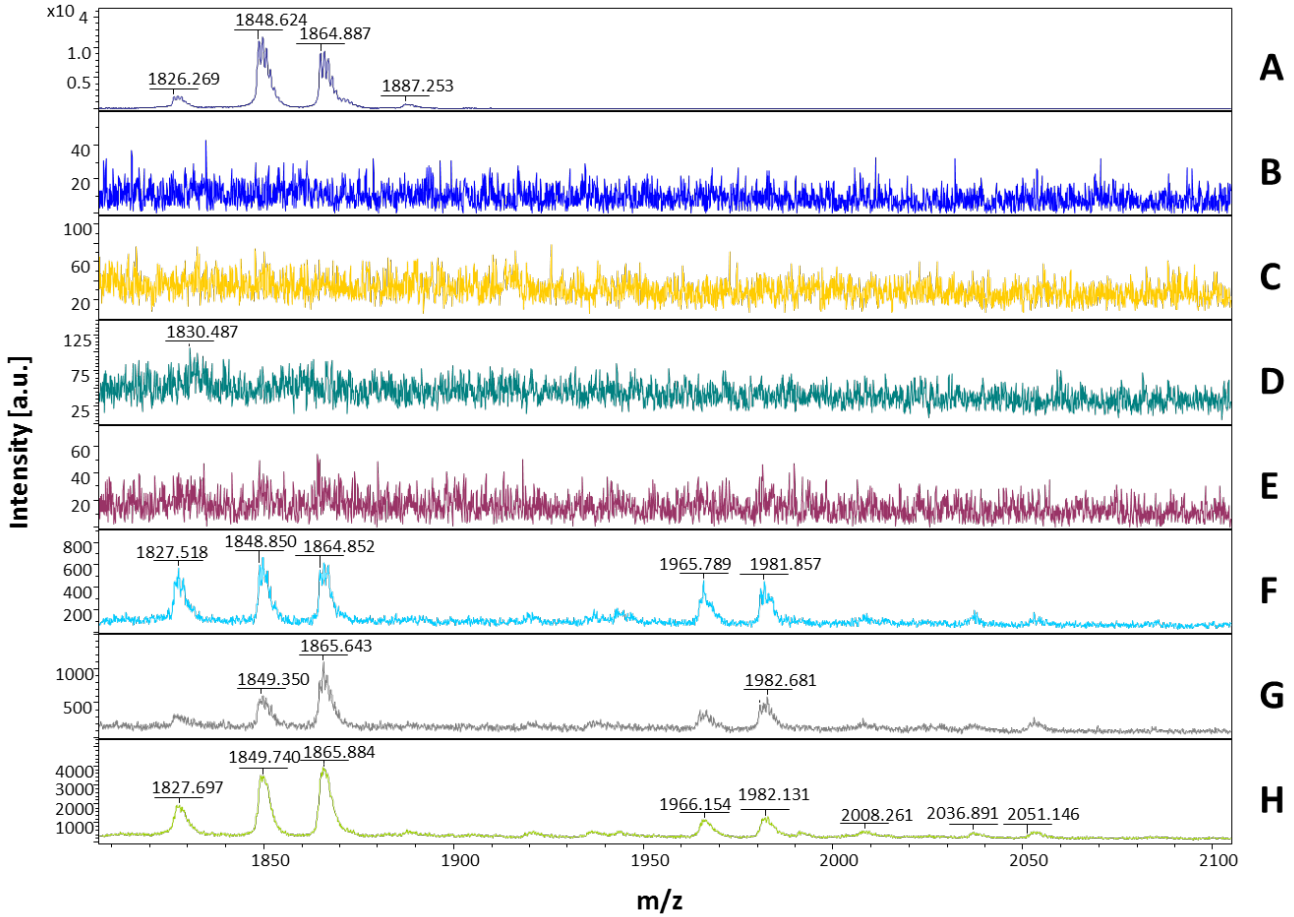

Figure 3.3.6: Comparative MALDI-TOF analysis of culture supernatants of *B. amyloliquefaciens* FZB42 (B), mrs1 (C) and the isogenic mrs35 mutant (D), mrs1 pOPAR1 (E, F) and pPAR1 (G, H) in comparison to purified mersacidin (A). In contrast to culture supernatants of *B. amyloliquefaciens* FZB42 and the mrs1 mutant, substances with MWs corresponding to mersacidin-derived masses could be detected in both strains that harbor *in trans* completed gene clusters when cultivated in presence of chloramphenicol (F, H). In the absence of chloramphenicol, mersacidin production was detected in supernatant of pPAR1 mutant only (H).

Table 3.1: Calculated masses of mersacidin variants that differ in their (incomplete) modification and processing states. Calculated MWs that matched, considering 3 Da in difference, with mass signals that were detected in MALDI_TOF spectra of the culture supernatant of mersacidin-expressing mutants, are highlighted in red.

Mersacidin peptide/ variant	Calculated MW [Da]					
CTFTLPGGGGVCTLTSECIC	**mersacidin**	**K adduct**	**Na adduct**	**lacking AviMeCys**	**K adduct**	**Na adduct**
0 dehydration	1914.3	1953.3	1937.3	1962.3	2001.3	1985.3
1 dehydration	1896.3	1935.3	1919.3	1944.3	1983.3	1967.3
2 dehydrations	1878.3	1917.3	1901.3	1926.3	1965.3	1949.3
3 dehydrations	1860.3	1899.3	1883.3	1908.3	1947.3	1931.3
4 dehydrations	1842.3	1881.3	1865.3	1890.3	1929.3	1913.3
5 dehydrations	1824.3	1863.3	1847.3	1872.3	1911.3	1895.3
A CTFTLPGGGGVCTLTSECIC						
0 dehydration	1937.4	1976.4	1960.4	1985.4	2024.4	2008.4
1 dehydration	1919.4	1958.4	1942.4	1967.4	2006.4	1990.4
2 dehydrations	1901.4	1940.4	1924.4	1949.4	1988.4	1972.4
3 dehydrations	1883.4	1922.4	1906.4	1931.4	1970.4	1954.4
4 dehydrations	1865.4	1904.4	1888.4	1913.4	1952.4	1936.4
5 dehydrations	1847.4	1886.4	1870.4	1895.4	1934.4	1918.4
AA CTFTLPGGGGVCTLTSECIC						
0 dehydration	2056.4	2095.4	2079.4	2104.4	2143.4	2127.4
1 dehydration	2038.4	2077.4	2061.4	2086.4	2125.4	2109.4
2 dehydrations	2020.4	2059.4	2043.4	2068.4	2107.4	2091.4
3 dehydrations	2002.4	2041.4	2025.4	2050.4	2089.4	2073.4
4 dehydrations	1984.4	2023.4	2007.4	2032.4	2071.4	2055.4
5 dehydrations	1966.4	2005.4	1989.4	2014.4	2053.4	2037.4
EAA CTFTLPGGGGVCTLTSECIC						
0 dehydration	2185.6	2224.6	2208.6	2233.6	2272.6	2256.6
1 dehydration	2167.6	2206.6	2190.6	2215.6	2254.6	2238.6
2 dehydrations	2149.6	2188.6	2172.6	2197.6	2236.6	2220.6
3 dehydrations	2131.6	2170.6	2154.6	2179.6	2218.6	2202.6
4 dehydrations	2113.6	2152.6	2136.6	2161.6	2200.6	2184.6
5 dehydrations	2095.6	2134.6	2118.6	2143.6	2182.6	2166.6

However, a clear assignment of these mass signals to specific mersacidin-variants was impossible, as the latter often showed nearly identical MWs and, thus, cannot be distinguished based on mass spectra only.

Chloramphenicol was used in routine cultures *of B. amyloliquefaciens* pPAR1 and pOPAR1. Since the antimicrobial activity of chloramphenicol in culture supernatants interferes with the antimicrobial activity of heterologous expressed mersacidin in agar diffusion assays, cultivations were additionally investigated in absence of chloramphenicol. The resulting supernatants were analyzed by MALDI-TOF. Here, mersacidin-derived masses were only detected in culture supernatant of the pPAR1 mutant (figure 3.3.6). Additionally, plasmid

maintenance was analyzed by plasmid preparation and gel electrophoresis. In concert with the mass spectrometry results, the plasmid pOPAR1 was lost in cultivations that were grown in absence of its selection marker chloramphenicol, whereas pPAR1 was still detectable after 24 h of cultivation.

Although mersacidin production was detected in supernatants of the pPAR1 mutant by MALDI-TOF analysis, inhibition zones against *M. luteus*, *S. simulans* and *B. megaterium* in agar well diffusion assays were not significantly larger than those of the non-mersacidin-producing strains *B. amyloliquefaciens* FZB42 or *B. amyloliquefaciens* mrs1 (figure 3.3.7).
It might be possible, that this was due to production of another antibiotic substance. *B. amyloliquefaciens* FZB42 is known to produce various antimicrobial and antifungal substances, as seen in table 3.1, including polyketides (bacillaene, difficidin, macrolactin), lipopeptides (surfactin, fengycin, bacillomycin D), antimicrobial and antifungal dipeptides chloroetaine and bacilysin and the TOMM peptide plantazolicin (Rückert *et al*., 2011; Idriss *et al*., 2002 and Arguelles-Arias *et al*., 2009).

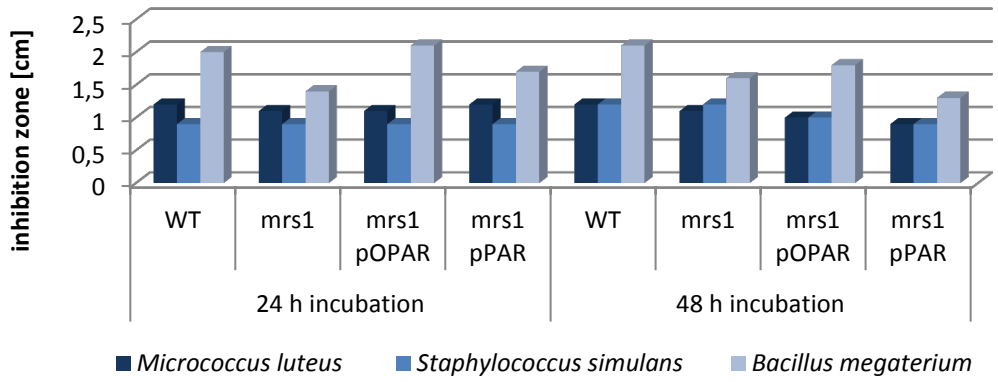

Figure 3.3.7: Inhibition zones of culture supernatant of *B. amyloliquefaciens* mrs1 pPAR1 / pOPAR1 in comparison to *B. amyloliquefaciens* wild-type (WT) and the mrs1 strain. For all three *B. amyloliquefaciens* strains inhibition zones (wells with a diameter of 0.7 cm) of comparable size were detected against the indicator strains *M. luteus*, *B. megaterium* and *S. simulans*.

Table 3.1: Overview over antimicrobial and antifungal substances produced by *B. amyloliquefaciens* strains (according to Arguelles-Arias *et al.,* 2009)

Type of antibiotic	Name	Masses [Da]		
		+H	+Na	+K
polyketides	bacillaene A	583.5	-	-
	bacillaene B	583.5	605.5	-
	difficidin	559.2	-	-
	macrolactin A28	403.4	425.4	-
	macrolactin A 7-o-malonyl	489.4	511.4	-
	macrolactin A 7-o-succinyl	503.4	525.4	-
	macrolactin D	629.3	-	-
lipopeptides	surfactin C13	1008.8	1030.8	1046.8
	surfactin C14	1022.8	1044.8	1060.8
	surfactin C15	1036.8	1058.8	1074.8
	fengycin Ala6 C15	1449.9	1471.9	1487.9
	fengycin Val6 C16	1491.9	1513.9	1529,9
	fengycin Ala6 C16	1463.9	1485.9	1501.9
	fengycin Ala6 C17	1477.9	1499.9	1515.9
	fengycin Val6 C17	1505.8	1527.8	1543.8
	bacillomycin D	1044.1	1066.1	-
	iturins C15 A	1057.1	1079.1	-
non-ribosomally expressed di-peptides	chlorotetaine	289.2	-	-
	bacilysin	270	-	-
TOMM	plantazolicin	1335	-	-

Comparative MALDI-TOF spectra of *B. amyloliquefaciens* FZB42 and its mutant strains were recorded in order to identify production of these antibiotic substances under conditions permitting lantibiotic production. Mass spectrometric analysis of culture supernatants of *B. amyloliquefaciens* and its mutant strains revealed the presence of signals with masses of approximately 1500 Da and 1050 Da, indicating production of the lipopeptide surfactin and the antifungal compounds fengycin and bacillomycin D (figure 3.3.8). This notion was supported by the finding that surfactin activity could be detected in hemolysis assays on Columbia blood agar plates (figure 3.3.9). Thus, antimicrobial activity of mersacidin was most probably masked by the activity of surfactin in the agar well diffusion assays.

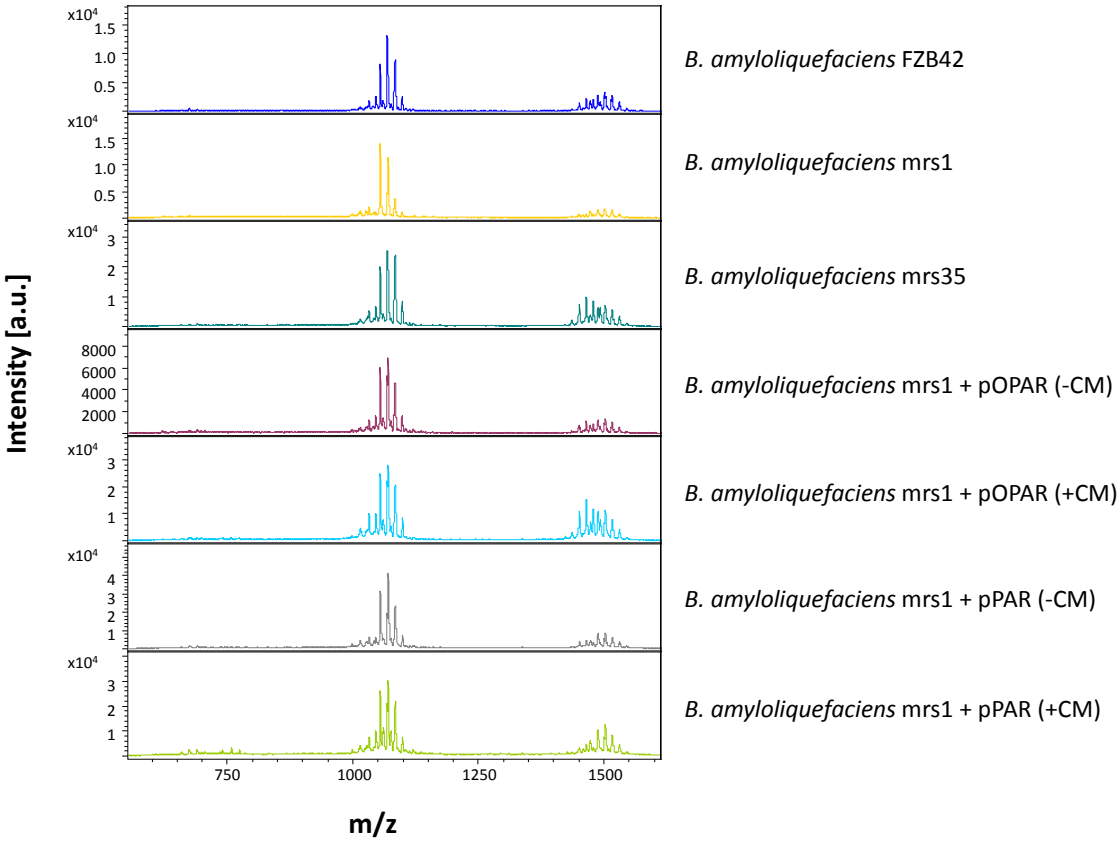

Figure 3.3.8: Comparative MALDI-TOF analysis of the culture supernatants (24 h incubation, in presence (+CM) or absence (-CM) of chloramphenicol) of *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* mrs1 and the isogenic mrs35 strain, *B. amyloliquefaciens* mrs1 pOPAR1 and *B. amyloliquefaciens* pPAR1 indicated production of the lipopeptide surfactin and the antifungal compounds fengycin and bacillimycin D.

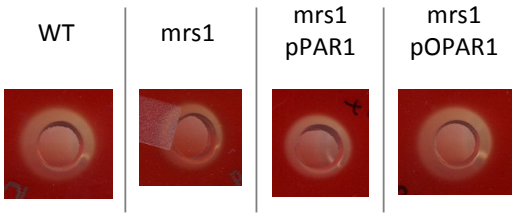

Figure 3.3.9: Detection of a haemolytic activity of culture supernatants (-CM) of *B. amyloliquefaciens* FZB42 (WT), *B. amyloliquefaciens* mrs1, *B. amyloliquefaciens* mrs1 pOPAR1 and *B. amyloliquefaciens* pPAR1 on Columbia agar plates also indicated production of a surfactant.

HPLC purification of mersacidin produced by *B. amyloliquefaciens* mrs1 pPAR1 and determination of antimicrobial activity of the purified peptide

Since determination of the effective antimicrobial activity of heterologous expressed mersacidin failed in agar diffusion assays employing crude supernatants, HPLC-purified mersacidin was used in subsequent assays.

In order to verify that mersacidin produced by *B. amyloliquefaciens* mrs1 pPAR1 is correctly modified and active, mersacidin was purified by two consecutive HPLC runs. Notably, HPLC peaks containing mersacidin were significantly larger when supernatants of chloramphenicol-containing cultures were analyzed compared to that of chloramphenicol-free cultures. In addition, mersacidin containing HPLC fractions were more active against *M. luteus*. A control run with 2xBPM containing 20 mg/ml chloramphenicol resulted in an active fraction (3.2 cm against *M*. *luteus*) that was eluted at min 4-6. Thus, the enhanced antimicrobial activity observed on mersacidin-containing fractions of chloramphenicol-containing cultures (eluted at min 17-18) can be attributed to mersacidin itself and is not an effect of chloramphenicol.

In the first purifications (figure 3.3.10), culture supernatant of *B. amyloliquefaciens* mrs1 pPAR1 was applied to a POROS II RP-HPLC column and an active substance was eluted in a gradient of 30-42% acetonitrile (containing 0.1% TFA). HPLC-fractions that eluted between min 17-18 (36.5- 38% acetonitrile) were active against *M. luteus* (1.9 cm) and corresponding MALDI-TOF spectra showed mersacidin-related masses. However, these fractions also showed a hemolytic activity on Columbia agar plates and masses assigned to surfactin. Likewise, corresponding HPLC fractions of the mrs1 mutant supernatant (negative control) contained surfactin-related masses and hemolytic activity, but exhibited no or only a weak (0.9 cm) antimicrobial activity against *M. luteus* (data not shown). These results indicated that mersacidin is responsible for the antimicrobial effect of the active fraction of the mersacidin producing mutant.

For these first HPLC purification runs, 85 ml supernatant were used and resulted in 17 active fractions. A total volume of approx. 30 ml was subsequently lyophilized and yielded in 500 mg white powder. Lyophilized pellets were resuspended in 15 ml 5% acetonitrile containing 0.1% TFA, but complete dissolution, as proved by centrifugation, was impossible, even after addition of 50 µl, 100 µl or 150 µl chloroform. The latter treatment still resulted in an insoluble yellow-orange pellet that was finally removed by centrifugation. The resulting solutions (5% acetonitrile) showed antimicrobial activity with an inhibition zone of 2.3 cm against *M. luteus*. MALDI-TOF analysis confirmed the presence of mersacidin and surfactin. The sample was further analyzed by a second HPLC run (figure 3.3.10).

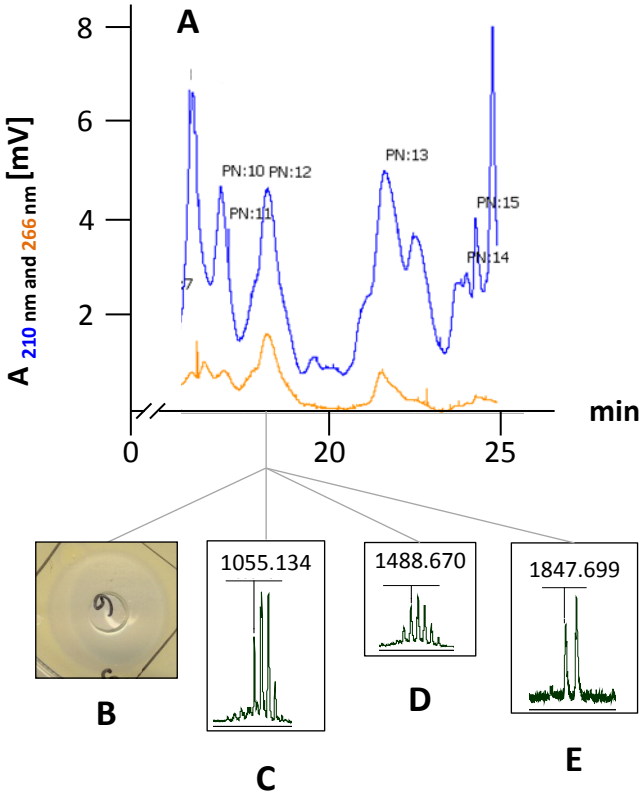

Figure 3.3.10: HPLC pre-purification of mersacidin from culture supernatant of *B. amyloliquefaciens* mrs1 pPAR1 (A). Fractions were assayed for antimicrobial activity in agar well diffusion tests against *M. luteus* (B) and analyzed by MALDI-TOF (C-E). Active fractions eluted after 17-18 min in a gradient of 50-65% acetonitrile (0.1% TFA) and were characterized by the presence of an 1847 Da mass signal representing the sodium adduct of mersacidin (E) and surfactin (1055 Da) (C) and fengycin-derived (D) mass signals (1488 Da).

In order to prove that mersacidin produced *by B. amyloliquefaciens* exhibits antimicrobial activity, the sample was further purified by applying to a Nucleosil C18 column. Active fractions were eluted after 20-21 min in a gradient of 50-65% acetonitrile (0.1% TFA) and showed the typical adsorption spectrum of mersacidin (3.3.12 A). Antimicrobial activity of these fractions was demonstrated in agar diffusion assays against *M. luteus,* in which they caused inhibition zones with diameters up to 3.2 cm (figure 3.3.12 C). Furthermore, MALDI-TOF spectra displayed mass signals characteristic of mersacidin and typical surfactin-derived mass signals were not present (figure 3.3.12 B). In line with this, no hemolytic activity was detected on blood agar plates (figure 3.3.12 C).

In conclusion, these results indicated, that the mutant *B. amyloliquefaciens* mrs1 pPAR1 had produced mersacidin which is active and fully modified.

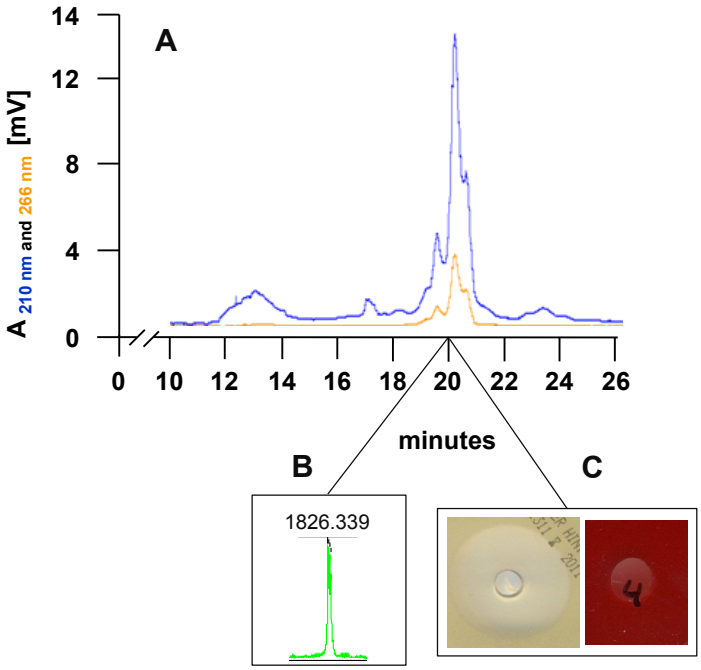

Figure 3.3.12: Purification of mersacidin. (**A**). Active fractions were assayed for antimicrobial activity in agar well diffusion tests against *M. luteus* (**C**) and analyzed by MALDI-TOF (**B**). Active fractions eluted after 20-21 min in a gradient of 50-65% acetonitrile (0.1% TFA) and were characterized by presence of a mersacidin signal (1826 Da). Surfactin could neither be detected in mass spectra nor in hemolysis assays.

Phylogenetic classification of the mersacidin producer within the species *B. amyloliquefaciens*

In previous experiments A.M. Herzner had demonstrated, that the mersacidin producer most likely belongs to the species *B. amyloliquefaciens* and postulated that this strain might rather be related to *B. amyloliquefaciens* FZB42 than to the DSM7 type strain. Very recently, these two strains have been reported to be typical representatives of two *amyloliquefaciens* subspecies (Borriss *et al*., 2010): 1. the subgroup of plant associated *B. amyloliquefaciens* subspec. *plantarum* and 2. the non-plant associated subgroup *B. amyloliquefaciens* subspec. *amyloliquefaciens*.

In order to further clarify the degree of relatedness between the mersacidin producer *B.* sp. HIL Y-85,54728 and other bacilli of the *amyloliquefaciens* group, a phylogenetic tree based on the *gyrA* sequence, which codes for the gyrase subunit A, was calculated. To this end, parts of the *gyrA* gene of the mersacidin producer were amplified by PCR using previously published primers (Reva *et al*., 2004). The resulting products were sequenced. Subsequently, the obtained DNA sequences were compared in an alignment based taxonomic tree

calculation to previously annotated *gyrA* sequences of different members of the *plantarum* and *amyloliquefaciens* subspecies, as well as to other *Bacillus* strains of the *cereus, subtilis* and *licheniformis* groups.

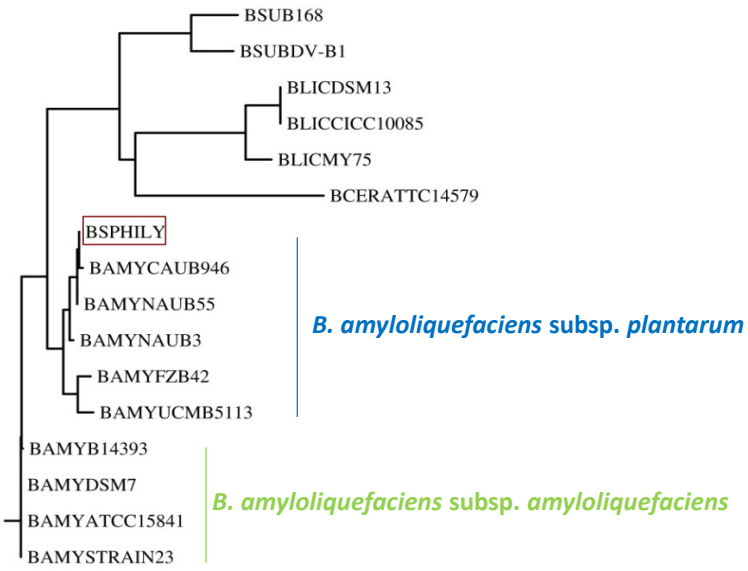

Figure 3.3.13: **Phylogenetic tree based on the partial nucleotide sequence of the *gyrA* gene.** The tree was calculated based on the *gyrA* nucleotide sequences of the mersacidin producer (BHILY, marked by a red box) and different members of the genus *Bacillus* (NCBI accession numbers in brackets) [*B. amyloliquefaciens* = BAMY strains: FZB42 (CP000560), CAUB946 (FN652789), S23 (FN652780), *ATCC* 15841 (FN662838), DSM 7 (FN597644), NAUB3 (FN652783), NAUB55 (FN652801), UCMB5113 (AY212974); *B. licheniformis* = BLIC strains: MY75 (EU073420), DSM13 (BLi00007), CICC10085 (GQ355995); *B. subtilis* = BSUB strains: 168 (BSU00070), DV1-B1 (EF134416) and *B. cereus* = BCER strain: ATCC14579 (BC0006)]. The mersacidin wild-type producer is placed among members of the subspecies *B. amyloliquefaciens* subsp. *plantarum* forming a cluster that is strictly separated from all other bacilli strains.

In this taxonomic tree (figure 3.3.13), the mersacidin producer strain is found in a cluster formed by members of *B. amyloliquefaciens*. In detail, the strain clustered with strains that belong to the *plantarum* subspecies and was clearly distinct from strains related to *B. amyloliquefaciens* type strain DSM7 and to the other bacilli.

These results verified the high degree of relatedness of the wild-type mersacidin producer to *B. amyloliquefaciens* FZB42. Most likely this strain itself is a member of the genus *B. amyloliquefaciens* and appeared to belong to the plant-associated *B. amyloliquefaciens plantarum* subgroup.

Discussion

Emergence and spread of resistance to widely used antimicrobial therapeutics among clinical relevant bacteria has become one of the most challenging tasks in treatment of infectious diseases. Over the past decades, many resistance mechanisms have been emerged in pathogens (Wright, 2012). At the same time, only few new antibiotics have reached the pharmaceutical market, thus resulting in a dramatic shortage in the availability of potent therapeutics in future (Bassetti *et al*., 2011). Infections caused by resistant pathogens such as methicillin-resistant *Staphylococcus aureus* strains, lead to prolonged hospitalizations, increased costs and lethality, thus representing a significant burden of the health care sector worldwide. This necessitates search for and development of new antimicrobial compounds with new modes of action and targets to overcome the growing problem of antibiotic resistance (Wright, 2012).

Advances in various high-throughput techniques, such as next generation sequencing, resulted in an explosion of genomic data and new bioinformatic tools are revolutionizing biology (Lee *et al*., 2010; Wecke and Mascher, 2011). In this context, the mining of bacterial genomes for potential antibiotic substances has opened the door to a new era in natural product drug discovery (Haney *et al*., 2002; Gross, 2009) and has already resulted in the identification of various new antibiotics and antibiotic classes (de Jong *et al*., 2010; Taylor and Wright, 2008).

Lantibiotics are among the most promising candidates for future antimicrobials due to their capacity to inhibit the growth of clinically relevant pathogenic bacteria, even of multidrug resistant strains, by mechanisms of action not shared by other antibiotics in use (v. Heel *et al*., 2011). Enzymes involved in the introduction of class specific modifications of lantibiotics share conserved aa sequences and structures and, therefore, are amenable to *in silico* identification of homologous proteins (de Jong *et al*., 2010), thus leading to the prediction of novel lantibiotic gene clusters.

This thesis aimed at the identification of novel lantibiotic producers employing *in silico* screening for as yet unknown lantibiotic modification enzymes in publicly available bacterial genome sequences. This bioinformatic approach resulted in prediction of lantibiotic-like gene clusters in *B. licheniformis* DSM 13, *N. punctiforme* ATCC 29133 and *C. bescii* DSM 6527.

The novel two-peptide lantibiotic lichenicidin is produced by *Bacillus licheniformis* DSM 13

A closer bioinformatic inspection of the predicted lantibiotic gene cluster of *B. licheniformis* DSM 13 indicated that this cluster encodes a two-peptide lantibiotic, lichenicidin, with structural and sequence similarity to known two-peptide lantibiotics such as haloduracin. So far, nine two-peptide lantibiotics have been described, eight of which are closely related with regard to their structures and aa sequences. Interestingly, lichenicidin and haloduracin, both produced by two different *Bacillus* strains, share the highest similarity and their clusters have a similar organization e.g. corresponding gene arrangement.

MALDI-TOF analyses of whole cells and isopropanol cell wash extracts identified two cell-surface-associated peptides with size of 3251 and 3021 Da that were suggested to represent the fully modified and active lichenicidin peptides. In a comparable genome mining approach, Begley *et al*. (2009) identified the same lichenicidin gene cluster in the isogenic *B. licheniformis* ATCC 14580 strain. The same MWs were predicted for both lichenicidin peptides based on results of colony mass spectrometry.

In this thesis, mass prediction for both lichenicidin peptides was verified by disruption of the lichenicidin modification enzymes (LicM1; LicM2) via integration mutagenesis. In subtractive MALDI-TOF analyses, the antimicrobial activity of the cell wash extracts could be attributed to production of a lantibiotic encoded by the predicted gene cluster and a mass of 3251 Da for active Licα and a mass of 3021 Da for the active Licβ-peptide was confirmed.

LicA1 exhibits aa sequence similarity to LanA1 peptides of other two-peptide lantibiotics. With exception of a Lys residue, that probably is located within a third thioether ring (position +25 which is taken by His in some other peptides), all aa found in Licα are present in at least one other peptide of the LanA1 group. A mass of 3251 Da is consistent with seven dehydrations and indicated that one residue of LicA1 escapes dehydration. This unmodified hydroxy-aa most likely is the Ser residue at position 30, especially since analogous Ser residues in the α-peptides of haloduracin and plantaricin W escape dehydration (McClerren *et al*., 2006; Holo *et al*., 2001).

In addition, the location of the typical LanA1 motif (Cotter *et al*., 2006) responsible for formation of the conserved C-terminal three rings, indicated that the C-terminal thioether bridging pattern of Licα might be identical to that of other α-peptides with a solved structure (lacticin 3147 and haloduracin). This close similarity does not extend to the N-terminus, where, just as in mersacidin, a fourth (Me)Lan might be formed, since a Cys and several hydroxy-aa are present additionally.

In 2010, Shenkarev *et al*., demonstrated that lichenicidin is also produced by *B. licheniformis* VK21. Caetano *et al*. (2011) additionally identified a lichenicidin gene cluster in the strain *B.*

licheniformis I89. Prepeptides of these two lichenicidin producers are, with regard to their aa sequences, identical to lichenicidin produced by the DSM 13 strain. Partial sequencing of the lichenicidin gene cluster of *B. licheniformis* VK21 revealed identical DNA sequences for both structural genes and the structural genes found in the DSM 13 strain, whereas aa sequences coding for the LicA1 modifying enzyme (LicM1) differ in five aa (Shenkarev *et al*., 2010).
In both studies, structures of fully modified lichenicidin peptides (figure 4.1 and 4.2) were elucidated by NMR (Shenkarev *et al*., 2010), site directed mutagenesis and MS/MS fragmentation (Caetano *et al*., 2011).

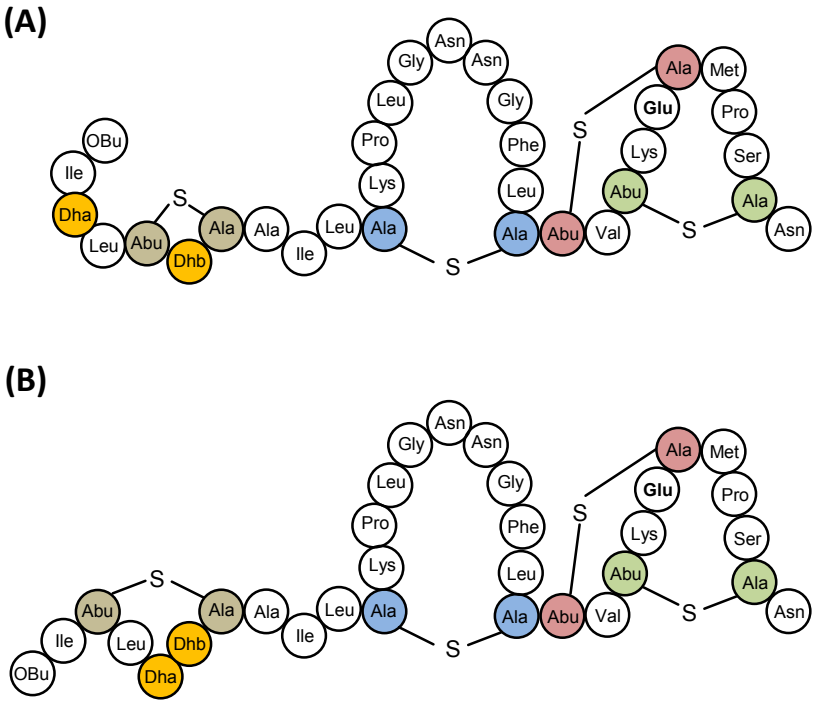

Figure 4.1: Structure of the lichenicidin α-peptide according to (A) Caetano *et al*. (2011) and (B) Shenkarev *et al*. (2010). Four thioether aa are present in active Licα-peptides. The most N-terminal Thr residue is modified to an oxobutyryl residue. C-terminal rings are formed as in haloduracin. An additional, N-terminal MeLan ring is present. For this ring, two different structures have been published: (A) a three aa spanning ring (Thr5-Cys7) by Caetano *et al.* (2011) and (B) a five aa spanning ring (Thr3-Cys7) by Shenkarev *et al*. (2010).

As predicted, the Ser at position +30 is not dehydrated in the active LicA1 peptide. Additionally, these structure resolutions confirmed the herein predicted C-terminal thioether bridging pattern of Licα and formation of an additional, N-terminal MeLan ring structure. According to Shenkarev *et al*. (2010) this N-terminal MeLan is formed by Thr at

position +3 and Cys at position +7. In contrast, the structure published by Caetano *et al*. (2011) contains a shorter MeLan ring, which is formed by a thioether linkage between Thr (+5) to Cys (+7).
The mass of 3251 Da indicated that leader cleavage occurs after the anticipated proteolytic cleavage sequence (GG; aa 41/42, position -1; -2) in LicA1, resulting in an N-terminal Dhb residue (+1), which was demonstrated to be modified to oxobutyryl in both studies. N-terminal Dhb residues are instable in aqueous solutions and undergo spontaneous and rapid hydrolysis that results in a 2-oxobutyryl residue by desamination (Kellner *et al*., 1989). Such a modification has previously been demonstrated for N-terminal Dhb residues found in the α-peptide of lacticin 3147 and in the single-peptide lantibiotic Pep5 (Ryan *et al*., 1996; Bierbaum *et al.*, 1996).

As seen for LicA1, a conserved two-peptide lantibiotic motif, the LanA2 motif (Cotter *et al*., 2006), was observed in LicA2, strongly supporting formation of the three conserved C-terminal rings. Its N-terminus deviates from other lantibiotic peptides and contains several additional hydroxy-aa. Furthermore, Licβ-peptides are characterized by masses consistent with eleven or twelve of fifteen possible dehydrations and an additional N-terminal processing of six aa. Such a second processing has also been demonstrated for Halβ and the β-peptide of plantaricin W (Holo *et al*., 2001; McClerren *et al*., 2006). This step occurs extracellularly after the proteolytic leader cleavage site by an additional protease and results in the removal of the N-terminal six aa of the propeptide sequence. In all three cases, the mature peptides gain a positive net charge by removal of two negatively charged aa. The protease encoded by BLi4124 contains a secretion signal and might well be involved in this second extracellular processing reaction.
The N-terminus of LicA2 contains a further Cys and seven hydroxy-aa, suggesting formation of a fourth ring. The insensitivity of the antibacterial activity in the cell wash extract to treatment with chymotrypsin strongly indicates presence of such a fourth, N-terminal ring structure, since a chymotrypsin cleavage site (TTPATTSSW↓TC) is located in this area.
The predicted thioether ring pattern suggest an elongated peptide structure for the mature Licβ-peptide, resembling peptide structures of pore-forming class I lantibiotics. Nisin, the prototype of class I lantibiotics, harbors an internal linear region, the so called flexible hinge region, that is involved in transmembrane orientation of the C-terminus of the peptides during pore formation (Wiedemann *et al*., 2001). Based on the predicted ring pattern of LicA2, a linear region may be located between aa I12 - V18. A proteinase K cleavage site within this linear region (ITA↓GVTV) might account for loss of activity seen upon treatment with this protease, thereby strongly supporting a linear peptide structure in this region.

Indeed, Caetano *et al*. (2011) and Shenkarev *et al*. (2011) found such an elongated structure for the mature LicA2, that is, in large parts, consistent with the structure proposed here. As predicted, both studies revealed a C-terminal lanthionine bridging pattern corresponding to haloduracin and plantaricin W and confirmed presence of a fourth Lan ring in the N-terminus, which involves Ser at position 7 and Cys at position 11. After processing, the resulting peptide retains a free Dhb at its N-terminus, that is spontaneously hydrolyzed and desaminated to 2-oxobutyryl.

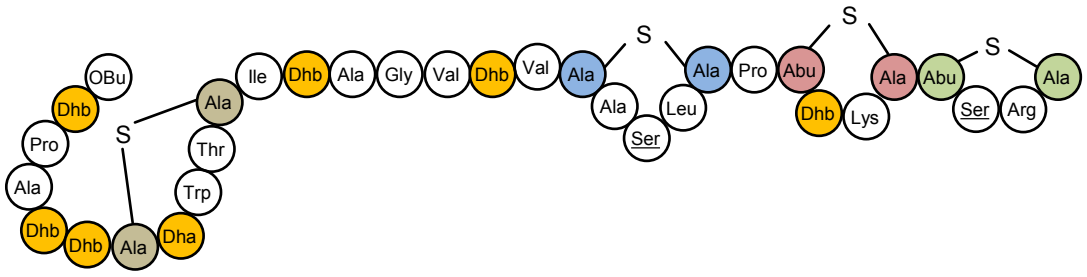

Figure 4.2: Structure of the mature LicA2 peptide of the lantibiotic lichenicidin according to Shenkarev *et al*. (2010) and Caetano *et al*., 2011.

Licβ contains two MeLan and one Lan ring in its C-terminus with the same connectivity as confirmed for haloduracin. An additional N-terminal Lan ring is formed between Ser (7) and Cys (11). A second proteolytic cleavage removes six N-terminal aa resulting in an N-terminal Dhb residue, which is subsequently modified to a 2-oxobutyryl residue.

With the exception of cytolysin, all two-peptide lantibiotic gene clusters harbor two LanM modification enzymes, which display little sequence similarity. McClerren *et al*. (2000; 2006) demonstrated that each lacticin 3147 and haloduracin peptide is modified separately by one of these LanM enzymes and it has been proposed that this is a common feature in the modification of two-peptide lantibiotics. A similar modification strategy was observed for both lichenicidin peptides here. Inactivation of the LicA1 modifying enzyme LicM1 resulted in loss of Licα expression, whereas expression of the fully modified Licβ-peptides was not affected. As previously reported for mature lacticin 3147 peptides (McClerren *et al*., 2000), export and processing of mature Licβ-peptide was even observed in absence of Licα indicating that transport and leader cleavage of both structural peptides occurs independently of each other.

Remarkably, the antimicrobial activity of lichenicidin targeted a broad range of MRSA strains that cause problematic infections in the hospital setting. The test panel included clinical

isolates of epidemic strains, which are currently widespread in Germany. For the two-peptide lantibiotics haloduracin and lacticin 3147 a dual mode of action principle has been described that combines inhibition of peptidoglycan biosynthesis by binding and dislocation of lipid II with pore formation in bacterial membranes (Wiedemann *et al*. 2006; Oman *et al*., 2011). Both functions are assigned to two distinct lantibiotic peptides. The globular α-peptide with homology to mersacidin binds to lipid II. As previously shown for nisin, Halα binds lipid II in a 2:1 ratio and, thereby, inhibits cell wall biosynthesis at the stage of transglycosylation catalyzed by the penicillin binding protein PBP1 (Oman *et al*., 2011). Elongated β-peptides bind to the Lanα-lipid II complexes and subsequently results in pore formation in bacterial membranes that finally leads to cell death.

Structural and sequence data of lichenicidin suggested, that it might act by a similar lipid II-involving mechanism. The Licα-peptide harbors the lipid II binding motif, which is believed to be important for lipid II binding of mersacidin-like peptides (Sahl and Bierbaum, 2009; Böttiger *et al*., 2009). Such a MeLan ring is also found in other LanA1 peptides and in the N-terminus of one-peptide lantibiotics of the lacticin 481 subgroup. Based on NMR data, Shenkarev *et al*. (2010) proposed an N-terminal ring structure in Licα, that resembles a part of the lipid II binding motif of nisin-like lantibiotics (Hsu *et al*., 2004), which is absent in other LanA1 peptides. Formation of three H-bridges between amides of the Licα-peptide backbone and oxygen atoms of the pyrophosphate moiety of lipid II was predicted. To this end, the authors suggested that Licα might bind lipid II via two motifs, the N-terminal nisin-like and the C-terminal mersacidin-like motif.

It is worth mentioning, that a recent publication reporting the identification of the new two-peptide lantibiotic enterocin, suggested a synergistic effect of two membrane active peptides (Sawa *et al*., 2011). This is in clear contrast to the above described MoA, that was thought to hold true for two-peptide lantibiotics in general. Although both enterocin peptides harbor typical Lanα- and Lanβ- motifs, including the lipid II binding site of class II lantibiotics, and are highly similar to plantaricin W, both were demonstrated to act on model membranes without docking molecules in nanomolare concentrations. A membrane-targeting effect of enterocin was determined by tryptophan fluorescence spectroscopy, light scattering and calcein leakage tests using egg yolk liposomes, which mimic gram-positive cell membranes. Activity of either peptide alone was barely detected and an optimal efficacy required addition first of α-peptide and then enterocin Wβ, suggesting that the order of peptide binding to membranes might be important for antimicrobial activity.

In this thesis, it was clearly demonstrated, that both lichenicidin peptides are necessary for an optimal antimicrobial activity, indicating a synergistic effect of Licα and Licβ. This observation was also described by Shenkarev *et al.* (2010) and, in addition, the authors demonstrated a maximal synergistic effect at a 1:1 ratio of both lichenicidin peptides.
For some two-peptide lantibiotics, an activity of the single Lanα- and Lanβ-peptides was demonstrated. The alpha-peptides of plantaricin W and lacticin 3147 and β-peptides of haloduracin and lacticin 3147 exhibit low level of activity, but this is only observed in higher concentrations (µM) (McClerren *et al*., 2006; Holo *et al*., 2001; Wiedemann *et al*., 2006). This effect is most likely due to a lipid II binding-mediated inhibition of the cell wall biosynthesis. LicA1is likely to have a certain degree of activity on its own, as HPLC fractions that only contain Licα were active against *M. luteus*. Similarly, an activity was also observed in HPLC fractions containing Licβ, which probably was due to formation of ion-conducting pores. µM concentrations of nisin in lipid II free liposomes indicated, that pore formation occurs also in absence of the target molecule and might be due to binding of cationic peptides to anionic lipids within the membrane (Wiedemann *et al*., 2001 and 2004).

Starón *et al*. (2011) and Burkhard and Stein (2008) established four *B. subtilis* whole cell biosensors that test the interference of peptide antibiotics with the lipid II cycle which is essential for peptidoglycan biosynthesis. Promotor-*lacZ* fusion mutants were generated in order to monitor induction of detoxification systems by different cell-wall-acting antibiotics such as the single-peptide lantibiotics mersacidin, actagardine, duramycin, gallidermin, nisin and subtilin. Each of these peptides was sensed by at least one of these indicator strains. It was observed that sensing was specific for each detoxification system and specificity did not appear to be correlated to the MoA or the structure of tested peptides. Thus, closely related lantibiotics, like mersacidin and actagardine, are not sensed by the same system (Starón *et al*., 2011).
An inhibition of all envelope biosyntheses by the lichenicidin peptides could not be verified using these biosensors and, therefore, it was hypothesized that none of the indicator strains is able to sense two peptide-lantibiotics. This notion was supported by results of the bioassays with culture supernatant of the haloduracin producer *B. halodurans* C-125. This two-peptide lantibiotic showed no induction in the four biosensors as well, although it has been demonstrated to act by lipid II binding and pore formation (Oman *et al*., 2011).

Taken together, a MoA similar to that of lacticin 3147 and haloduracin, with exception of the 2:1 lipid II binding stoichiometry, is likely for lichenicidin. Further MoA studies will focus on the lipid II binding and the pore formation capacity of the lichenicidin peptides.

The question, why lichenicidin is exclusively present in cell wash extract, remains open, but an explanation might be an attachment of these peptides to the cell-surface of the producer strain. A location in the cell wall has also been described for haloduracin and lacticin 3147 (Lawton *et al*., 2007; Cotter *et al.,* 2006). For the lacticin peptides, studies with various mutants indicated that the LtnA2 peptides might be tethered to the cell wall of producing cells via the LtnA1 peptides (Cotter *et al*., 2006). Such a tethering effect was not observed for Licα, since Licβ is still located on the cell-surface in absence of Licα, as indicated by MALDI-TOF analysis of culture supernatants and cell wash extracts of the LicM1 insertion mutant (figure 5.1, appendix, page 168).
Proteolytic degradation of lichenicidin was supposed to be an alternative explanation for the absence of active lichenicidin peptides in the culture supernatant, but this notion was disproven by the observation that lichenicidin was stable against the proteases excreted by the producer strain. An attachment of the lichenicidin peptides to cell surfaces might alternatively be mediated by a cell capsule. *B. licheniformis* is able to produce a glutamyl polypeptide capsule (Troy, 1973). Diffusion of the positively charged lichenicidin peptides (Licα +1, Licβ +2) might be restricted by capsule formation, especially as incubation of another strain of this species, *B. licheniformis* DSM 641, on nisin containing agar plates selected nisin resistant mutants with increased capsule formation (Bierbaum, unpublished results). These results suggested that capsules indeed inhibit diffusion of, or can bind, cationic lantibiotics.

On the whole, such a cell-surface location seems inappropriate, considering that the overwhelming majority of lantibiotics is regarded as bacteriocins. These molecules function in defense of producer strains and, thus, the peptides need to be present in the surrounding medium. On the other hand, some morphogenetic peptides, e.g. SapB and SapT of *Streptomyces* strains are lanthionine-containing peptides, too, and possess only very low antimicrobial activity (Kodani *et al*., 2004 and 2005). In recent years, other lantibiotic peptides that do not exert antimicrobial effects have been identified, e.g. the labyrinthopeptins and prochlorosin (Müller *et al*., 2010; Li *et al*., 2011). These peptides form a new class of lantibiotics, called lantipeptides, comprising lanthionine-containing peptides with functions other than antimicrobial activity (Willey and v. d. Donk, 2007).

Interestingly, the majority of lantipeptides was identified in filamentous bacteria, e.g. strepto- and actinomycetes (Kodani *et al*., 2005; Kodani *et al*., 2006; Li *et al*., 2011; Müller *et al*., 2010; Goto *et al*., 2010). Some of these peptides were supposed to fulfill intracellular as well as intercellular signalling functions. A signalling function has also been described for antimicrobially active lantibiotics like nisin and mersacidin: these peptides are autoinducers

of their own biosynthesis in quorum sensing systems (Schmitz *et al*., 2006; Kuipers *et al*., 1995). Thinking of the cell surface association of lichenicidin, it is intriguing to speculate about further functions and roles played by antimicrobially active lantibiotics, too, e.g. signalling functions involved in cell morphogenesis such as sporulation.
In contrast to the DSM 13 producer strain, lichenicidin produced by *B. licheniformis* VK21 and I89 was additionally detected in culture supernatants (Shenkarev *et al.* 2010; Caetano *et al*., 2011). Furthermore, neither publication mentions production of an additional, lichenicidin-independent antimicrobial activity, which might be due to different cultivation conditions or variations in the genomic background of these *B. licheniformis* strains.

For lantibiotic producers, it is obvious that production of a lantibiotic is nearly always restricted to specific strains and not a common feature of the species. This is supported by the observation that lantibiotic gene clusters often appear to be gained by horizontal gene transfer. In agreement with this notion, neither presence of a lichenicidin coding gene cluster nor production of an antimicrobial substance was found in the *B. licheniformis* strain I5 in this study. Lichenicidin is produced by at least four different *B. licheniformis* strains e.g. *B. licheniformis* DSM 13/ATCC 14580, I89 and VK21 and a *Bacillus* strain that was isolated from infested soil in this study. Previous studies reported that due to the ability to form endospores, *B. licheniformis* strains can be isolated from soils and plant material all over the world (Sneath *et al*., 1986). In line with this, the lichenicidin producers were isolated from different sources and ecological niches: the isogenic *B. licheniformis* DSM 13 and ATCC 14580 strains were isolated from soil samples (Rey *et al*., 2004); whereas the I89 and VK21 strains are isolates from hot springs in the Azores (Portugal) or of the Kamchatka Peninsula (Russia), respectively (Mendo *et al*., 2004; Temirov *et al*., 2003). These findings indicate that lichenicidin producing *Bacillus* strains are widely distributed in nature and are found in different ecological niches. In contrast, the morphogenetic prochlorosin lantipeptides are widely distributed in strains of the same or related species found in the same ecological niche and appeared to fulfill more general intra- or extracellular functions (Li *et al*., 2010). Complete genome sequences of *B. licheniformis* I89 and VK21, that would give more insights into relatedness of all three lichenicidin producers, are not yet available.

Potential applications for lantibiotics are food conservation and novel therapeutics. An obstacle that has hampered medical application of many lantibiotics is their missing resistance to proteases; this is especially characteristic for elongated pore-forming peptides like nisin, epidermin and Pep5. These peptides possess a linear hinge region, that is essential for pore-formation and which is susceptible to proteolytic degradation by, for example,

chymotrypsin or trypsin. Stabilization of Pep5 by a further ring structure prevented its digestion by chymotrypsin; however, the antibacterial activity was also decreased (Bierbaum *et al*., 1996). In contrast, peptides that are characterized by formation of intertwined thioether rings and do not have elongated linear stretches, e.g. mersacidin, show higher protease resistance. One reason that *B. licheniformis* DSM 13 was chosen for further investigations is, that it is most prominent in protease production. Seven protease genes are annotated in the genome of this strain (BLi00340, BLi04019; BLi01123; BLi02863; BLi02862, BLi01109, BLi01909). Among those are alkaline serine proteases (subtilisins) e.g. Subtilisin Carlsberg, a protease that is employed in detergents. Therefore, the antibacterial activity excreted by the DSM 13 strain was expected to show higher protease resistance than lantibiotics produced by members of the genera *Staphylococcus* and *Lactococcus*. Indeed, the protease treatments confirmed, that lichenicidin is resistant against trypsin, chymotrypsin and proteases excreted by the producer making lichenicidin a potential antibiotic for medical applications.

In conclusion, the data presented here, establish that *B. licheniformis* DSM 13 produces a novel two-component lantibiotic that was attributed to the presence of a lantibiotic gene cluster, that was found in a bioinformatic approach. This novel, cell surface associated two-peptide lantibiotic lichenicidin displays interesting features for medical applications such as protease resistance and activity against (multiresistant) human-pathogenic strains.

***Bacillus licheniformis* DSM 13 produces an additional bacteriocin**

In the course of this thesis, *B. licheniformis* DSM 13 was shown to produce two antibiotic substances, one present in cell wash extract and an additional in the culture supernatant. Performing agar well diffusion assays showed that both exhibit antimicrobial activities against Gram-positive clinical isolates and were inactive against Gram-negatives. Interestingly, both suspensions displayed a slightly different spectrum of activity indicating that two independent antibiotic substances were produced under chosen conditions. This suggestion was underlined by results of peptide stability assays showing that the substance in the culture supernatant is less stable to heat treatment than the lantibiotic lichenicidin, the substance in the cell wash extract. Furthermore, differences in growth assays with *M. luteus* and *B. subtilis* based whole cell biosensor assays (Staron *et al*., 2011) were observed. The latter suggested that the substance present in culture supernatant causes a cell envelope stress, whereas lichenicidin does not activate the pLIA reporter system. Finally, studies with lichenicidin knock out mutants, demonstrated that the antimicrobial activity in

the *B. licheniformis* culture supernatant is not associated to the lichenicidin gene cluster and, instead, is caused by an independent, unknown substance.

Bacillus species are known to produce a large number of peptide antibiotics with various chemical structures and their genomes very often harbor more than one gene cluster coding for biosynthesis of antibiotic substances (von Doehren *et al*., 1995). In *B. subtilis* 168, different polyketide synthetases exist, that cover almost 4% of its genome. Interestingly, these genes were not identified in the genome of the close relative *B. licheniformis* DSM 13, although it exhibits a marked co-linearity with the genome of *B. subtilis* (Veith *et al*., 2004; Lapidus *et al*., 2002). In contrast to other *B. licheniformis* strains, e.g. *B. licheniformis* ATCC 10716, genes coding for biosynthesis machinery of the cyclic polypeptide bacitracin are absent in the DSM 13 strain (Flickinger and Perlman, 1979; Rey *et al*., 2004).

Besides the gene cluster coding for lichenicidin, four genes (BLi00401- BLi00404) encoding subunits of non-ribosomal peptide synthesis complexes involved in biosynthesis of lichenysin were identified during annotation of the genome (Veith *et al*., 2004). Lichenysin is a cyclic anionic lipopeptide resembling surfactin, an amphipathic biosurfactant, which is produced by *B. subtilis* strains (Yakimov *et al*., 1995; Grangemard *et al*., 2001). Based on its membrane-disruptive effect, surfactin exhibits an antimicrobial activity against Gram-positive bacteria and causes hemolysis of erythrocytes (Arima *et al*., 1968; Nakano *et al*., 1988). Analog activities were also reported for lichenysin (Yakimov *et al.,* 1995) and production of at least six slightly different lichenysin variants (lichenysin A, B, C, D, G and BL86) with a MW between 979-1091 Da, has been observed for *B. licheniformis* strains (Nerukar, 2010).
B. licheniformis DSM 13, is not thought to produce lichenysin itself, although it harbors the corresponding biosynthesis genes. Transcription of the lichenysin biosynthesis machinery is activated by the phosphorylated form of the competence factor ComA (Yakimov *et al*., 1997). However, in *B. licheniformis* DSM 13, the gene of the kinase ComP, that is necessary for activation of the factor ComA, has suffered insertion of an IS element (IS3Bli1) (Lapidus *et al*., 2002; Rey *et al*., 2004) and this gene inactivation might account for the failure to produce lichenysin. In line with this and contrary to the surfactin producer *B. subtilis* 168, no hemolytic activity could be detected for *B. licheniformis* DSM 13 when streaked on Columbia agar plates. In addition, the antimicrobially active substance in the culture supernatant lacks a hemolytic activity indicating that this substance does not represent lichenysin.

In 2009, D. Haft identified, based on bioinformatic studies, existence of a new subfamily of thiazole- and oxazole-modified microcins (TOMMs), the heterocycloanthracins. An

analogous, not yet annotated, structural gene was found in the genome of *B. licheniformis* ATCC 14580, which is isogenic to the DSM 13 strain (figure 4.3 (A)).

TOMM peptides are highly modified bacteriocins, that undergo extensive post-translational modifications resulting in introduction of class specific heterocyclic oxazole and thiazole rings. In a first step, the aa side chains of Cys, Ser and Thr residues are joined to the carbonyl group contributed by the peptide bond with the immediately N-terminally located residue, which often is glycine. The reaction eliminates a water molecule from the amide backbone, thereby, introducing the heterocyclic rings, thiazolines and oxazolines, and is catalyzed by an ATP-dependent cyclodehydratase. An FMN-dependent dehydrogenase removes two electrons and protons to form the class specific aromatic rings, thiazoles and oxazoles. Both modification enzymes are co-organized and form a trimeric modifying protein complex with an additional scaffold protein (Ghilarov *et al*., 2011; Melby *et al*., 2010).

The heterocycloanthracin precursor of *B. licheniformis* is characterized by twelve repetitive cysteine-rich motifs in its C-terminal sequence (figure 4.3). Eight of these repeats are tripeptides characterized by a CXS motif and five of them share the related "CWS" sequence. A similar tetrapeptide "CWSC" repeat is present in the class IIa bacteriocin thuricin S produced by *B. thuringiensis* (Haft, 2009; Chehimi *et al*., 2007). In this cationic and hydrophobic peptide, the CWSC motif also occurs within a repetitive CXX sequence. Based on the aa sequences of the precursor, introduction of up to thirteen thiazoline/thiazoles and ten oxazolines/oxazoles would be possible

MQPFHDELQSLEMDHFQADDMTHWDPDRHHANVKH CAS CWS CGS CAS CWS CAGHS CWS CMGHS CWS CAGH CCWS CWHGGM

Figure 4.3: AA sequence of the predicted heterocycloanthracin precursor identified in the genome of *B. licheniformis* DSM 13. The putative heterolycloanthracin precursor of *B. licheniformis* DSM 13 is characterized by repetitive CXX motifs (**orange**).

In vicinity of the putative bacteriocin precursor, Haft identified two ORFs (BLi01044 and BLi01045) that are similar to enzymes and proteins involved in post-translational modifications of TOMM peptides. Although this arrangement is commonly found in gene clusters coding for bacteriocins, a co-localization of precursor and corresponding modification enzymes was not found universally in genomes of predicted producers of heterocycloanthracins (figure 4.3 (B)).

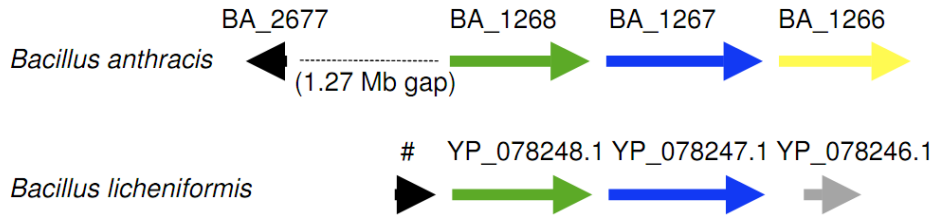

Figure 4.4: Genomic regions in *B. anthracis* Ames and *B. licheniformis* ATCC 14580 predicted to encode bacteriocin precursors of the heterocycloanthracin group. Putative structural genes are black and other genes are marked as follows: **cyclodehydratases**, putative **docking scaffold proteins**, **dehydrogenases** and **protein with unknown function** (Haft, 2009).

ORF BLi01044 codes for a cyclodehydratase and BLi01045 is a scaffold-like protein. In contrast to other TOMMs, even to other putative heterocycloanthracin producers, a thiazole/oxazole-producing dehydrogenase was not identified in the genome of *B. licheniformis* (Haft, 2009), a finding that has previously been described for producers of the TOMM peptides of the cyanobactin subfamily, too. Absence of modification enzymes resembling FMN-dependent dehydrogenases in these producers results in synthesis of peptides with thiazoline and oxazoline rings. Subsequently, these residues are partially converted into their aromatic heterocycles by so far uncharacterized cellular enzymes (McIntosh and Schmidt, 2010; Ghilarov *et al*., 2011). However, some of these oxazolines and thiazolines still remain unmodified in the otherwise completely modified peptides, e.g. patellamides A and trunkamide (Irland *et al*., 1982; Wipf *et al*., 2000). Interestingly, for members of other TOMM peptide subfamilies, e.g. microcin B17 and streptolysin S, variants containing unoxidized thiazolines or oxazolines were not detected (Ghilarov *et al*., 2011). These findings open the following questions regarding the structure of the putative heterocycloanthracin peptide found in *B. licheniformis* DSM 13:

1. Does this bacteriocin contain the unoxidized oxazoline and thiazoline residues instead of oxazole and thiazole rings?
2. Are these residues (partially) converted into oxazoles and thiazoles by an unknown, additional modification enzyme?
3. Is this gene cluster a complete heterocycloanthracin gene cluster that confers ability to produce the fully modified peptide?

The antimicrobial substance in the *B. licheniformis* DSM 13 culture supernatant might represent this new TOMM-like bacteriocin. As demonstrated in *B. subtilis* whole cell biosensor assays, the substance in the supernatant most likely targets the bacterial cell

surface. Interestingly, such an effect has not been described for any antimicrobially active peptide of the TOMM family so far. Nevertheless, a cell membrane affecting MoA has been described for thuricin S, which possesses only little aa similarity to the putative *B. licheniformis* heterocycloanthracin and apparently belongs to another class of bacteriocins (Chimini *et al*., 2007; 2010; Haft, 2009).

No bacteriocin of the predicted heterocycloanthracin subfamily has been purified or experimentally characterized up to now. Therefore, putative molecular weights, modification states or MoA could not be predicted for these new bacteriocins based on *in silico* analysis.

However, MALDI-TOF analysis of the culture supernatant revealed different mass signals within the TOMM relevant range (2-10 kDa, see appendix page 168). At least one of these signals might represent the modified bacteriocin. This hypothesis awaits experimental confirmation. To this end, cyclodehydratase knock out mutants will be generated in order to correlate antimicrobial activity of *B. licheniformis* DSM 13 culture supernatant to the predicted bacteriocin-coding gene cluster. Additionally, future studies will focus on purification, structural characterization and MoA analysis of this promising substance.

Caldicellulosiruptor bescii DSM 6752 and *Nostoc punctiforme* ATCC 29133 are putative producers of lanthionine-containing peptides

Detailed bioinformatic analysis identified complete lantibiotic-like gene clusters in *N. punctiforme* and *C. bescii*. However, both strains display features uncommon for producers of lantibiotics and lantibiotic peptides:

(A) *Caldicellulosiruptor bescii* DSM 6752 (besciin)

The genes of the lantibiotic-like gene cluster of *C. bescii* are highly similar to corresponding genes of the lichenicidin gene cluster. This sequence similarity extends to the C-terminal part of the besciin propeptide and led, together with the presence of a mersacidin-like binding motif, to the assumption that the encoded lantibiotic peptide might 1. hold a conserved lanthionine-bridging pattern as seen in LanA1 peptides of two-peptide lantibiotics and 2. be antimicrobially active due to lipid II binding and inhibition of the cell wall biosynthesis.

On the other hand, the propeptide sequence is extremely long, compared to other lantibiotics, and leader cleavage at the conserved processing site would lead to the largest lantibiotic peptide identified so far (52 aa). Similar to other predicted lantibiotic gene clusters, this cluster contains a gene coding for an FMN-depended reductase that might exert a so far unknown role in modification of the lantibiotic besciin.

(B) *Nostoc punctiforme* ATCC 29133 (puncticin)

The predicted lantibiotic gene cluster of *N. punctiforme* ATCC 29133 contains two almost identical putative lantibiotic structural genes (PunA1; PunA2), a modification enzyme of the LanM-type (PunM), an exporter (PunT(P)) typical for class II lantibiotics in addition to a second exporter lacking an internal protease domain and genes coding for membrane proteins. The genome additionally harbors three further LanM-like proteins and six ORFs coding for short peptides that exhibit sequence similarity to the leaders of PunA1 and PunA2. Three of those putative LanA coding ORFs (Npun_F3224 – 26) are clustered and two share almost identical aa sequences. A *lanM*-gene was not found in direct vicinity of these, but ~20 kb upstream. The other LanAs are widely distributed within the genome and two of them (Npun_R3313; Npun_AF077) are flanked by a LanM-like protein. The sixth ORF is not co-localized with a LanM and apparently is no lantipeptide precursor, since Cys are absent in its C-terminus.

Irrespective of the culture conditions chosen, no production of an antimicrobial activity could be detected for *Nostoc punctiforme* (diploma thesis, J. Dischinger, 2008). MALDI-TOF analysis of supernatants and isopropanol cell wash extracts did not identify expression of peptides attributable to the predicted puncticin precursors. For this reason, this study aimed at the establishment of expression systems for heterologous expression of the predicted precursors and modification enzymes with focus on the *lanA* and *lanM* genes found in the puncticin cluster.

Of note, cyanobacteria like *Nostoc* are referred to as Gram-negatives, which had not been considered as potential lantibiotic producers until characterization of the cyanobacterial lantipeptide prochlorosin (Li *et al*., 2010) that was described during preparation of this study. Prochlorosin is produced by the single-cell, planktonic, marine cyanobacterium *Prochlorococcus* MIT9313. A total of 29 different structural genes was identified, all have leader sequences that include GG or GA cleavage sites. Seven structural genes are found clustered with the gene coding for ProcM. The other 22 *procA* genes are found elsewhere in the genome, 20 of which also cluster in three further regions. Propeptide sequences of these precursors are diverse in regard to their length and aa composition. Even the aa precursors for lanthionine and methyllanthionine, Ser, Thr and Cys, are unevenly distributed in these peptides (Li *et al*., 2010).

In contrast to the leaders of prochlorosin peptides which are highly conserved with almost identical aa sequences, the putative structural genes identified in *N. punctiforme* have similar (33-65%) leader sequences. Exceptions are PunA1 and PunA2: both sequences are only almost identical, since their leaders differ in two positions and 1 aa exchange is found in

their propeptides. To this end, an evolution of these peptides by gene duplication is likely, especially since the intergenetic regions flanking both genes are highly similar, as well. Evolution by gene duplication of two almost identical lantibiotic precursors has been described for the lantibiotic gene cluster coding for streptococcin AM49 (Hynes *et al*. 1994).

In *in vitro* enzyme assays, 17 prochlorosin peptides were modified by a single, promiscuous LanM enzyme (ProcM) (Li e*t al*., 2010). In addition, production of at least three prochlorosins was demonstrated *in vivo* and occurred in late-exponential growth stage. Resulting lantipeptides are polycyclic, conformationally constrained products with highly diverse ring topologies. None of the analyzed peptides had aa sequence similarity or an analogous thioether ring pattern to known lantibiotics or lantipeptides. The peptides are believed to play habitat- and/or community-specific roles and were assigned to exert intracellular functions. Genomic data mining for similar lantipeptides in metagenomic databases of ocean samples showed that corresponding sequences occur in variable amounts across the oceans, with a hot spot in a Galapagos hypersaline lagoon (Li e*t al*., 2010). First data suggest an involvement of cyanobacterial lantipeptides in nitrogen metabolism. Whole genome expression studies showed that transcription of *procA* genes is down-regulated by nitrogen starvation, but does not respond to phosphate or iron starvation (Li *et al*., 2010).
Wang *et al*. (2011) showed, using genome mining, that filamentous heterocyst-forming cyanobacterial strains, such as *Nostoc* strains with larger genomes, tend to possess more bacteriocin gene clusters than unicellular marine strains. *N. punctiforme* is able to differentiate into different cell forms: nitrogen-fixing heterocysts, spore-like akinetes, and motile hormogonium filaments. Identification of global gene expression patterns in heterocyst-containing cultures and at specific time points during differentiation of akinetes and hormogonia (Campbell *et al*., 2007), showed that nitrogen-fixing conditions upregulate expression of a histidine kinase and its corresponding transcriptional regulator, that both were thought to be part of the puncticin gene cluster. This might indicate an involvement of puncticin in heterocyst differentiation and nitrogen assimilation.

Sequence similarity of predicted leader sequences found in *N. punctiforme* and Nif11 proteins, which code for nitrogen fixing proteins provide an additional link of cyanobacterial lantipeptides to nitrogen metabolism. In agreement with these data, certain prochlorosin precursors resemble Nif11 proteins, too (Li *et al*., 2010). Existence of bacteriocins containing Nif11-like leader sequences in cyanobacteria was discovered by Haft *et al*. (2010) and Wong *et al*. (2011) additionally identified different classes of bacteriocins in cyanobacteria by genomic data mining. Some of these predicted bacteriocin gene clusters include typical

lantibiotic modification enzymes of the LanM-type, others are co-localized with genes coding for enzymes involved in modification of TOMM-like bacteriocins.

Fourteen bacteriocin-coding gene clusters have been predicted for *N. punctiforme* ATCC 29133 (Wang *et al*. 2011. The authors reported four lantipeptide-like gene clusters in this organism, indicating that beside the putative precursors identified in this thesis, other putative lantibiotic-like precursors exist, which share no sequence similarity with PunA1 and PunA2.

Based on the data presented, it is assumed that the predicted structural genes of *N. punctiforme* code for lantipeptides without antimicrobial activity, too. Existence of different LanM enzymes and structural genes suggests that more than one lantipeptide is expressed in this organism, forming a complex lantipeptide-mediated signal network. Future studies will further explore heterologous expression of these *lanM* and *lanA* genes. *In vitro* reconstitution of lantibiotic modifications will be performed to confirm their lanthionine-containing structure.

(C) heterologous expression

For both putative producers, *N. punctiforme* and C. *bescii*, expression of biosynthesis enzymes and structural genes in a heterologous system to subsequently assess *in vitro* reconstitution of lantibiotic biosynthesis was tested. Recombinant vectors coding for N-terminally HIS-tagged precursors and C-terminally HIS-tagged modification enzymes were generated successfully. By site directed mutagenesis, engineered factor XA protease cleavage sites were introduced into the precursors to enable site specific *in vitro* processing. Heterologous expression of the precursors PunA1_XA and AnaA_XA was successfully achieved in *E. coli* Bl21 following the LanA NI-NTA purification protocol published by McClerren *et al*. (2006). Heterologous expression of PunM and AnaM was not successful so far and, thus, remains in the focus of ongoing studies. Using complementary approaches, overproduction of N-terminally HIS-tagged MrsM, the modification enzyme of mersacidin, was achieved successfully. First experiments in which PunM and AnaM were expressed in *E. coli* indicated overexpression of these proteins, but resulted in cell lysis due to a toxic effect. This challenge will be tackled by co-expression of chaperones *in E. coli* strains optimized for expression of toxic proteins.

Successful heterologous production and *in vitro* modification have previously been shown for other lantibiotics and lantipeptides, e. g. haloduracin, prochlorosin, nisin (McClerren *et al*., 2006; Li *et al*. 2010; Chen *et al*., 2007). *In vitro* modification systems hold certain

advantages in discovery and characterization of new lantibiotics and also in bioengineering of lantibiotic peptides. Thus, these systems allow rapid production and characterization of highly modified lantibiotic variants. *In vivo*, expression of engineered peptide variants often fails, since these peptides are not recognized by innate immunity, regulatory or transporter systems of producers and, hence, cannot be secreted or are toxic for producing strains. Moreover, these systems are promising tools for utilizing lantibiotic modification enzymes for incorporation of thioether rings into other medical peptides in order to increase their chemical and proteolytic stability (Kuipers *et al*., 2005; Bosma *et al*., 2011). Additionally, *in vitro* reconstitution of lantibiotic biosynthesis opens the door for a detailed investigation of new lantibiotics that were identified in silent or incomplete gene clusters or in metagenomic sequences.

The genome of *B. amyloliquefaciens* FZB42 harbors the mersacidin immunity genes that confers resistance against the lantibiotic mersacidin

Genome sequence annotations indicated that lantibiotic immunity operons are widely distributed in bacterial genomes. Typically, lantibiotic immunity operons are co-located with other genes involved in lantibiotic production and together they form lantibiotic gene clusters (Siezen *et al*., 1996). In bacteria producing lantibiotics, immunity proteins confer resistance against the lantibiotic produced, a phenomenon which is commonly referred to as immunity or producer self-protection. Mining of bacterial genomes revealed orphan immunity operons that did not appear to be associated with production of a corresponding lantibiotic in these hosts. Some of these immunity proteins have a high aa sequence similarity to previously described immunity systems and, thus, they might offer protection against the same lantibiotic or closely related peptides by homologous protein functions. This suggestion is supported by results obtained by transfer of immunity providing proteins to sensitive indicator strains. Expression of epidermin immunity transporters in *Staphylococcus carnosus* resulted in a 6-fold higher resistance to gallidermin, an epidermin analog (Otto *et al*., 1998).

Annotation of the *B. amyloliquefaciens* FZB42 genome revealed presence of the two mersacidin immunity operons (Chen *et al*., 2007). These include the subunits of the mersacidin immunity transporter, MrsEFG, and a two-component regulatory system comprising the transcriptional regulator MrsR2 and the histidine kinase MrsK (Altena *et al*., 2000). The latter is involved in transcriptional regulation of the immunity operon in the wild-type producer and induces expression of MrsEFG in presence of mersacidin (Schmitz *et al*., 2006; Guder *et al*., 2002). Involvement of the ABC-transporter subunits MrsEFG in resistance

against mersacidin was clearly demonstrated by heterologous expression of these proteins in *Staphylococcus carnosus* TM 300 (R. Reinartz, diploma thesis, 2005).
In silico analysis showed, that immunity proteins and their intergenic regions found in the FZB42 strain are highly similar to corresponding sites in the mersacidin gene cluster. Additionally, this cluster is located in the same genetic context as in the mersacidin producer *B. sp.* HIL Y-85,54728. These findings support the idea that *B. amyloliquefaciens* FZB42 might have lost the biosynthetic part of the mersacidin cluster during evolution.
In this study, the mersacidin immunity system in *B. amyloliquefaciens* FZB42 was demonstrated to confer resistance against mersacidin. In MIC determinations this strain was at least as resistant to mersacidin as the wild-type producer. *B. amyloliquefaciens* FZB42 produces a vast array of antibacterial and antifungal compounds that are thought to provide competitive advantage in its natural habitat, the rhizosphere. Presence of genes, which are almost identical to the immunity genes of mersacidin provides additional protection against competing strains that excrete this lantibiotic.

Recently, a gene locus *spiEFG*, resembling the subunits of the immunity ABC transporter (NsuEFG) of nisin U, was identified in *Streptococcus infantarius* (Draper *et al*., 2012). As seen for *B. amyloliquefaciens* FZB42, this gene locus also contains genes coding for the two-component regulatory system which is involved in regulation of nisin U biosynthesis and expression of the dedicated immunity system in the wild-type producer *Streptococcus uberis*. Heterologous expression of SpiEFG in *Lactococcus lactis* conferred resistance against nisin U and its variants as well, and verified a correlation of this transporter to nisin U resistance (Draper *et al*., 2012).
In agreement with this, “immune mimicry” has been described for proteins homologous to LanI referring resistance to lantibiotics due to natural presence or acquisition of homologs of lantibiotic immunity proteins (Draper *et al*., 2009). The authors demonstrated presence of orphan LanI peptides, that have sequence similarity to the LtnI peptide, which is associated with immunity to the two-component lantibiotic lacticin 3147. Orphan LanI peptides were identified in non-lantibiotic producers such as *E. faecium* DO and heterologous expression of these peptides resulted in development of resistance against lacticin 3147 in sensitive strains (Draper *et al*., 2009).
Presence of such immunity protein homologs, that confer innate resistance in pathogenic bacteria, might become an immense problem when lantibiotics are developed for clinical application. Additionally, transfer of these immunity systems by horizontal gene transfer among pathogenic strains might facilitate emergence of lantibiotic-resistant strains.

However, the lantibiotic nisin has been used as food preservative for more than 50 years and development of resistance has rarely been observed. If it occurs, the resistance generally relies on target modification such as changes in membrane composition (Kaur *et al*., 2011). On the other hand, lantibiotic immunity proteins are highly specific and cross immunity is a rare phenomenon, even among producers of structurally highly similar lantibiotics. Thus, possession of lantibiotic immunity proteins might confer resistance to only one lantibiotic or closely related variants thereof. This implies, that immune mimicry is not a general mechanism of resistance to lantibiotics.

Mersacidin is heterologous expressed in *B.amyloliquefaciens* FZB42

The mersacidin wild-type producer *B. sp.* HIL Y-85,54728 is not naturally competent. This means, that genetic manipulation of the lantibiotic gene cluster could only be achieved by protoplast transformation, which is time consuming and yielded only low transformation frequencies. This study, aimed at transferring the complete mersacidin gene cluster into a naturally competent *Bacillus* strain and the subsequent heterologous expression of mersacidin by this host.

Successful heterologous production has previously been shown for several lantibiotics, e. g. for subtilin (Liu *et al*., 1991) and nisin (Yuksel *et al.*, 2007) by *B. subtilis* 168, for lacticin 3147 by *Enterococcus faecalis* (Ryan *et al*., 2001), or for epicidin 280 by *Staphylococcus carnosus* (Heidrich *et al*., 1998). Very recently, production of active lichenicidin has even been achieved in *E. coli* (Caetano *et al*., 2011). However, heterologous production of a lantibiotic cannot be taken for granted and remains difficult. For example, epicidin 280 and Pep5 are two closely related lantibiotics, but Pep5 shows a higher antibacterial activity than epicidin 280. In contrast to epicidin 280, Pep5 cannot be expressed in *S. carnosus*, which is susceptible to this agent in a nanomolar range, indicating that toxicity of the product may be a problem here (G. Bierbaum, unpublished data).

Lantibiotics generally target essential and conserved cell structures such as bacterial cell membranes or the cell wall precursor lipid II, structures that are present in producing strains, as well. This necessitates, that these targets have to be protected by dedicated producer-self-protection systems to inhibit self-destruction of the producers. This common feature has to be considered when production of lantibiotics is aimed to be transferred into a heterologous expression host. Thus, for successful and high-level expression of mersacidin in a heterologous host, two hurdles have to be overcome: functional producer self-protection and transfer of the biosynthetic gene cluster to the new host.

Here, it was demonstrated that *B. amyloliquefaciens* FZB42 is resistant to mersacidin due to existence of homologous mersacidin immunity genes. Therefore, this strain was chosen as amenable to heterologous production of mersacidin. After transfer of the biosynthetic part of the gene cluster, successful expression of mersacidin was obtained from plasmids *in trans.* As demonstrated by MALDI-TOF analysis, HPLC based peptide purification and agar well diffusion assays, the expressed mersacidin was antimicrobially active and fully modified.

In conclusion, it could be shown here, that it is possible to produce mersacidin in *B. amyloliquefaciens* FZB42. The successful production of fully modified and active mersacidin by this strain provides an appropriate *in vivo* expression system for the construction and expression of mersacidin analogs and modified variants, as previously demonstrated for the *in vivo* expression system for nisin (Kuipers *et al.*, 2005; Bosma *et al*., 2011). Production of mersacidin in this strain additionally allows genetic manipulation and downstream modification of the whole biosynthesis gene cluster.

The Mersacidin producer *B. sp.* HIL Y-85,54728 belongs to the species *B. amyloliquefaciens*

Presence of the mersacidin immunity operon in *B. amyloliquefaciens* FZB42 raised the question whether the natural, so far uncharacterized, mersacidin producer might also belong to the species *B. amyloliquefaciens*.

In previous studies of our research group, various gene sequences obtained from the mersacidin producer were compared to those of the publicly available genome sequences of *B. amyloliquefaciens* FZB42, DSM 7 and *B. subtilis* 168 (A.M. Herzner, diploma thesis, 2008). In these *in silico* analyses, a high similarity between *B. amyloliquefaciens* FZB42 and the producer strain of mersacidin was obvious. All analyzed sequences, including the intergenic regions, showed about 98.5% nucleotide sequence identity to the FZB42 strain, whereas these sequences had about 93.5% and 77.4% identity to *B. amyloliquefaciens* DSM 7 and *B. subtilis* 168, respectively. Existence of a gene cluster coding for the non-ribosomal peptide antibiotic bacillaene was described for both *amyloliquefaciens* strains (Chen *et al*., 2007; Rueckert *et al*., 2011) and presence of the gene *baeD*, coding for a malonyl-CoA-transacylase/oxidoreductase involved in bacillaene synthesis, was also demonstrated in *B. sp.* HIL Y-85,54728. These findings indicated that the mersacidin producing strain at least carries parts of the bacillaene cluster.

Additionally, biochemical identification tests demonstrated that in contrast to *B. subtilis* 168, *B. amyloliquefaciens* FZB42 and *B. sp.* HIL Y-85,54728 were able to metabolize xylose, lactose and starch. Both strains neither grew at 50°C nor in presence of 10% sodium chloride.

Furthermore, both strains did not produce acid from trehalose and mannitol, but differed in their ability to metabolize gelatin. To clearly classify *B. sp.* HIL Y-85,54728, 16S rRNA coding genes were sequenced. NCBI BLAST searches employing the obtained sequences indicated a close similarity to *B. amyloliquefaciens* FZB42 (A.M. Herzner, diploma thesis, 2008).

B. amyloliquefaciens FZB42 was isolated from the plant rhizosphere and has recently been defined as the type strain of a group of growth-promoting and plant-associated *B. amyloliquefaciens* strains. Members of this group form a distinct cluster within the *amyloliquefaciens* species, the subspecies *B. amyloliquefaciens* subspec. *plantarum*. In addition to their ability to colonize plant roots, members of the *plantarum* subspecies are discriminated from the subspecies *B. amyloliquefaciens* subspec. *amyloliquefaciens* by differences in *gyrA* and *cheA* nucleotide sequences, hydrolysis of cellulose and an increased ability to produce non-ribosomal secondary metabolites like fengycin and difficin (Borriss *et al*., 2010; Chen *et al*., 2009; Chen *et al*., 2007; Rueckert *et al*., 2011).
Here, the *gyrA* gene of the mersacidin wild-type producer, which codes for the subunit A of the gyrase, was sequenced. A blast analysis in the NCBI database verified a close similarity of *B. sp.* HIL Y-85,54728, to *B. amyloliquefaciens* species. In a taxonomic tree, that was calculated based on *gyrA* sequences of the mersacidin producer and different members of the genus *Bacillus*, the producer strain is located in the cluster formed by members of the *plantarum* subspecies, suggesting a close association between the mersacidin wild-type producer and plant-associated strains. This similarity was not confined to the *gyrA* sequence, but was also reflected in the generally higher nucleotide sequence identity of *B. sp.* HIL Y-85,54728 and *B. amyloliquefaciens subspec. plantarum* FZB42 in comparison to the values reached by the DSM 7 type strain of the *B. amyloliquefaciens subspec. amyloliquefaciens* group.

A distinguishing feature of the subspecies *B. amyloliquefaciens* subsp. *plantarum* is the ability to colonize *Arabidopsis* roots (Borriss *et al*., 2010). In fact, nearly all members of this subspecies were isolated from plants, plant roots or, like *B. amyloliquefaciens* FZB42, from infested soil. The only exception is represented by the strain UCMB5113 that was isolated from soil. In contrast, the producer strain of mersacidin originates from soil of a salt pan in Mulund, India, and unfortunately association of the original isolate with plants was not mentioned in the first report (Chatterjee *et al*., 1992). However, the strain did not grow in presence of 10 % salt in the laboratory, indicating that the salt pan might not be its natural biotope and that its presence in the sample might rather be due to its ability to form long-lived spores.

In conclusion, the mersacidin wild-type producer was clearly demonstrated to belong to the species *B. amyloliquefaciens* and apparently is closely related to the plant-associated subspecies *plantarum*. Therefore, *B. sp.* HIL Y-85,54728 should be renamed *Bacillus amyloliquefaciens* HIL Y-85,54728.

General conclusion and comments

The data presented here, demonstrated the advantage of genomic data mining and bioinformatic analysis in the discovery of new antibiotic substances over classical antibiotic identification methods. The gene cluster encoding lichenicidin was identified by bioinformatics and its identification is an additional example of how publicly available bacterial genome sequences support discovery of new lantibiotics. On the other hand, structure elucidation of lichenicidin peptides showed, that prediction of lanthionine bridging pattern based on aa sequence similarity is not always correct and has to be verified experimentally.

Besides identification of new lantibiotic producers, genomic data mining of lantibiotic immunity proteins resulted in identification of a *Bacillus* strain immune to mersacidin. The strategy employed here, i.e. use of an organism that already possesses immunity genes of a lantibiotic, for production of the same substance, was successful and allowed production of active and fully modified mersacidin in an *in vivo* expression system using the naturally competent *B. amyloliquefaciens* FZB 42.

Bioinformatic prediction of new lantibiotic producers revealed that lanthionine-containing peptides are more widely distributed among bacteria than assumed. This finding is in line with recent publications reporting the identification and prediction of new bacteriocin producers, including bacterial taxa that were not assigned to be putative lantibiotic producers (Li *et al*., 2010). Characterization of antimicrobially inactive lanthionine-containing peptides and their distribution in nature suggested other than antimicrobial functions, like morphogenetic or signalling activity. This indicates that we are just starting to learn why bacteria produce these fascinating peptides.

Summary

Lantibiotics are microbial peptide antibiotics that are characterized by presence of the thioether aa (methyl-)lanthionine. Lantibiotics possess structural genes which encode inactive prepeptides. During maturation, the prepeptides undergo post-translational modifications including 1. introduction of rare aa such as (methyl-)lanthionine; and 2. proteolytic removal of the leader. The structural gene (*lanA*) and the other genes involved in modification of lantibiotics (*lanM*, *lanBC, lanP*), regulation (*lanR*, *lanK*), export (*lanT(P)*) and immunity (*lanEFG*) are organized in gene clusters.

The focus of the present studies was the identification of novel lantibiotic producers by genomic data mining. Lantibiotic modification enzymes share conserved structures and sequences. To this end, they are useful blueprints for bioinformatic identification and prediction of novel lantibiotic gene clusters.

Here, BlastP searches identified three putative lantibiotic producers, *Bacillus licheniformis* DSM 13, *Nostoc punctiforme* ATCC 29133 and *Caldicellulosiruptor bescii* DSM 6725, that were chosen for detailed analyses in this thesis.

For *B. licheniformis* DSM 13 a two-peptide lantibiotic cluster was predicted. Here, production of two antimicrobial substances was demonstrated for this strain, one present in the culture-supernatant and the other in the cell wash extract. Both exhibited differences in the activity spectra and displayed different biochemical properties.

By site-directed mutagenesis the antimicrobial activity of the cell extract could be attributed to production of a two-peptide lantibiotic, lichenicidin, encoded by the predicted gene cluster. Subtractive MALDI-TOF analysis of mutants confirmed masses of 3020.6 and 3250.6 Da for active lichenicidin. Both peptides were shown to exhibit activity, but antimicrobial action was enhanced when both were combined indicating a synergistic effect. Lichenicidin showed interesting activities against Gram-positives including multi-resistant *Staphylococcus aureus* strains. Its efficacy was comparable to that of other lantibiotics, some of which are promising candidates for medical application.

Antimicrobial activity of the culture supernatants was unaffected in these knock out mutants and apparently is not based on the lichenicidin cluster. During preparation of this study, the existence of a structural gene coding for a bacteriocin of a new family of thiazole-oxazole-microcin B17-like peptides (TOMM) was predicted (Haft, 2009) and this gene product might represent the uncharacterized antimicrobial substance of the supernatant. Future work will focus on characterization of this substance.

For the lantibiotic-like gene clusters found in *N. punctiforme* and *C. bescii*, features uncommon for lantibiotic producers are evident: 1. cyanobacteria like *Nostoc* are referred to

as Gram-negatives, which had not been considered lantibiotic producers until identification of the cyanobacterial lantipeptide prochlorosin (Li *et al*., 2010), and 2. the predicted lantibiotic precursor of *C. bescii* shares similarity to known lantibiotics, but is extremely long compared to other lantibiotic propeptides and its gene cluster might harbor a new modification enzyme. Furthermore, the active besciin peptide would be the largest lantibiotic known.

In this thesis, heterologous expression of biosynthesis enzymes and structural genes of both producers was aimed for the subsequent *in vitro* reconstitution of lantibiotic biosynthesis. Recombinant vectors coding for N-terminally HIS-tagged precursors and C-terminally HIS-tagged modification enzymes were successfully generated. Site directed mutagenesis introduced an engineered factor XA protease cleavage site into the precursors to enable site-specific *in vitro* processing. Heterologous expression of the HIS-tagged puncticin and besciin precursors was successfully achieved, whereas expression of the corresponding modification enzymes was not successful so far and this challenge is the subject of ongoing studies.

The second project aimed at constructing an *in vivo* lantibiotic expression system to produce mersacidin in a naturally competent *Bacillus* strain. *B. amyloliquefaciens* FZB42 harbors the mersacidin immunity genes and could be shown to be resistant to mersacidin. Therefore, *B. amyloliquefaciens* FZB42 was chosen for heterologous expression. After transfer of the biosynthetic part of the cluster, production of fully modified and antimicrobially active mersacidin was achieved *in trans*. The new mersacidin producer allowed genetic manipulation of the lantibiotic cluster. Furthermore, the mersacidin wild-type producer was demonstrated to be a member of the species *B. amyloliquefaciens* and, therefore, it was renamed *B. amyloliquefaciens* HIL Y-85,54728.

Both projects support the usefulness of genomic data mining and bioinformatics in identification of genes associated with lantibiotic production or immunity and enabled the discovery and characterization of the new two-peptide lantibiotic lichenicidin and the establishment of an *in vivo* mersacidin expression system in *B. amyloliquefaciens* FZB42.

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Appendix

AA sequence of the N-terminally HIS-tagged PunA1

- 11.922 kDa
- present in pET28PunA1
- additional aa that are not present in the original PunA1 sequence are highlighted in **bold**
- putative cleavage sites are underlined

MGSSHHHHHHSSGLVPRGSHMLHQIKELLQNAQLQQQVKAAANQAEAIKVLAIASAEKGYNFTVEAIS
QMLAELTSVASDELSEEELLSVSGGAVSESHVHMSCCNDCPTR*

AA sequence of the N-terminally HIS-tagged PunA1_XA

- 12.077 kDa
- present in pET28PunA1-XA
- additional aa that are not present in the original PunA1 sequence are highlighted in **bold**
- the XA-factor cleavage site is underlined

MGSSHHHHHHSSGLVPRGSHMLHQIKELLQNAQLQQQVKAAANQAEAIKVLAIASAEKGYNFTVEAIS
QMLAELTSVASDELSEEELLSIEGRAVSESHVHMSCCNDCPTR*

AA sequence of C-terminally HIS-tagged PunM

- 124.828 kDa
- present in pET22bPunMAB
- additional aa that are not present in the original AnaM sequence are highlighted in **bold**

MSQLFVQSSQQIPLSDLATIVTKASFLWERLNTKQFAIAAGLLNEQKIDHRLERWCQVVAQGNWNTLQ
KRLEWEGLDFDTIRPQLGTVEFNAGQPLPKWAETLQKIIQTATEFQPELETFLPTDSENPIPFEDIFL
PIIKVARLELRTRLEEHLPQLLLTETAYRNLERSLLQRLATFCTKTLHFEFSQARPFGQNLLNLLGLE
TESEKTKTHYNKFVNQLLQDGLMAFFCKYPVLGRLVATVVNFWVDFTAEFLQRLAEDKADIQQIFGEF
GSEQPDYLGKVAEIKTSLSDPHKQGRTVILLTFESGLKLVYKPKDLGLEVSFNQFLDWCNQHSHLLDF
KVIQVLNRNNYGWVEYVEHQACIDEAAAERFYQRAGMLLCVLYTLRGTDCHHENLIASGEHLVLIDTE
TLLHHEANLIENSPDMQGSEANAEQQLGNSVLRSGLLPRWDFSSDRRVAYDVSGLGSTDPQQAPQKVP
RWGLVNTDEMHLGYEFITLPIQKNVPYIGEIGLSPNDYQAQIAAGFEQMYRFLMDNKDMLFQPKSPLT
VMGKQQVRFVFRPTRVYSVILQKIWASDYLKDGIDYSIELERLSCAFLVAQEKPNAWHILSAELRAME
QLDIPFFTANAASDELSVSDDLSIPHYFKQPSYHHVLRQLQAMSKTDLARQIAIIQGSFHAKLAQTSS

EESEQWDVESAPLLSSAQLIEEAGAIANALEKTAILDPDGSINWIGLNYVPEAERFRLQVLGSGLYDG
RSGVALFLAALNKVAGEPRLRDLALRTLQPLRRQIQTFDLESQQRMSRLMGIGGGAGLGSLIYAFVKV
RQFLNDETLLADAQALAEWMTPELIAADKKLDIMSGAAGAILGLLSLYEVTKDATVLEKAIACGQHLL
SRQVSYKGAPKAWQTLGEQPLTGFSHGTAGISYALLRLYTVTQNQDYYEAALAGIEYERSVFCESQGN
WPDFRSAEMNQPPGFPVRWCHGATGIGLGRLGSLGVIDSPEIEQEIEIALQTTQNYSLQDSDHLCCGN
LGRIEVLLVGAQRCSRSDWHQIALQNAANVVAKAKQTGAYQMFTNLPSSVFHPSLFRGTAGIGYELLR
LATDDLPSVLLWE**LEHHHHHH***

AA sequence of the N-terminally HIS-tagged AnaA

- 12.491 kDa
- present in pET28AnaA
- additional aa that are not present in the original AnaA sequence are highlighted in **bold**
- putative cleavage sites are underlined

MGSSHHHHHHSSGLVPRGSHMKESTIIKNPVLRNKVNAKIYNPAGDIVKEIQEQNLPEQAGGGTPTVV
VGVISAVTAVTNLAFSIDQAITKYYACSLVYTYSAECRSDGRSCRMR*

AA sequence of N-terminally HIS-tagged AnaA_XA

- 12.633 kDa
- present in pET28AnaA_XA
- additional aa that are not present in the original AnaA sequence are highlighted in **bold**
- the XA-factor cleavage site is underlined

MGSSHHHHHHSSGLVPRGSHMKESTIIKNPVLRNKVNAKIYNPAGDIVKEIQEQNLPEIEGRGTPTVV
VGVISAVTAVTNLAFSIDQAITKYYACSLVYTYSAECRSDGRSCRMR*

AA sequence of C-terminally HIS-tagged AnaM

- 122.567 kDa
- present in pET22bAnaMAB
- additional aa that are not present in the original AnaM sequence are highlighted in **bold**

MMKQDNNWLIYNLSTDKEFTFDDVINYWLTLFPEIKKKEELEKLLQEISNTNFEKIREYYYSTEKHGE
KFLGKYCNNIELVLELERLLAIIHNEYKWVYFFKPIIRTYFEYIYELTQRSEFIHDKNEFMIQILKLA
IENLYKIAYRVLILELNIARIESKLVGETPEQRANYFSNILLRNDDYIEKLYMEYNELTRLMDLCMRN
FCNYLREIIENTEREEKQLSKKLLDGKHLGKLKSIEFGAGDMHNGGRSVAVLYFDSGVKLIYKPRALD

LEVKFGEFIEWLNNQCIPNFYSLKTCRTYTIESAGWVEFIEYKECCEIDEIKRFYYRAGEILCILYTL
NARDMHFENIIAEGENPVLIDLETLFHPDLFDINRTETFASTEVLRILNNSVRGIGLLPTQIVNFKSG
KVFEIGGLCAEDEQEAPFHSLFVNNYNTDEIKIEYDYAIIKPKSNNPIMKGKRIKSSEYVNEIINGFV
NTYKWILKNKNEYIKKVREKFQNCKCRVIFKPTSIYAQLLATSYHPDLLRNSIDRKVFLHRIGLVPYE
EEKRIVLSEIEDMLNGDVPYFTTFLNCNSIINSKGEKIEPCYKQTPLDYVIKKIESMNEKDLERQVAL
INMSYLNKVTYDMGYHTNIRFKTCNNIKKFDKQKFLDIAKKIGDLILEKSIMGKRNGKNERSWIGSIG
IDDKFYFITDVGYDLYSGNSGIALFLAFLGAITGSKKYKLAAIEAMVPVISYIENLGKVSNSKINRIG
AFSGVCGYFYALFHIGKCLDIKEFLDIVYGKIGILSKAELITENHDIISGLSGTLGVLLSIYDKTAEE
KIREELLSICTKILYAINEKSNKCKEGITWGEEGYVGYSHGNAGVTSQLIRLFYINHNSNIIDLIRLS
LTYERNMFDKRNNNWRRSLLEDGFSFGWCHGAPGILLSRINLIEKGYHDDYCFEEIKKAIEITKKYAF
GKDYCLCHGDIGNLRILYYTAKVLKDEELKISCLATLNEFLDRYFMGRWCNGEFNKIENYGLMTGLSG
VGYAFLQFCEYVQMPEILCLE**LEHHHHHH***

AA sequence of N-terminally HIS-tagged MrsM

- 123. 23 kDa
- present in pET28MrsM
- additional aa that are not present in the original MrsM sequence are highlighted in **bold**

MGSSHHHHHHSSGLVPRGSHMHTKFKRNSVWNRSSSISERKVRRSLNTNWDELTNRRFERWKSLVESD
EGIRIEDVLATQNIDEETLKHTINAKEVEFINEGDHQGWLEIIQLVDEQSYKNVNIEVRKDILFFSFI
KPFLKIARGKLEEVLYSHSTKSLIKEELSPSVIDDLLNNLGETLSAISSRILILELNVARVSGKLRGE
TSEERASYFNQALLNDPAYVRSIREEYIVLTRLLATKTMYWIQNTSDLLVRFHQDKGILESEFSNGQK
LGKIISIDTGSGVSDTHNKGKTVAILNFETGIKIVYKPRSLEIDVKFNKFVNYLNGKNLSFDLKTVHT
LNKKSYGWTQFISYKECQEELQIGKFYWRIGSYLAILYAMNAVDFHMQNLIADGEYPILVDLESLFHN
NSTYTDTSAFSRAQEHIERSVLRIGLLPRKINSKAGFEGIDLSALGAQEGQVSPHKTSTIVDRDKDTV
RIEEKNFPIPVSQHRPMLHGQIINTVAYEGNIIKGFEETYFLFMKYKQDMLEQIDSFKGVTVRQILRG
TSRYANLLKISLHPDFMRDGLDREMILDKLWLDTKLNPRLNQVVNSEKEGLFLGDIPYFTSKPESTNM
WDSSGRKINNFFKTSALNETKEKINEMNESDCHEQVSFIKTAILVIKDSYRKHKVFDINPRLHVFNPK
DFFQEAIKIGDFLASRAIEGEQLDGQEDVSWIGSFVDNQREDQFKISAANSSLYEGVGGISLFLAYLG
RLSNNEKYTKLSKKALVAVHKNMSASSDLGAFGGIASYLYLLDHLSKLWNDEQLLKNELYSALNKLDS
LIERDENNDILTGVAGTAVILINLFKRYKEEQILNLITKCGNRLIQNINVMEKGVGWKVPANPTPASG
FAHGASGIIWALYEIYAITKQTVFKEVAEKALEFERTLFIPEKNNWADIKLENGQFRNDNFVAWCNGA
AGIGLSRILILPHNQNELIKDEAHVAINTTLKYGFEHDQSLCHGDLGNLDILMYAAENFNKKLSVNVT
ELSHKILNDIKLRGWLTGFEKNNESPSLMMGYAGIGLGLLKIFAPVEVPSVLRLQSPLELKL

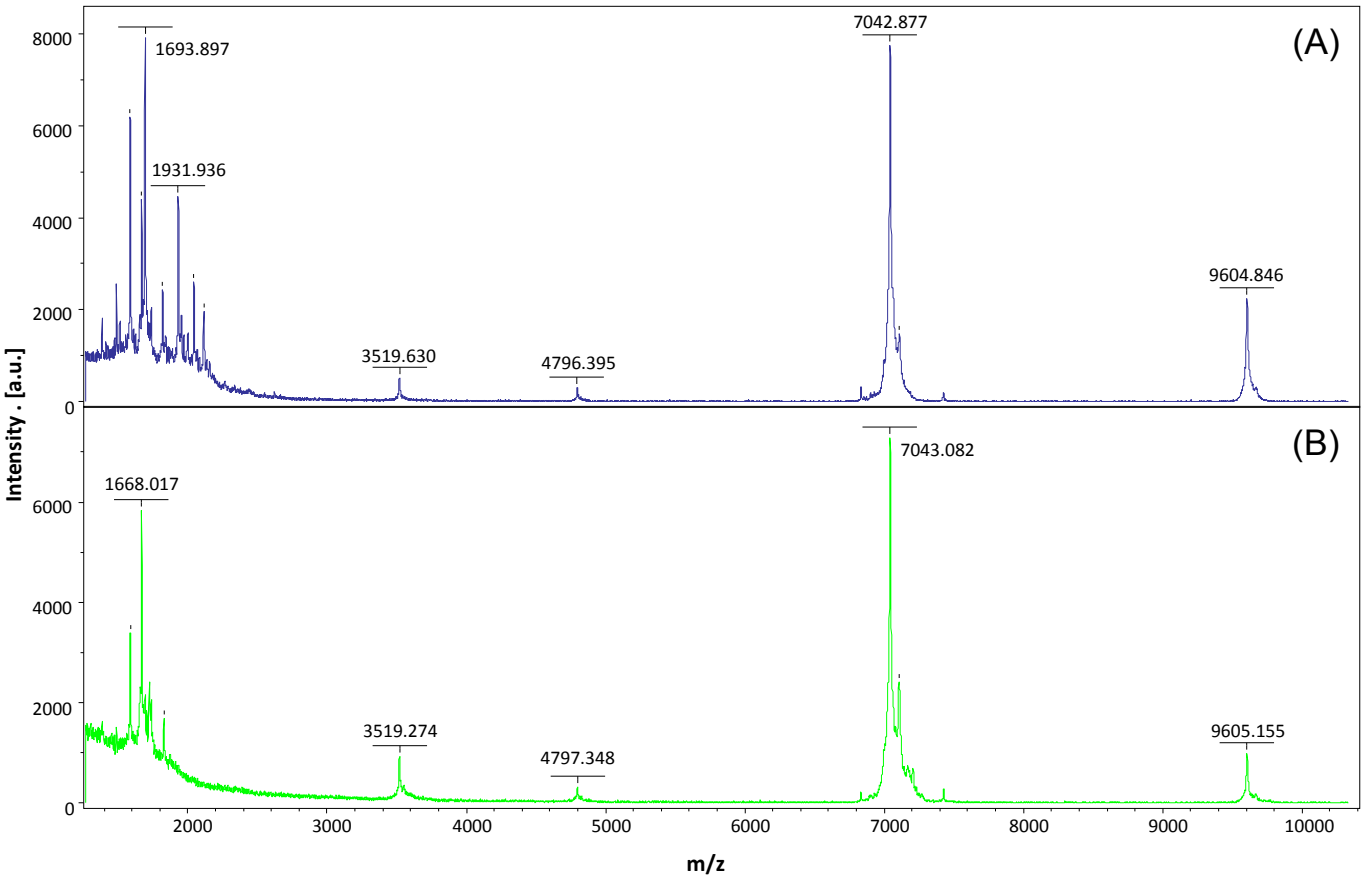

Figure 5.1: MALDI-TOF spectra of culture supernatant of (A) *B. licheniformis* MW3 and (B) its LicM2 insertion mutant. Both spectra showed existence of similar mass signals, which are not associated to the lichenicidin gene cluster. In conclusion none of these peaks represent active Licα- or Licβ-peptides. Both samples showed antimicrobial activity in agar diffusion assays, that was hypothesizes to be due to expression of a predicted novel TOMM-like bacteriocin, heterocycloanthracin, which might be supposed to be represented by at least one of observed mass signals shown in A and B. Validation of this notion will be focused in future experiments.

Publications

Journal articles

Expression of the lantibiotic mersacidin in *Bacillus amyloliquefaciens* FZB42
Herzner AM, Dischinger J, Szekat C, Josten M, Schmitz S, Yakéléba A, Reinartz R, Jansen A, Sahl HG, Piel J, Bierbaum G.; PLoS One. **2011**;6(7)

Production of the novel two-peptide lantibiotic lichenicidin by *Bacillus licheniformis* DSM13
Dischinger J, Josten M, Szekat C, Sahl HG, Bierbaum G; PLoS ONE. **2009**; 4(8)

Book chapters

Handbook of biologically active peptides, chapter lantibiotics
Dischinger J, Wiedemann I, Bierbaum G and Sahl HG, editor: Abba J. Kastin, Academic Pr Inc, second edition, **in press**

Oral presentations

Mining for new lantibiotic producer in microbial genome sequences
Dischinger J, Josten M, Herzner AM, Yakéléba A, Oedenkoven M, Sahl HG, Piel J and Bierbaum G
Annual Conference of the Association for General and Applied Microbiology (VAAM), **2012, abstract accepted**

Posters

Characterization of an antimicrobial substance produced by *Bacillus pseudomycoides* DSM 12442
Chipalu S, Dischinger J, Josten, M, Sahl HG and Bierbaum G
Annual Conference of the Association for General and Applied Microbiology (VAAM), **2012, abstract accepted**

Discovery of novel lantibiotic producers by genomic data mining
Dischinger J, Josten M, Herzner AM, Yakéléba A, Oedenkoven M, Sahl HG, Piel J and Bierbaum G
Annual Conference of "Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM)", **2011**

Mersacidin production by *Bacillus amyloliquefaciens* FZB42
Dischinger J, Herzner AM, Josten M, Hoffmann A, Sahl HG, Piel J and Bierbaum G
Annual Conference of the Association for General and Applied Microbiology (VAAM), **2011**

Discovery of new lantibiotic producers by genomic data mining

Dischinger J, Josten M, Sahl HG and Bierbaum G

Annual Conference of "Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM)", **2010**

Characterization of an antimicrobial substance produced by *Bacillus pseudomycoides* DSM 12442

Chipalu S, Dischinger J, Josten M, Szekat C, Sahl HG and Bierbaum G

Annual Conference of the Association for General and Applied Microbiology (VAAM), **2010**

Characterization of an antimicrobial substance produced by *Bacillus cereus* ATCC 14579

Feger S, Dischinger J and Bierbaum G

Annual Conference of the Association for General and Applied Microbiology (VAAM), **2009**

***Bacillus licheniformis* DSM 13 produces an antimicrobial substance that is based on a putative lantibiotic gene cluster**

Dischinger J, Josten M, Sahl HG and Bierbaum G

Annual Conference of the Association for General and Applied Microbiology (VAAM), **2009**

Production of a new two-peptide lantibiotic by *Bacillus licheniformis* DSM 13

Dischinger J, Josten M, Sahl HG and Bierbaum G

Annual Conference of "Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM)" and ProkaGenomics Conference, **2008**

Lichenicidin: A novel two-component lantibiotic produced by *Bacillus licheniformis* DSM 13

Dischinger J, Szekat C, Sass P and Bierbaum G

Annual Conference of the Association for General and Applied Microbiology (VAAM), **2008**

Discovery of a putative two-component lantibiotic gene cluster in *Bacillus licheniformis* DSM 13

Dischinger J, Szekat C, Sass P and Bierbaum G

Annual Conference of "Deutsche Gesellschaft für Hygiene und Mikrobiologie (DGHM)", **2008**

Analysis of the new two-component lantibiotic Lichenidicin

Dischinger J, Szekat C, Sass P and Bierbaum G

VAAM Meeting „Biology of bacteria producing natural compounds", **2008**

Acknowledgments (Danksagung)

Bei allen, die durch ihre fachliche und/oder persönliche Hilfe und Unterstützung zum Gelingen dieser Arbeit beigetragen haben, möchte ich mich an dieser Stelle herzlichst bedanken.

An erster Stelle geht mein herzlicher Dank an Frau Prof. Dr. Gabriele Bierbaum für die Überlassung des spannenden Themas, die intensiven und inspirierenden Diskussionen, sowie für ihre Unterstützung und für die gewährte Freiheit und das Vertrauen bei der Verwirklichung eigener Ideen und Ansätze.

Herrn Prof. Dr. Hans-Georg Sahl danke ich herzlich für sein Interesse an meiner Arbeit, für seine Unterstützung und die Bereitschaft als Zweitbetreuer tätig zu sein.

Herrn Prof. Dr. Witke und Frau Prof. Dr. König danke ich sehr für ihre Bereitschaft, sich als Mitglieder der Prüfungskommission zur Verfügung zu stellen, und für das Interesse, das sie meiner Arbeit entgegengebracht haben.

Herrn Prof. Dr. Borriss und Herrn Prof. Dr. Jörn Piel danke ich für die Bereitstellung des *Bacillus amyloliquefaciens* FZB42. Herrn Prof. Dr. Meinhardt danke ich für die Bereitstellung des *Bacillus licheniformis* MW3 Stamms. Für die Bereitstellung des pET28MrsM Vektors danke ich Herrn Jesus Cortés und der Firma Novacta, sowie Frau Dr. Tanja Schneider für den Vektor pET28b.

Diese Arbeit wurde im Rahmen der DFG-Forschergruppe FOR854 „Post-genomische Strategien für neue antibiotische Wirkstoffe und Zielstrukturen“ angefertigt. Der Deutschen Forschungsgesellschaft danke ich für die finanzielle Unterstützung.

Bei Frau Michaele Josten möchte ich mich herzlich für die Anfertigung der MALDI-TOF Analysen sowie ihre Hilfe bei allen Fragen und Nöten rund um die HPLC und die Peptidaufreinigung bedanken. Ein besonderer Dank, nicht nur für ihre Unterstützung und Hilfe bei der Anfertigung der Puncticin Überexpressionsklone, geht an Frau Marion Oedenkoven. Frau Christiane Szekat danke ich herzlich für ihre Hilfe bei allen allgemeinen Labor- und „*Bacillus*-Fragen“. Herrn Dr. Michael Türck möchte ich für seine tatkräftige Unterstützung und seine Ideen bei der heterologen Proteinexpression sowie für seine stete Diskussions- und Hilfsbereitschaft danken. Ebenfalls bedanken möchte ich mich bei Frau

Kirsten Braun, die sich im Rahmen ihrer Projektarbeit mit der heterologen Expression der Lantibiotikabiosynthese-Gene beschäftigt hat.

Frau Dr. Katharina Lückerath, Herrn Dr. Michael Nagel, Frau Tina Reuter und Frau Ines Dischinger danke ich herzlich für das kritische Korrekturlesen dieser Arbeit, ihre Unterstützung und Aufmunterungen. Herrn Wolfgang Dischinger danke ich für seine Unterstützung bei der Erstellung der PDF-Dateien.

Bei allen aktiven und ehemaligen Mitgliedern der Arbeitsgruppen Bierbaum und Sahl möchte ich mich für die angenehme Arbeitsatmosphäre und die gute Zusammenarbeit bedanken.

Meiner Familie und meinen Freunden danke ich herzlich für ihre Unterstützung und ihre Nachsicht, vor allem in den letzten Monaten. Ein ganz besonderer Dank geht hierbei an Herrn Matthias Rickmeyer, besonders für seine Geduld und seinen ansteckenden Optimismus.

Declaration (Eidesstattliche Erklärung)

An Eides statt versichere ich, dass

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Teile der Dissertation sind vorab an den unter „Publications“ aufgeführten Stellen auszugsweise veröffentlicht worden.

Bonn, März 2012

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Jasmin Dischinger